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An Investigation into the Effects of Current Immunosuppressants and Novel Compounds on Animal Models of Asthma and Delayed Type Hypersensitivity

By

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A thesis presented to the University of Dublin

for the degree of Doctor of Philosophy

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2006

Declaration

This thesis has not been submitted as an exercise for a degree at any other University. The work described is entirely my own work. I agree that the library may lend or copy this thesis upon request.

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Summary

Asthma is a disease with a worldwide distribution and of increasing prevalence. There are two forms of the disease; intrinsic, which is believed to follow a Th1 cytokine profile and extrinsic, which follows a Th2 response. A problem with many current treatments is the side effects they cause at high doses. For this reason, a number of novel compounds were evaluated in animal models of delayed type hypersensitivity (DTH) and asthma.

In extrinsic asthma, an allergen stimulates a Th2 response, IgE production from B cells and mast cell degranulation, causing the release of eicosanoids and various cytokines. This stimulates the recruitment of leukocytes such as eosinophils and neutrophils to the lungs and ultimately leads to hypersensitivity of the airways. Bronchial hyper-reactivity also occurs following leukocyte recruitment in intrinsic asthma although it may follow a Th1 response and has not been shown to involve mast cells or IgE production.

A number of novel compounds were tested in three animal models of DTH, namely; the methylated bovine serum albumin (mBSA) model, the sheep red blood cell (SRBC) model and the oxazolone model. 3C8 and 3C9 potently reduced inflammation in both the mBSA and SRBC models ($P<0.05$). Since a Th1 response is generated in each model, such compounds may be useful in treating intrinsic asthma. Compounds were next evaluated in rat and mouse models of asthma. While many animal models of extrinsic asthma have been established by generating a Th2 response, the addition of Freunds complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T)) as a

Th1 inducer would hopefully enable us to mimic the intrinsic form of the disease. Sensitising animals to OVA using AlOH as a Th2 inducer and FCA(T) as a Th1 inducer would hopefully mimic extrinsic and intrinsic asthma respectively. A third model was set up using both adjuvants for sensitisation, which would theoretically generate a mixed Th1/Th2 response. 3C8 potently reduced bronchoalveolar lavage (BAL) fluid eosinophil levels in both the Th2 and mixed Th1/Th2 models in rats and mice ($P<0.05$). This is of particular significance due to the role that eosinophils play in the pathogenesis of asthma through the release of various cytotoxic proteins, eicosanoids and cytokines. Eosinophil peroxidase (EPO) and neutrophil myeloperoxidase (MPO) levels generally correlated well with levels of eosinophils and neutrophils, from which they originate.

Levels of the cytokines interleukin-4 (interleukin; IL), IL-5, IL-10 and interferon gamma (IFNγ) were measured in mouse BAL fluid and lung extracts. 3C8 and 3C9 reduced levels of all four cytokines in one or both asthmatic models (Th2 and mixed Th1/Th2), with 3C8 found to significantly reduce levels of all four cytokines in the mixed response model, and 3C9 having this effect in the Th2 model ($P<0.05$). This highlights the potential suitability of these compounds for the treatment of asthma, particularly due to the significant role of IL-4 in the Th2 response and IFNγ in the Th1 response.

Results for these novel compounds, in particular 3C8 and 3C9, are very promising. Due to their significant impact on both Th1 and Th2 models, they may be suitable for treating asthma, organ transplantation rejection and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.

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Abbreviations

5-HT	5-hydroxytryptamine
5-LO	5-Lipoxygenase
AA	Arachidonic Acid
AHR	Airway Hyper-Responsiveness
AlOH	Aluminium Hydroxide
APC	Antigen Presenting Cell
β_2-agonists	beta$_2$-Adrenergic Stimulants
BAL	Bronchoalveolar Lavage
BN	Brown Norway
BSA	Bovine Serum Albumin
BSS	Buffered Salt Solution
^{o}C	Degrees Centigrade
Ca^{2+}	Calcium Ion
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanine Monophosphate
CHS	Contact Hypersensitivity
Cl^-	Chloride Ion
cLTs	Cysteinyl Leukotrienes
CMC	Carboxymethylcellulose
COX	Cyclo-oxygenase
CRAC	Calcium Release-Activated Calcium Channels
CTAC	Cetyltrimethylammonium chloride solution
CycA	Cyclosporin A

DAD	Disease Activated Drugs
DAG	Diacylglycerol
dH20	Distilled Water
DTH	Delayed Type Hypersensitivity
e.c.	Extracellular
ECP	Eosinophil Cationic Protein
ELAM	Endothelial Leukocyte Adhesion Molecule
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Eosinophil Peroxidase
ER	Endoplasmic Reticulum
FCA(B)	Freunds Complete Adjuvant (containing *Mycobacterium butyricum*)
FCA(T)	Freunds Complete Adjuvant (containing *Mycobacterium tuberculosis*)
FKBP	FK-binding protein
FLAP	5-Lipoxygenase Activating Protein
GDP	Guanine Diphosphate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GTP	Guanine Triphosphate
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulphuric Acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid
HETE	Hydroxy-eicosatetraenoic acid
HOCL	Hypochlorous Acid
HPETE	Hydroxy-peroxy-eicosatetraenoic acid
HRP	Horse Radish Peroxidase
i.c.	Intracellular

ICAM	Intracellular Cell Adhesion Molecule
i.d.	Intradermal
IFA	Incomplete Freunds Adjuvant
IFNα	Interferon alpha
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
i.p.	Intraperitoneal
IP_3	Inositol Triphosphate
i.v.	Intravenous
KBr	Potassium Bromide
KCl	Potassium Chloride
KH_2PO_4	Potassium dihydrogen phosphate
LFA	Lymphocyte Function-Associated Antigen
LPS	Lipopolysaccharides
LT	Leukotriene
MBP	Major Basic Protein
mBSA	Methylated Bovine Serum Albumin
MCP-1	Monocyte Chemoattractant Protein 1
MHC	Major Histocompatibility Complex
MIF	Macrophage Migration Inhibitory Factor
MIP-1α	Macrophage Inflammatory Protein 1α
MPO	(Neutrophil) Myeloperoxidase
MW	Molecular Weight

Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-AT	Nuclear Factor of Activated T Cells
NK	Natural Killer
ng	nanogram
NK	Natural Killer
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric Oxide Synthase
O_2^-	Superoxide anion
OH^-	Hydroxy Free Radical
$OONO^-$	Peroxynitrite Anion
OPD	*o*-Phenylenediamine, Dihydrochloride
OVA	Ovalbumin
PAF	Platelet Activating Factor
PAPase	Phosphatidate Phosphohydrolase
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PG	Prostaglandin
pg	Picogram
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol Bisphosphate
PKC	Protein Kinase C

PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
ROC	Receptor Operated Calcium Channel
rpm	revolutions per minute
RSV	Respiratory Syncytial Virus
S1P	Sphingosine-1-Phosphate
s.c.	Subcutaneous
SD	Sprague Dawley
SEM	Standard Error of the Mean
SPF	Specific Pathogen Free
SRBC	Sheep Red Blood Cells
TBS	Tris Buffered Saline
Tc1	Cytotoxic T Cell
TCR	T Cell Receptor
TGFβ	Tumour Growth Factor beta
Th	T Helper Cell
Th0	Naive T Helper Cell
Thp	Precursor T Helper Cell
TNFα	Tumour Necrosis Factor alpha
TNFβ	Tumour Necrosis Factor beta
TX	Thromboxane
VCAM	Vascular Cell Adhesion Molecule
VLA	Very Late Antigen
VOC	Voltage Operated Channels

Chapter 1

Introduction

Chapter 1: Introduction

Extrinsic and intrinsic asthma are believed to involve a Th2 and Th1 cytokine profile respectively. This concept and the aetiology of both forms of the disease will be detailed later on. Delayed type hypersensitivity (DTH) responses, as explained in **section 1.5**, generally involve a Th1 response and are often used to examine T cell mediated immune responses *in vivo*. There may therefore be a link between intrinsic asthma, also thought to involve a Th1 response, and DTH. For this reason, animal DTH models were, in addition to animal models of both forms of asthma, used to examine the effects of the test compounds.

1.1: Asthma

Asthma is a chronic inflammatory disease, which despite our improving knowledge, is increasing in terms of its prevalence and mortality in many countries (Sidebotham, Roche 2003; Pallapies 2006). The incidence of asthma has increased by almost two-fold in the past two decades (Umetsu, McIntire et al. 2002). It is the most common chronic inflammatory disorder of the airways, affecting approximately twenty million Americans, one third of whom are children. The prevalence of asthma has increased dramatically in developed countries in the past decade, killing around five thousand annually in the United States (Bharadwaj and Agrawal 2004). The highest incidence of the disease in adults aged between 18 and 44 years old occurs in Ireland, where there are approximately 400,000 sufferers in total. Asthma is defined as bronchoconstriction and inflammation, which is reversible with time or with

treatment, ultimately leading to hypersensitivity of the airways. Patients suffer from eosinophilic inflammation, mucus hypersecretion, reversible airflow limitation, wheezing and coughing (Holgate 1993; Bochner, Undem et al. 1994; Busse, Neaville 2001).

There are two clinically defined forms of asthma; intrinsic and extrinsic. Intrinsic asthma is associated with a Th1 (Th; T Helper Cell) type cytokine profile whereas extrinsic asthma follows a Th2 cytokine profile (Virchow, Kroegel et al. 1996). Sufferers of both forms of asthma experience bronchoconstriction as well as epithelial shedding, tissue remodelling and influx of inflammatory cells, particularly eosinophils to the lungs. Eosinophils contribute further to the inflammatory process though the release of cytotoxic proteins, cytokines and leukotrienes and are believed to contribute largely to the development of airway hyper-responsiveness (AHR).

1.1.1: Clinical signs And Background

1.1.1.1 Extrinsic Asthma

Extrinsic asthma is also referred to as allergic or atopic asthma because patients show a positive result to skin prick tests to common aeroallergens, resulting in IgE production (Johansson, Hourihane et al. 2001). Sufferers experience coughing and wheezing as a result of airflow limitation and mucus production, caused in part by the influx of inflammatory cells (Holgate 1993; Bochner, Undem et al. 1994). It has an early and a late phase. The early phase involves the stimulation of the Th2 response, immunoglobulin E (immunoglobulin; Ig) production from the B cells and mast cell

degranulation, leading to bronchoconstriction, vasodilation, increased capillary permeability and chemotaxis. This early phase occurs within five to fifteen minutes of exposure and lasts for up to an hour. The late phase involves the influx of the inflammatory cells, such as the eosinophils, leading to mucus hypersecretion, epithelial shedding and bronchial hyper-reactivity. This occurs two to six hours after exposure, peaking at about nine hours, with the airway obstruction resolved in twelve to twenty four hours (Peters, Zangrilli et al. 1998; Varner, Lemanske 2000).

1.1.1.2 Intrinsic Asthma

Less is known about the intrinsic form of the disease. Intrinsic (or non-allergic, non-atopic) asthma usually develops later on in life and is associated with female predominance and a greater degree of severity (Rackeman 1947; Bentley, Menz et al. 1992; Humbert, Grant et al. 1996; Romanet-Manent, Charpin et al. 2002). It has been reported that intrinsic asthma comprises approximately 10% of the total asthmatic population. However, according to the Swiss SAPALDIA survey which included more than eight thousand adults between eighteen and sixty years of age, one third of the asthmatic population was intrinsic (Godard, Bousquet et al. 1997, Wuthrich, Schindler et al. 1995).

As is the case in patients with extrinsic asthma, there is an influx of inflammatory cells, in particular eosinophils to the lungs as well as epithelial shedding, tissue remodelling and the development of AHR. Sufferers have more prominent eosinophilia than extrinsic asthmatics, with more severe symptoms and a worse prognosis. They also show a negative result to skin prick tests to common allergens

such as dust mites and grass pollen. This is a widely accepted method for distinguishing intrinsic or non-allergic asthma from extrinsic or allergic asthma (Romanet-Manent, Charpin et al. 2002).

1.1.2 Aetiology

1.1.2.1 Extrinsic Asthma

There are many possible causes for extrinsic asthma, one major factor being immediate hypersensitivity to allergens such as dust mites and pollen. There is also a significant genetic predisposition to the development of asthma in humans, with monozygotic twins of sufferers being four times more likely to develop the disease, and dizygotic twins being twice as likely to have asthma compared to the general population (Duffy, Martin et al. 1990). Respiratory syncytial virus (RSV)-induced bronchiolitis in infants is associated with an increased risk of asthma development (Sigurs, Bjarnason et al. 1995; Stein, Sherrill et al. 1999). This may be related to the fact that IFNγ secretion in the peripheral blood mononuclear cells of these patients is reduced (Renzi, Turgeon et al. 1999). IFNγ downregulates IL-4 production and receptor expression, therefore inhibiting the Th2 response (Paludan 1998). Thus, a reduced production of IFNγ as seen in patients with RSV-induced bronchiolitis may facilitate the Th2 response and the development of extrinsic asthma.

As stated earlier, extrinsic asthma can be divided into the early phase and the late phase response. The early phase occurs following allergen exposure in a sensitised asthmatic patient. It involves the initiation of the Th2 response, leading to IgE

production from the B cells and subsequent mast cell degranulation. A number of inflammatory mediators are then released from the mast cells, including histamine, eicosanoids and cytokines such as IL-3, IL-4, IL-5, IL-6, GM-CSF and TNFα. The result is bronchoconstriction, vasodilation, increased capillary permeability and chemoxtaxis. This early phase occurs within five to fifteen minutes of exposure, lasting for up to an hour.

Two to six hours after exposure, the late phase occurs and lasts for twelve to twenty-four hours. This involves the influx of the inflammatory cells (eosinophils, neutrophils, monocytes, macrophages, T lymphocytes), leading to mucus hypersecretion, epithelial shedding and bronchial hyper-reactivity (Peters, Zangrilli et al. 1998; Varner, Lemanske 2000).

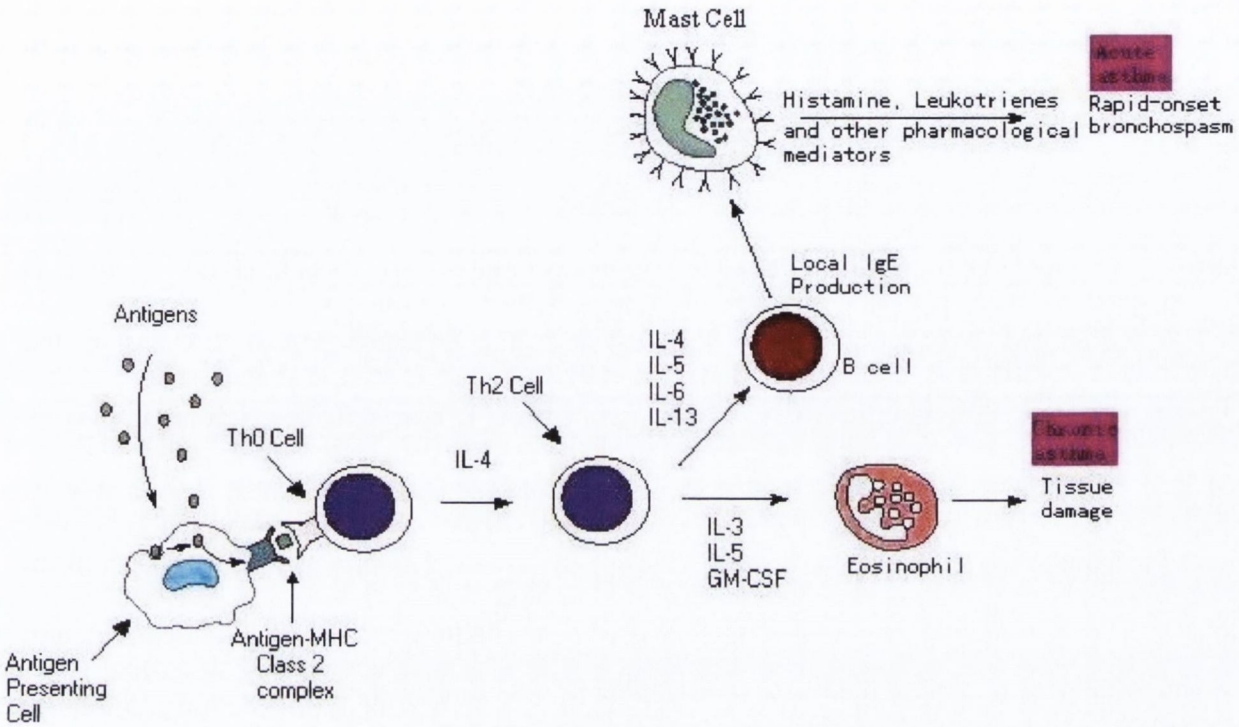
1.1.2.1.1 Early Phase

1.1.2.1.1.1 Th2 response and IgE production from B cells

The presentation of antigen, stimulation of the Th2 response and subsequent isotope switching of the B cells to produce IgE is illustrated in **figure 1.1.2.1.1.1**. Upon allergen exposure, the antigen is processed by an antigen presenting cell (APC). Examples of such cells include macrophages, monocytes, dendritic cells, Langerhans cells and B lymphocytes. Fragments of the antigen bind to the major histocompatibility complex (MHC) molecules of the APC, forming the antigen-MHC class two complex. This complex interacts with the T cell receptor (TCR) of the naive T helper cell (Th0 cell). IL-4 is produced and released from the Th0 cell, stimulating

the maturation and proliferation of Th2 cells. More IL-4 is then produced from the Th2 cells, along with the cytokines IL-3, IL-5, IL-6, IL-9, IL-10, IL-13 and GM-CSF (Krishna, Chauhan et al. 1996). IL-4 is the main cytokine involved in the stimulation of the Th2 response. It is also one of the most crucial components in stimulating the isotope switching of the B cells to produce IgE and plays a role in upregulating the endothelial adhesion molecules necessary for leukocyte recruitment in the late phase (Vercelli, Geha 1992; Herz, Bunikowski et al. 1998). IL-5, IL-6 and IL-13 also contribute to the differentiation of the B cells to IgE producing cells. IL-5 may have an additive effect (Pene, Rousset et al. 1988) while IL-6 appears to have an obligatory effect on IL-4-induced IgE production (Lacy, Levi-Schaffer et al. 1998). IL-13 can stimulate B cell growth and differentiation with equal potency to IL-4 although IL-4 can stimulate greater IgE production. It may act to sustain IgE production when IL-4 has been downregulated since it is produced earlier from the Th2 cells and sustained for longer than IL-4 (Robinson 1996; Bousquet, Yssel et al. 1997). Apart from these cytokines, a T cell-B cell interaction is also necessary for IgE production, allowing for a second signal to be sent from the T cell to the B cell (Krishna, Chauhan et al. 1996).

Figure 1.1.2.1.1.1: Stimulation of the Th2 response and subsequent IgE production from B cells



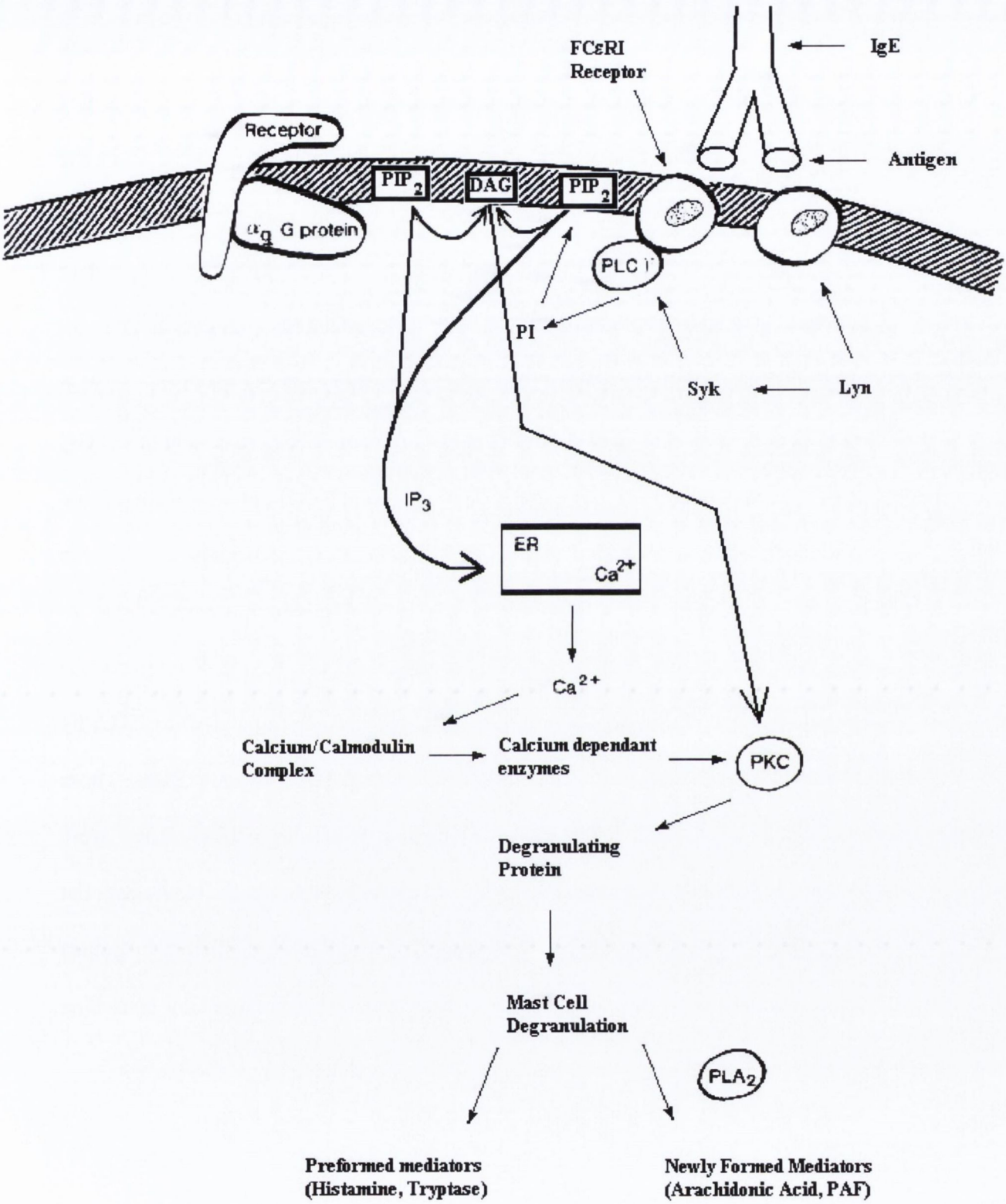
[Adapted from (Humbert, Menz et al. 1999)]

1.1.2.1.1.2 Mast Cell Degranulation Pathway

Following production of the IgE specific to the antigen, the antigen-IgE complex is formed and cross links with the FCεRI mast cell receptor. This triggers a chain of events leading to degranulation of the mast cells and the release of inflammatory mediators (Kobayashi, Ishizuka et al. 2000; Galli, Wedemeyer et al. 2002). This mast cell degranulation pathway is illustrated in **figure 1.1.2.1.1.2**. Following cross

linkage, protein tyrosine phosphorylation takes place, occuring within five to fifteen seconds (Benhamou and Siraganian 1992; Hamawy, Mergenhagen et al. 1995; Suzuki, Takei et al. 1997). The β and γ subunits of the FCεRI receptor are phosphorylated at the tyrosine residue by a tyrosine kinase called lyn. Lyn then phosphorylates another tyrosine kinase called syk, thus activating syk which activates phospholipase Cγ (PLCγ), again via phosphorylation. PLCγ catalyses the hydrolysis of phosphatidylinositol (PI) to phosphatidylinositol bisphosphate (PIP_2), which is converted to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts on the endoplasmic reticulum (ER), causing an increase in intracellular calcium (i.c. Ca^{2+}). Following the mobilisation of the Ca^{2+} from i.c. stores, possibly by means of sphingosine-1-phosphate (S1P) (Choi, Kim et al. 1996), more Ca^{2+} enters from extracellular (e.c.) sources by means of the calcium release-activated calcium channels (CRAC) and this is chloride ion (Cl^-) dependant. The calcium/calmodulin complex is then formed, which activates Ca^{2+} dependant enzymes. These enzymes, together with DAG activate protein kinase C (PKC), which activates a degranulating protein by means of phosphorylation, leading to mast cell degranulation.

Figure 1.1.2.1.1.2: Mast cell degranulation pathway

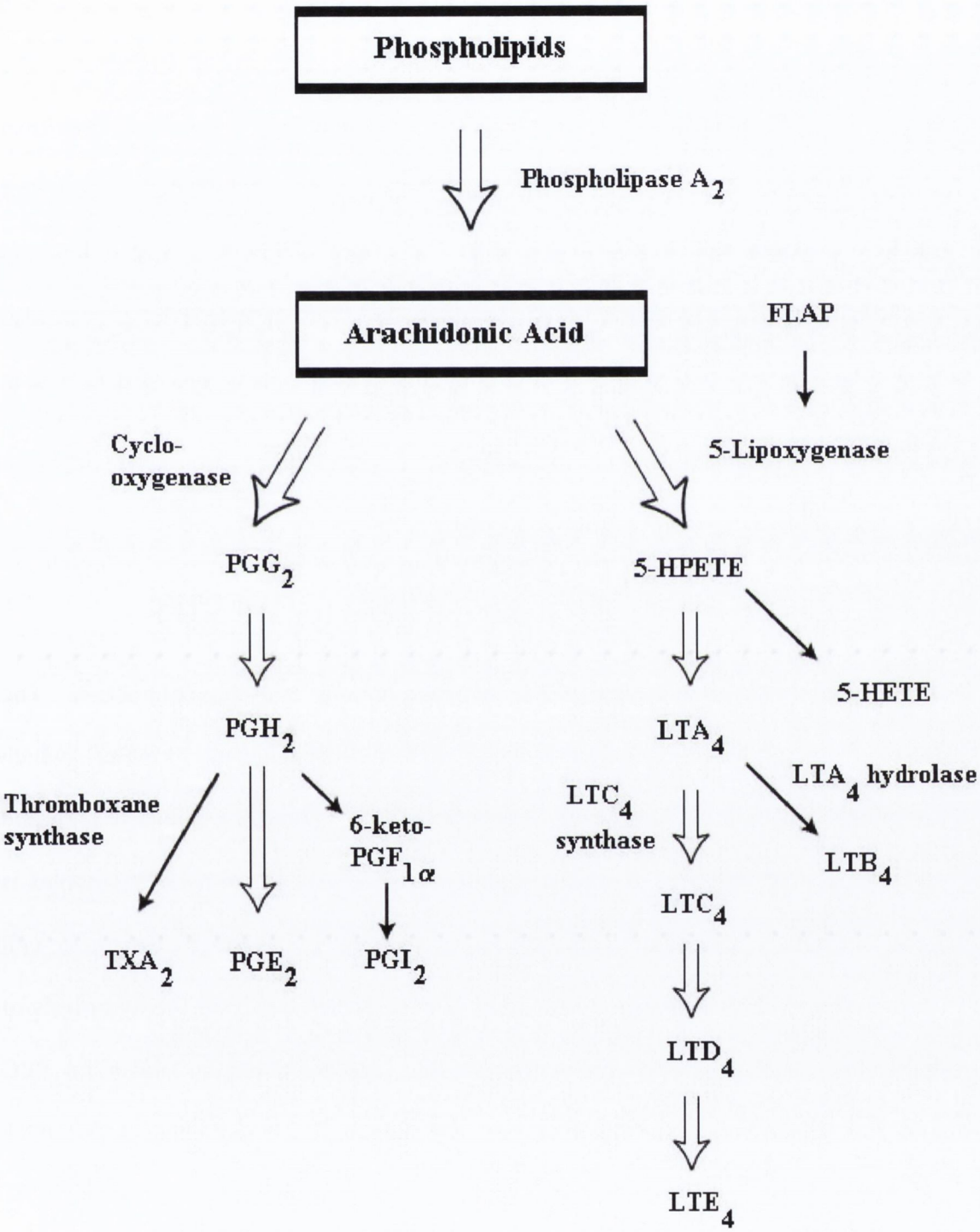


[Adapted from (Benhamou and Siraganian 1992; Hamawy, Mergenhagen et al. 1995; Bondeson 1997)]

1.1.2.1.1.3 Formation of Mast cell Products

Following degranulation, a number of preformed and newly formed mediators are released from the mast cells. This is again illustrated in **figure 1.1.2.1.1.2**. Preformed mediators include histamine and tryptase. Newly formed mediators come from the breakdown of the membrane phospholipids to arachidonic acid (AA) and platelet activating factor (PAF), either by phospholipase A_2 (PLA_2) in a Ca^{2+} dependant manner or through the phospholipase D- phosphatidate phosphohydrolase-diacylglycerol lipase (PLD-PAPase-DAG lipase) pathway. AA can be further broken down through the cyclo-oxygenase (COX) pathway, forming the prostaglandins (PGs) and thromboxanes (TXs), or though the 5-Lipoxygenase (5-LO) pathway, forming the leukotrienes (Bell, Harris et al. 1997; Lazarus 1998). These two pathways for AA breakdown are illustrated in **figure 1.1.2.1.1.3**. 5-LO, once activated by 5-Lipoxygenase Activating Protein (FLAP), breaks down the AA to leukotriene A_4 (leukotriene; LT). This can be converted to LTB_4 by LTA_4 hydrolase, or to LTC_4 by LTC_4 synthase. LTC_4 can be further broken down to LTD_4 and then LTE_4. These three LTs (LTC_4, D_4 and E_4) are the cysteinyl leukotrienes (cLTs). They were originally termed the slow-reacting substance of anaphylaxis due to the slow onset but prolonged and powerful contractions they caused upon allergen challenge in guinea pig ileum as opposed to the rapid onset but brief contractions caused by histamine (Lazarus 1998).

Figure 1.1.2.1.1.3: Arachidonic Acid formation and breakdown through the COX and 5-LO pathways in mast cells



[Adapted from (Bondeson 1997)]

1.1.2.1.1.4 The role of Mast cell Products in the Early Phase

The preformed product histamine, released from the mast cells upon degranulation causes bronchoconstriction through H1 receptor stimulation, and also causes vasodilation and increased capillary permeability (early phase characteristics). The cLTs are potent bronchoconstrictors, with over a thousand times greater potency than histamine and the PGs. TXA_2 is a potent vasoconstrictor while PGE_2 is a vasodilator which also increases vascular permeability and inhibits T-cell proliferation and production of the Th1 cytokine IL-2 (Chouaib, Welte et al. 1985). PGE_2 can also inhibit the induction of TNFα, a pro-inflammatory cytokine that plays a crucial role in leukocyte recruitment in the late phase.

1.1.2.1.1.5 Bronchoconstriction

Calcium and calmodulin are essential for smooth muscle contraction. The calcium/calmodulin complex activates myosin light chain kinase, which phosphorylates light chain myosin. This results in cross bridging between actin and myosin and therefore smooth muscle contraction. The calcium/calmodulin complex is formed through a similar pathway as was already described for mast cell degranulation. This involves the activation of PLC, which catalyses the hydrolysis of PI, leading to IP_3 and DAG production. In this case however, the trigger for PLC activation is different and involves receptor operated calcium channels (ROCs). Following binding of a bronchoconstrictor to a ROC, (for example the histamine H1 and leukotriene cys-LT1 receptors), the active form of the G-protein coupled receptor is formed. This involves the replacement of guanine diphosphate (GDP) with guanine

triphosphate (GTP), the dissociation of the β and γ subunits of the receptor and formation of the GTP-α subunit. The active GTP-α stimulates PLC.

The resulting IP_3 causes the initial large rapid contraction by acting on the ER and increasing i.c. Ca^{2+}. The contraction is sustained due to PKC, which is in turn activated by DAG and calcium dependant enzymes. PKC enhances the sensitivity to calcium, possibly by phosphorylating the contractile proteins. Depletion of i.c. calcium reduces the membrane potential, allowing for further calcium influx, thus also contributing to the sustained contraction (Ahmed, Foster et al. 1984; Hall, Donaldson et al. 1989). Depolarisation of the membrane as caused by KCL can also cause bronchoconstriction by triggering the opening of voltage operated channels (VOCs) (Foster, Okpalugo et al. 1984; Ahmed, Foster et al. 1985). This leads to calcium influx and contraction in the same way as for ROC-mediated contractions.

1.1.2.1.2 Late Phase

The late phase, which lasts for twelve to twenty-four hours involves the recruitment of leukocytes by means of rolling, activation, firm adhesion and trans-endothelial migration (Sallusto, Lanzavecchia et al. 1998). The result of this is mucus hypersecretion, epithelial shedding, subepithelial fibrosis, tissue remodelling and the development of bronchial hyper-reactivity (Peters, Zangrilli et al. 1998; Varner, Lemanske 2000). This hypersensitivity of the airways applies to a range of agents including bronchoconstrictors, cold air, ozone, pollutants and methacholine (Blease, Lukacs et al. 2000). Factors involved include cytokines such as IL-3, IL-4, IL-5, IL-6, GM-CSF and TNFα, the eicosanoids and NO, as explained in **section 1.1.2.1.2.1**.

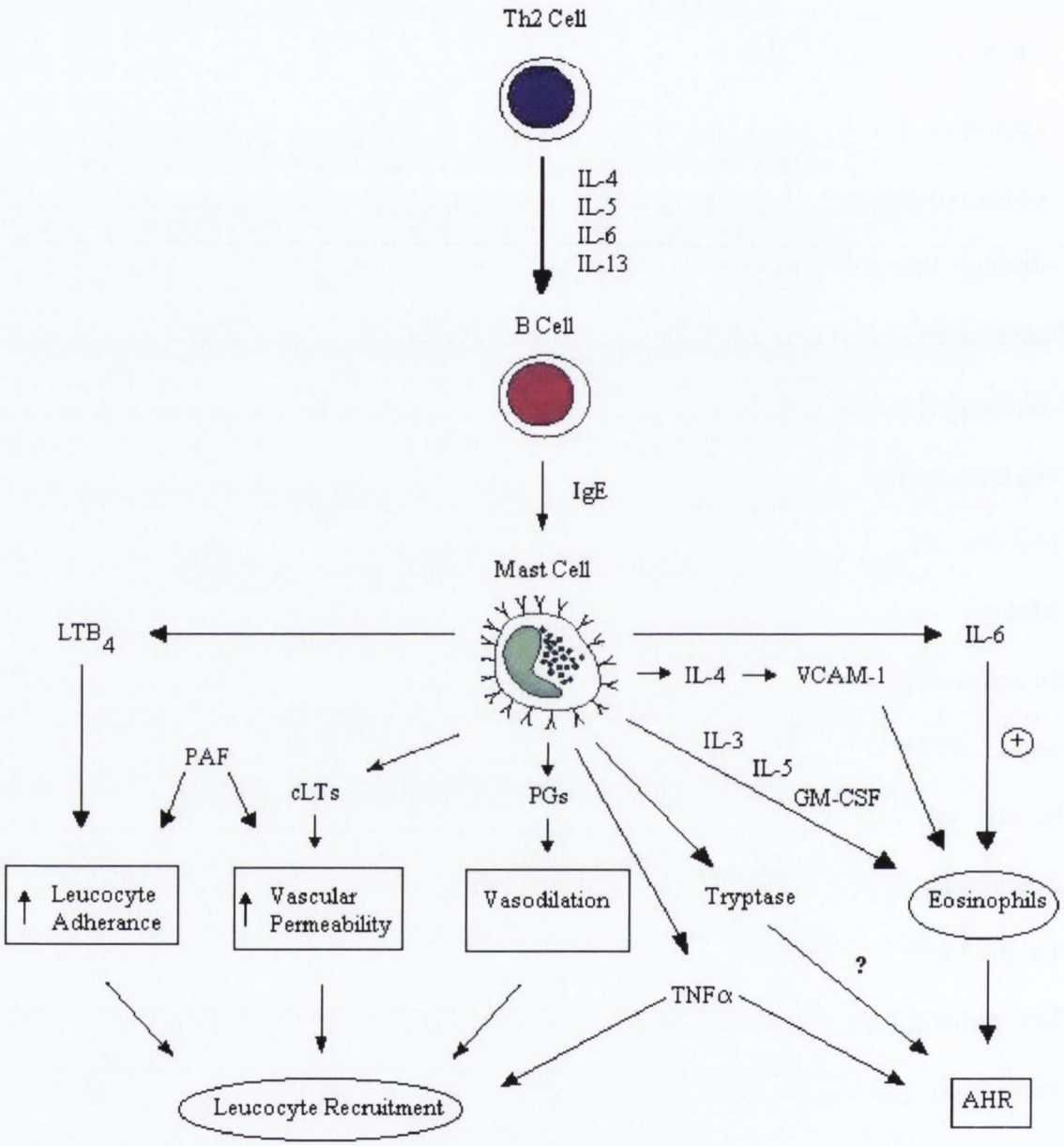
Eosinophil derived mediators such as ECP and MBP also play a role and are believed to contribute to the development of AHR. This is explained in more detail in **section 1.1.2.4.1**.

1.1.2.1.2.1 The role of Mast cell Products in the Late Phase

Cytokines produced from the mast cells include IL-3, IL-4, IL-5, IL-6, GM-CSF and TNFα. These cytokines along with many other mast cell products contribute to the development of the late phase response, involving leukocyte recruitment and the development of AHR, as illustrated in **figure 1.1.2.1.2.1**. PAF and LTB_4 increase leukocyte adherance (Bochner 1997), while PAF and the cLTS increase vascular permeability (Dahlen, Bjork et al. 1981), allowing for migration of the leukocytes, all of which contributes to leukocyte recruitment. Meanwhile, the PGs cause vasodilation, allowing for a greater concentration of leukocytes at the site of inflammation (Salmon and Higgs 1987; Miyake, Yamamoto et al. 1993). Tryptase, a preformed mediator, may contribute to hypersensitivity. IL-3, IL-5 and GM-CSF are eosinophil active cytokines, contributing to eosinophil differentation, activation and survival. IL-5 is the most important of the three, acting specifically on mature eosinophils, and also contributing to their chemotaxis and degranulation (Meng, Ying et al. 1997). TNFα is a fibrogenic cytokine with many pro-inflammatory effects. It upregulates the endothelial adhesion molecules necessary for leukocyte recruitment (E-selectin, P-selectin, ICAM1 for lymphocytes, VCAM1 for eosinophils, ELAM1 for neutrophils) (Krishna, Chauhan et al. 1996; Kumar, Dhawan et al. 1998) as well as upregulating IL-5 production in T-lymphocytes, which as mentioned is the most important of the eosinophil active cytokines. It also induces iNOS and increases

COX2 activity. NO is produced as a result of iNOS and can cause epithelial damage. This damage is greater when NO is converted to the peroxynitrite anion ($OONO^-$), which causes lipid oxidation, protein degradation and DNA damage. TNFα can also contribute directly to the late phase response by affecting fibroblasts and assisting in tissue remodelling. IL-4, the Th2 inducer, also contributes to the late phase response through upregulating VCAM1 (Krishna, Chauhan et al. 1996). VCAM1, which as mentioned is also upregulated by TNFα, binds to VLA4 on the eosinophil surface for eosinophil recruitment. Finally, IL-6 has positive feedback on the mast cells as well as on eosinophils.

Figure 1.1.2.1.2.1: Mast Cell Products and the Late Phase



It is clear from this evidence that the mast cells play a crucial role in the development of the allergic Th2 response in extrinsic asthma. Many of the mediators produced upon mast cell degranulation in the early phase contribute to the late phase response, including leukocyte recruitment and the development of bronchial hyper-reactivity.

1.1.2.2 Intrinsic Asthma

Intrinsic asthma is also referred to as post-infectious asthma since it has been known to develop following a viral respiratory tract infection. It is possible that the virus, acting as an antigen, would stimulate IgE production from the B cells and consequently lead to mast cell degranulation, as in the early phase of extrinsic asthma. However, B cell and mast cell involvement are unfounded in intrinsic asthma, and IgE levels specific to common allergens have not been found (Humbert, Menz et al. 1999). The exact cause of intrinsic asthma is unknown and could involve a hidden allergen. The disease appears to follow a Th1 type cytokine profile, involving interferon gamma (IFNγ), IL-2, IL-3, IL-5 and GM-CSF. The latter three cytokines are the three main eosinophil active cytokines. This may indicate how hypersensitivity develops in intrinsic asthma.

1.1.2.3 Cell infiltration

1.1.2.3.1 Extrinsic Asthma

As already mentioned, the late phase of extrinsic asthma involves the recruitment of inflammatory cells such as neutrophils and eosinophils. The recruitment of leukocytes involves a rolling phase, activation, firm adhesion to the endothelial cells and finally migration to the site of inflammation. Factors involved in cell infiltration in extrinsic asthma include cytokines such as TNFα, IL-4 and IL-5 as well as the PGs and LTs, all of which are produced from the mast cells as mentioned in **section 1.1.2.1.2.1**.

The rolling phase is a process involving adhesion molecules known as selectins. E-selectin and P-selectin are endothelial adhesion molecules, which when upregulated by TNFα bind to corresponding leukocyte adhesion molecules. L-selectin, a leukocyte adhesion molecule, is also involved in this process, the purpose of which is to slow down the flow rate of the leukocytes, allowing them to be activated (Carlos, Harlan et al. 1994; Kansas 1996). The selectins have been shown to play a vital role in eosinophil recruitment, where antibodies directed against either L-selectin or a combination of both E-selectin and P-selectin almost completely abolished eosinophil recruitment in lipopolysaccharide (LPS)-injected mice (Henriques, Miotla et al. 1996). Endothelial chemokines bind to their receptors on the leukocyte surface, thereby activating the leukocytes. Following activation by the chemokines, which causes the shedding of L-selectin and upregulation of other leukocyte adhesion molecules, integrin-mediated firm adhesion to the endothelial cells can take place (Picker, Butcher 1992; Springer 1994). Leukocyte adhesion molecules upregulated include LFA-1 and the eosinophil adhesion molecule VLA-4. These again bind to corresponding endothelial adhesion molecules, ICAM-1 for LFA-1 and VCAM-1 for VLA-4. Both ICAM-1 and VCAM-1 have been shown to be upregulated in the airways following antigen challenge (Bentley, Durham et al. 1993; Ohkawara, Yamauchi et al. 1995). The LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions are particularly important in the pathogenesis of inflammatory and autoimmune diseases due to their involvement in leukocyte recruitment. For this reason, LFA-1 and VLA-4 antagonists, including antibodies and peptides, have been developed for controlling inflammatory and autoimmune diseases (Yusuf-Makagiansar, Anderson et al. 2002). Following firm adhesion, the leukocytes migrate between the endothelial cells to the target area (Bochner 1997; Ward and Lentsch 1999). The destination of the

leukocytes depends on which adhesion molecules, chemokines and chemokine receptors are expressed. The chemokines are of particular importance, playing a role not only in the migration of the leukocytes but also in their activation (Sallusto, Lanzavecchia et al. 1998).

1.1.2.3.2 Intrinsic Asthma

Leukocyte infiltration takes place in intrinsic asthma through the same process that occurs in the late phase of extrinsic asthma. However, since intrinsic asthma seems to mimic a Th1 cytokine profile, the triggers are different. IL-4 for instance, a Th2 cytokine which upregulates VCAM-1 for firm adhesion of the eosinophils to the endothelial cells (Krishna, Chauhan et al. 1996) does not seem to be present in the intrinsic form of the disease. It is possible that TNFα may perform this function in this case. As already mentioned, it is a product of mast cells and plays a role in leukocyte recruitment through the upregulation of numerous endothelial adhesion molecules (Kumar, Dhawan et al. 1998). Although mast cell involvement has not been proven in intrinsic asthma, TNFα is also produced from epithelial cells, eosinophils, macrophages, T cells and B cells and contributes to leukocyte recruitment in both forms of asthma. Another cytokine specifically involved in intrinsic asthma is the Th1 cytokine IFNγ. It is capable of upregulating ICAM-1 and has been found to do so synergistically in the presence of TNF in cultured endothelial cells. It has also been found to have an additive effect on TNF-induced ELAM-1 expression, where it seems to accelerate and prolong transient expression of the molecule (Doukas and Pober 1990).

1.1.2.4 Eosinophilia

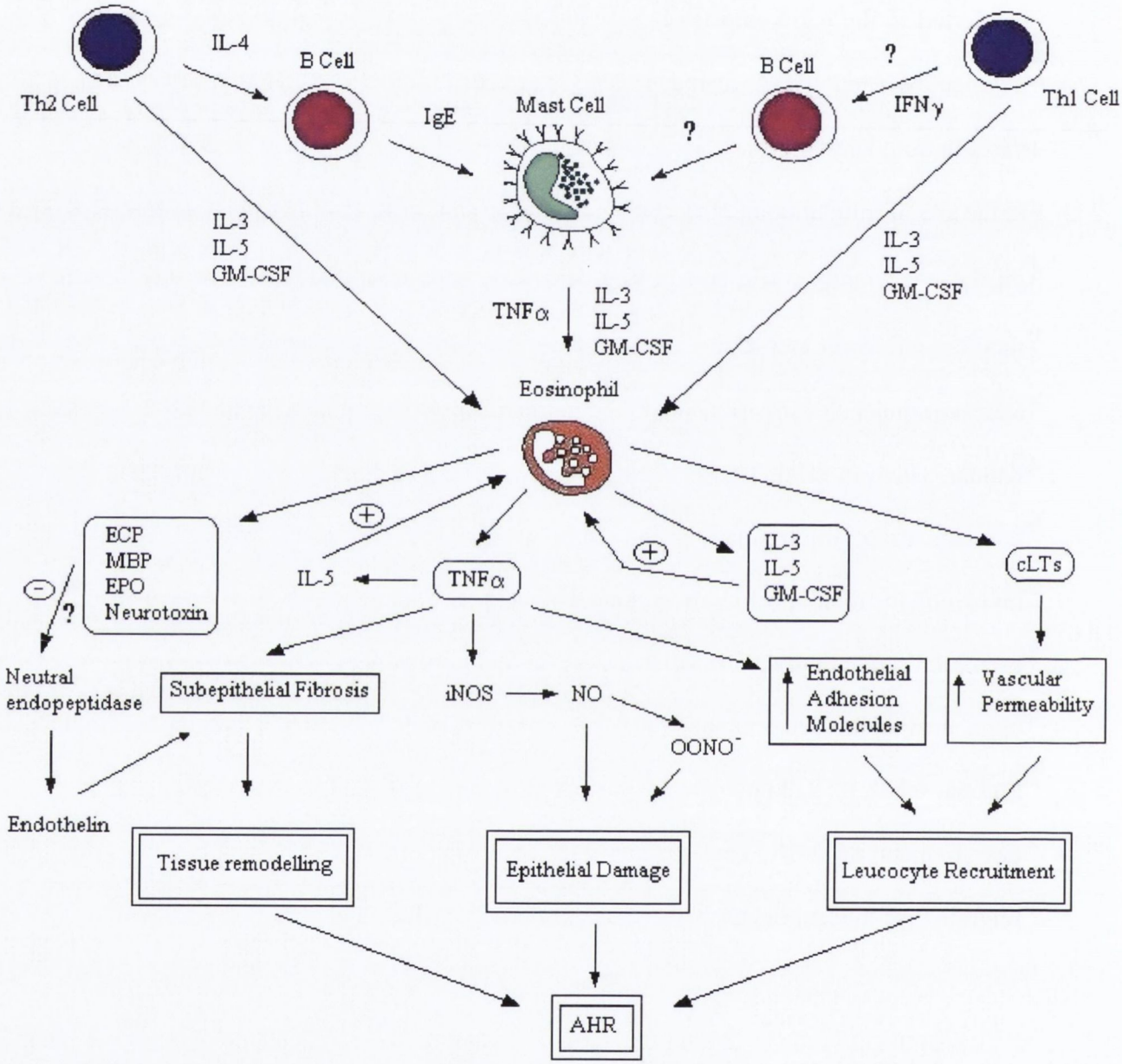
1.1.2.4.1 Extrinsic Asthma

Eosinophils are among the leukocytes recruited in the late phase of extrinsic asthma and play a significant role in the development of the disease (Reed 1994; Semanario, Gleich 1994; Peters, Zangrilli et al. 1998; Varner, Lemanske 2000). Upon degranulation, cytotoxic proteins, eicosanoids and cytokines are released, all of which contribute to inflammation (Abu-Ghazaleh, Fujisawa et al. 1989; Kroegel, Yukawa et al. 1989). The products of the eosinophils and their effects are illustrated in **figure 1.1.2.4**. Eosinophil-derived proteins include eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), major basic protein (MBP) and eosinophil-derived neurotoxin (Robinson 1996). These cytotoxic proteins are believed to contribute to the development of airway hyper-responsiveness (AHR), possibly through inactivating neutral endopeptidase, an enzyme that forms an important part of the metabolic barrier in the epithelium. Inactivation of this enzyme would allow potential bronchoconstrictors such as endothelin to act. If unrestricted, endothelin can cause subepithelial fibrosis. In this way, recruitment and activation of eosinophils can indirectly cause fibrosis and contribute to AHR. Levels of eosinophil-derived proteins have been found to be elevated in the BAL fluid of asthmatic patients (Durham, Kay 1985; Diaz, Gonzalez et al. 1989).

In addition to the effects of these eosinophil-derived proteins, cytokines released include IL-3, IL-4, IL-5, IL-6, GM-CSF and TNFα, all of which are also released from mast cells. Since TNFα can cause tissue remodelling and contribute directly to

subepithelial fibrosis, this is another mechanism for eosinophils to contribute to AHR. As previously mentioned, TNFα also induces iNOS and increases COX-2 activity. The resulting NO can cause epithelial damage, an effect that is more potent when it is converted to the peroxynitrite anion ($OONO^-$), which causes lipid oxidation, protein degradation and DNA damage. PGs produced in the COX pathway cause vasodilation, allowing for a greater concentration of eosinophils and other leukocytes at the site of inflammation, thereby amplifying the process. IL-6 has positive feedback on the eosinophils while both TNFα and IL-4 upregulate VCAM-1 for eosinophil recruitment, again amplifying the asthmatic process. TNFα upregulates a number of adhesion molecules involved in leukocyte recruitment (Krishna, Chauhan et al. 1996; Kumar, Dhawan et al. 1998). Meanwhile, IL-3, IL-5 and GM-CSF are important in the activation, differentiation and survival of the eosinophils, with IL-5 being involved in their proliferation and chemotaxis, acting specifically on mature eosinophils (Clutterbuck, Hirst et al. 1989; Sanderson 1992). Leukotrienes released upon eosinophil degranulation also play a significant role in the pathogenesis of asthma. The cLTS, known to be potent bronchoconstrictors also contribute to the development of AHR, causing increased vascular permeability, which allows for migration of the leukocytes between the endothelial cells.

Figure 1.1.2.4: The effects of eosinophil-derived mediators in extrinsic and intrinsic asthma



1.1.2.4.2 Intrinsic Asthma

Eosinophil recruitment, activation and the consequent release of cytotoxic proteins, eicosanoids and various cytokines as well as their effects are the same as described for extrinsic asthma, as illustrated in **figure 1.1.2.4**. The eosinophils therefore contribute largely to the pathogenesis of both forms of asthma. B cell and mast cell involvement have not been proven for intrinsic asthma. However, they cannot be ruled out since as mentioned earlier, viral respiratory tract infection, which may trigger the development of non-atopic asthma, may stimulate IgE production and mast cell degranulation. Levels of eosinophilia are even higher in the intrinsic form, meaning that sufferers experience more severe symptoms and are more difficult to treat.

1.1.2.5 Other Leukocytes

As well as eosinophils, neutrophils and basophils accumulate and contribute to the development of many inflammatory diseases. Activated neutrophils have been found to release chemoattractants involved in T cell and monocyte recruitment to the site of inflammation (Taub, Anver et al. 1996). They are capable of causing tissue damage through the release of oxygen metabolites, proteases and cationic materials. As phagocytes, they can also undergo one of two oxidant-generating pathways; the nitric oxide synthase (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathways (Ward and Lentsch 1999). The hydroxy free radical (OH^-) is produced in both these pathways. It is highly reactive and can cause tissue damage through lipid oxidation, protein degradation and DNA damage. Myeloperoxidase (MPO) is also released from activated neutrophils and contributes to the NADPH

oxidase pathway, converting hydrogen peroxide (H_2O_2) to hypochlorous acid (HOCL), which causes further oxidative tissue injury. Normal tissue can be damaged through these pathways since the action of the resulting free radicals is non-specific. Activated neutrophils can also generate eicosanoids and PAF (Lewis, Austin 1984). All of this would suggest that neutrophils probably contribute to the development of asthma. However, while eosinophils are known to contribute greatly to the development of both forms of the disease, a role for neutrophils has not been proven. While elevated neutrophil levels have been found in the BAL fluid of asthmatics, they may not necessarily be activated. In some cases, MPO levels in the BAL fluid of asthmatic patients have not been found (DuBuske 1995). It is possible that while neutrophils may accumulate in the airways of asthmatics, they may not be actively producing toxic metabolites. MPO levels have however been demonstrated in lung extracts taken from Brown Norway rats in a model of allergic pulmonary inflammation (Schneider and Issekutz 1996). It is possible that the lack of detection of MPO in other assays may be due to different experimental methods. However, the role of neutrophils in asthma remains unclear.

Basophils also contribute to inflammation and are similar to mast cells in their activity. Like mast cells, they degranulate upon binding of antigen-bound IgE to their receptors, causing the release of histamine-containing granules (Benhamou, Gutkind et al. 1990; MacGlashan, Miura et al. 2000). However, they have not been found to contribute to the pathogenesis of asthma (Kay 1991).

1.1.2.6 Bronchohypersensitivity

Bronchohypersensitivity or airway hyper-responsiveness (AHR) refers to excessive bronchial narrowing in response to certain stimuli such as bronchoconstrictors, cold air and methacholine (Blease, Lukacs et al. 2000). As previously mentioned, AHR develops in the late phase of extrinsic asthma as well as in intrinsic asthma and is a result of tissue remodelling and epithelial shedding (Peters, Zangrilli et al. 1998; Varner, Lemanske 2000). It can develop due to excessive shortening of the airway smooth muscle caused either by enhanced smooth muscle responsiveness (Fredberg, Inouye et al. 1997; Gunst, Wu 2001) or by thickening of the airway wall (Moreno, Lisboa et al. 1993). Enhanced airway closure caused by airway wall thickening seems to be the main cause of AHR in an asthmatic model in Balb/C mice (Wagers, Lundblad et al. 2004).

Factors that contribute to this bronchial hyper-reactivity include eicosanoids, endothelin and NO, all of which are produced in the epithelium, as well as histamine and various cytokines. Eosinophils also play a significant role in AHR, through the release of cytotoxic proteins, eicosanoids and various cytokines, as described earlier and illustrated in **figure 1.1.2.4**. Increased levels of eosinophils in asthma has been found to correlate consistently with increase in bronchial hyper-responsiveness (Kraneveld, Folkerts et al. 1997). Similarly, histamine levels in the airways of asthmatics have been found to correlate significantly with airway responsiveness to methacholine (Casale, Wood et al. 1987). There may also be a genetic component involved. In a study of randomly chosen Chinese families, the familial aggregation of AHR to methacholine was examined. A significant correlation was found for parent-

offspring and offspring-offspring pairs, but was not significant in father-mother pairs, thus implying a role for genetic factors in AHR (Hao, Chen et al. 2005).

The epithelium plays a particularly large role in AHR. It forms not only a physical barrier but also a metabolic barrier against bacteria, viruses and inflammatory mediators (Asano, Chee et al. 1994). Epithelial derived relaxing factors such as PGE2 and NO relax the smooth muscle. Tracheas lacking the epithelial layer have been found to contract in response to certain endogenous mediators whereas preparations with an intact epithelium have been found to relax following the same treatment (Folkerts and Nijkamp 1998). This would suggest that epithelial shedding and therefore removal of these relaxing factors may contribute further to AHR. Neutral endopeptidase, an enzyme contained in the epithelium is capable of inactivating bronchoconstrictors such as endothelin, which is also produced from epithelial cells. Endothelin may be capable of causing airway remodelling by inducing subepithelial fibrosis (Krishna, Chauhan et al. 1996). Levels of the ET-1 gene have been found to be significantly enhanced in sensitised Brown Norway rats in the early phase compared to controls, suggesting their involvement in bronchoconstriction (Sanchez-Cifuentes, Rubio et al. 1998). Furthermore, the ETA and ETB receptors have been found in the lung and both have been shown to mediate airway smooth muscle contraction in humans and animals (Goldie, Knott et al. 1996). Epithelial shedding and the inactivation of neutral endopeptidase would allow such agents to act, thus removing an important defensive barrier of the epithelium.

As discussed earlier, this is a possible mode of action for the eosinophil derived proteins. Through inactivating this enzyme, they could allow endothelin to contribute

directly so subepithelial fibrosis. However, the mechanism of action of the eosinophils in the development of AHR is not fully understood. It has been suggested that airway eosinophilia and therefore the development of AHR involves both IL-5 and the chemokine eotaxin. IL-5 is of particular importance since it is the main eosinophil active cytokine, contributing not only to their growth, differentiation and survival but also acting as an activator and primer. Eotaxin may also be involved in eosinophil chemotaxis and activation through binding to the eosinophil-specific CCR-3 receptor (Kraneveld, Folkerts et al. 1997). Furthermore, eotaxin levels have been found to correlate well with elevated eosinophil levels in the sputum of asthmatics while antibodies directed against eotaxin have significantly reduced eosinophil infiltration and reduced AHR in numerous animal models of asthma (Gonzalo, Lloyd et al. 1996; Yamada, Yamaguchi et al. 2000).

NO, also produced from the epithelium can cause epithelial damage. Increased levels of NO have been found in asthmatic individuals and in animal models of asthma (Kharitonov, Yates et al. 1994; Massaro, Gaston et al. 1995; Mehta, Lilly et al. 1997). In mice with a targeted deletion of type 1 neural NOS, a significant decrease in airway responsiveness to methacholine was found compared to wildtype mice. This would suggest that the neural NOS-derived NO contributes to this hyper-responsiveness (De Sanctis, Mehta et al. 1997). It can also be converted to the $OONO^-$ anion, which causes further tissue damage through protein degradation, lipid oxidation and DNA damage (Patel and Block 1986). Vasodilation caused by NO would allow for a greater concentration of eosinophils and other leukocytes to accumulate, contributing further to airway hypersensitivity.

IL1β, IL-6 and GM-CSF are among the cytokines produced from the epithelium and all contribute either directly or indirectly to bronchohypersensitivity. IL1β is capable of increasing the activity of the PLA_2 and COX2 enzymes, thus increasing eicosanoid and PAF production. As already discussed, these products cause vasodilation, increased vascular permeability and increased leukocyte adherance, all of which contributes to leukocyte recruitment (Krishna, Chauhan et al. 1996). IL-6 has an obligatory role in IgE production from B cells (Vercelli, Jabara et al. 1989). This will stimulate mast cell degranulation and further eicosanoid production as well as having positive feedback on eosinophils. GM-CSF is one of the eosinophil-active cytokines, contributing to their differentiation, activation and survival.

TNFα is another cytokine produced in epithelial cells as well as in mast cells, eosinophils, macrophages, T cells and B cells. It has been implicated in the development of AHR in a number of ways and may act through altering the contractile properties of the airway smooth muscle (Amrani, Chen et al. 2000). It semms to have a proliferating effect on fibroblasts and might therefore contribute directly to AHR (Galli and Costa 1995). It is also capable along with IL1β and IFNγ of inducing iNOS, thereby leading to increased NO production. NO contributes further to AHR through causing direct tissue damage and through formation of the peroxynitrite anion as described earlier. TNFα upregulates the endothelial adhesion molecules necessary for leukocyte recruitment. These include VCAM-1, which will facilitate eosinophil recruitment, therefore contributing to AHR. It has also been found to stimulate ICAM-1 expression as well as cytokine and chemokine secretion in airway smooth muscle (Johnson, Knox 1997). In addition to indirectly recruiting eosinophils by upregulating endothelial adhesion molecules, TNFα upregulates IL-5

production in T cells, which is the main eosinophil-active cytokine, contributing to their chemotaxis, maturation and activation (Kovacs 1991; Galli and Costa 1995). Inhibition of this cytokine has been found to completely abolish bronchial hyper-responsiveness in a sensitised guinea pig model (Renzetti, Paciorek et al. 1996) while treatment with TNFα has caused hyper-responsiveness to many contractile agonists in airway smooth muscle both *in vivo* and *in vitro* (Amrani, Panettieri et al. 1998). Acetylcholine-induced contractile responses have also been enchanced with 30 minutes TNFα treatment due to increased calcium sensitivity of the contractile elements in bovine tracheal smooth muscle (Nakatani, Nishimura et al. 2000).

As well as eotaxin, a number of other chemokines have also been implicated in the development of AHR. Their involvement may be linked to leukocyte recruitment and activation, collagen deposition and fibrosis (Boulet, Chakir et al. 1998). Briefly, chemokines can be divided into four supergene families based on the number and arrangement of conserved amino acid sequences at the N terminus. These are the CC, CXC, C and CX_3C families. Like eotaxin, RANTES is a CC chemokine and can therefore target many cell types including macrophages, eosinophils and basophils (Blease, Lukacs et al. 2000). Both of these chemokines, as well as macrophage chemoattractant protein-3 (MCP-3), macrophage inflammatory protein-1α (MIP-1α) and MCP-4 contribute to eosinophil migration and therefore also contribute indirectly to AHR (Lukacs, Standiford et al. 1996; Stafford, Forsythe et al. 1997).

1.1.3 Asthma Treatments

A number of drugs are currently on the market for asthmatic patients. While they may have differing modes of action, a common problem is the side effects that can result when administered at high doses. Thus, in many cases the therapeutic margin is narrow and the anti-asthmatic effects of the compounds at these low doses are often relatively weak. The search for new treatments is ongoing and is particularly important since although the prevalence and mortality rates for asthma may have stabilised or even declined in countries such as the United Kingdom, rates are still increasing in many countries (Sidebotham, Roche 2003; Pallapies 2006). Therapy for asthma has evolved in the past 10 years, with a greater emphasis placed on anti-inflammatory treatment as opposed to the use of bronchodilators (Lazarus 1998). This is due to the improved understanding of its pathogenesis and its recognition as an inflammatory disease. A suitable anti-asthmatic drug would be one which has good efficacy, relatively little toxicity and ease of administration for the patient.

1.1.3.1 Corticosteroids

Inhaled corticosteroids are largely recognised as being the only effective treatment for intrinsic asthmatics (Sheffer 1991). They have a wide range of effects due to their ability to either induce or inhibit the transcription of various genes. For instance, they have been shown to inhibit eicosanoid production both *in vivo* and *in vitro* in many cells including fibroblasts and macrophages (Fuller, Kelsey et al. 1984; Raz, Wyche et al. 1989). This effect may be due to the induction of the transcription of the gene for lipocortin, a protein which inhibits phospholipase A_2 (PLA_2) activity (Davidson,

Dennis et al. 1987). In this way, steroids could inhibit the breakdown of the membrane phospholipids to arachidonic acid (AA) and platelet activating factor (PAF) and prevent formation of the eicosanoids as seen in the early phase of extrinsic asthma. Leukocyte recruitment, as seen in the late phase and also in intrinsic asthma is inhibited due to the upregulation of IκBα (Auphan, Di Donato et al. 1995). This protein inhibits NF-κB, which as previously mentioned is activated by TNFα for upregulation of the endothelial adhesion molecules. TNFα-induced NF-κB activation is largely inhibited by steroids in many cells although this pathway has been found to be insensitive to dexamethasone pretreatment in airway smooth muscle cells (Auphan, Di Donato et al. 1995; Amrani, Lazaar et al. 1999). This may explain why the steroid was unable to inhibit NF-κB-dependant pathways and the subsequent cytokine-induced expression of intracellular cell adhesion molecule-1 (ICAM-1) in this cell type. TNFα is also directly inhibited by steroids along with other cytokines such as IL-3, IL-4, IL-5 and GM-CSF due to the inhibition of their transcription (Schwiebert, Beck et al. 1996). Inhibition of TNFα will not only inhibit leukocyte recruitment but also negatively impact the induction of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2), the upregulation of IL-5 in T cells and the effects on tissue remodelling as caused by the cytokine (Amrani, Chen et al. 2000). Inhibition of IL-4 will inhibit the development of the Th2 response and upregulation of vascular cell adhesion molecule-1 (VCAM-1) for eosinophil recruitment. Meanwhile, IL-3, IL-5 and GM-CSF are the eosinophil-active cytokines. Therefore, leukocyte recruitment and in particular eosinophil recruitment, activation and survival are reduced by steroids due to the inhibition or induction of the transcription of many genes.

Examples of inhaled corticosteroids include beclomethasone, flunisolide, fluticasone and budesonide. All have been shown to have anti-inflammatory properties and are effective anti-asthmatic agents (Laitinen, Haahtela et al. 1992; Duddridge, Ward et al. 1993; Booth, Richmond et al. 1995; McGill, Joseph et al. 1995). However, side effects of steroids include adrenal suppression, growth retardation in children, thinning of skin, osteoporosis, glaucoma, cataracts, hypertension and glucose intolerance (Wasserfallen and Baraniuk 1996; Lazarus 1998). When administered in daily doses of less than 600 to 800μg for adults or 400 to 600μg for children, these side effects are rare. While these low doses are effective in mild sufferers, higher doses may be necessary to alleviate the symptoms of chronic asthmatics. One solution to this problem may be combination therapy. Theophylline is a bronchodilator which acts by inhibiting phosphodiesterase (PDE), therefore preventing cyclic adenosine monophosphate (cAMP) breakdown and relaxing the smooth muscle. Side effects are again a problem at high doses due to the fact that it non-selectively inhibits all the PDE enzymes. However, administration of low doses of budesonide and theophylline have been found to be as effective as a high dose of budesonide (Page 1999). In this way, the efficacy versus toxicity ratio is improved.

The use of inhaled corticosteroids may reduce asthma mortality rates. In Argentina, the crude asthma mortality rate and the rate adjusted for 5 to 34 year olds were significantly reduced from 3.38% and 0.72%, respectively, in the 1980 to 1989 decade to 2.58% and 0.38%, respectively, in the following decade (Neffen, Baena-Cagnani et al. 2005). Meanwhile, there was a 479% increase in sales of inhaled corticosteroids in the country from 1990 to 1999, by far the biggest increase in sales of any anti-asthmatic drugs. The inverse correlation between these sales and both asthma

mortality rates were significant. The use of inhaled corticosteroids has also been found to inversely correlate with asthma mortality rates in other countries (Mormile, Chiappini et al. 1996; Goldman, Rachmiel et al. 2000; Suissa, Ernst et al. 2000).

1.1.3.2 Bronchodilators

As already stated, a greater emphasis is now being placed on anti-inflammatory treatments as opposed to the previously popular bronchodilators. The principal bronchodilators used to treat asthma are the β_2 adrenoceptor agonists. They act through increasing the synthesis of cAMP, thus relaxing the smooth muscle. PDE inhibitors such as the aforementioned theophylline are another type of bronchodilator and act through preventing cAMP breakdown. Salbutamol and salmeterol are examples of widely used β_2 agonists. Salbutamol has a short duration of action and is therefore often used to relieve an acute attack. Conversely, salmeterol has a long duration of action, with the bronchodilatory effect lasting for more than 12 hours, and is often given to prevent an attack (Ball, Brittain et al. 1991).

Another significant difference between these two drugs is the fact that salmeterol, in addition to being a bronchodilator also has significant anti-inflammatory properties. It has been found to inhibit eosinophil recruitment to the lungs in sensitised guinea pigs (Sanjar, Mc Cabe et al. 1991) and has also been found to suppress the development of bronchial hyper-reactivity in asthmatics (Twentyman, Finnerty et al. 1990). This would suggest that the drug may be a suitable alternative to steroids. Some studies suggest that the anti-inflammatory effects of salmeterol may be linked to the inhibition of eosinophil degranulation and/or mobilisation to the bronchial tissue since

serum levels of eosinophil cationic protein (ECP) were reduced in allergen-challenged asthmatics pretreated with the drug, without any change in total blood eosinophil levels (Di Lorenzo, Morici et al. 1995). However, other studies have shown that IL-5-induced superoxide anion (O_2^-) release was significantly reduced by salmeterol but not by salbutamol, while PAF-induced O_2^- release was significantly reduced by both drugs, the former being 20 times more potent. In addition, adherence induced by both PAF and IL-5 were again significantly reduced by salmeterol but not by salbutamol (Ezeamuzie and al-Hage 1998). These results would suggest that the greater anti-inflammatory effects of salmeterol compared to salbutamol may be due to the superior inhibition of both PAF-induced and IL-5-induced O_2^- release, adherence and subsequent eosinophil infiltration, as opposed to the inhibition of eosinophil degranulation. Also, the use of ICI 118551, a potent and selective β_2 adrenoceptor antagonist, had no significant impact on the effects caused by salmeterol but did reverse the effects of salbutamol. This would suggest that the actions of the former and not the latter are independent of the β_2 adrenoceptors.

In any case, salmeterol is a potent anti-inflammatory agent and inhibits eosinophil activity. It is therefore an effective anti-asthmatic drug since eosinophils contribute to the pathogenesis of asthma through the release of cytotoxic proteins such as ECP and major basic protein (MBP), eicosanoids and various cytokines, resulting in epithelial damage and the development of bronchial hyper-reactivity (Laitinen, Heins et al. 1985; Abu-Ghazaleh, Fujisawa et al. 1989; Kroegel, Yukawa et al. 1989). However, tolerance is a problem with chronic use of β_2 agonists (Kleerup 1997).

Theophylline is a PDE inhibitor which is similar to salmeterol in that it is also a bronchodilator with anti-inflammatory effects. IL-5 and GM-CSF are two inflammatory cytokines thought to contribute to granulocyte infiltration by delaying the apoptosis of granulocytes such as eosinophils. Theophylline has been found to significantly increase GM-CSF-induced delayed apoptosis of both eosinophils and neutrophils and also significantly increased the apoptosis of eosinophils as delayed by IL-5, in a dose-dependant manner *in vitro* (Yasui, Hu et al. 1997). The fact that the administration of cAMP-increasing agents such as rolipram actually inhibited granulocyte apoptosis would suggest that the apoptosis induced by theophylline is unrelated to PDE inhibition. These anti-inflammatory effects would be of further benefit in asthma treatment since unnecessary and prolonged survival rates of the granulocytes can cause damage to healthy tissue, while eosinophils are believed to play a large role in bronchial hyper-reactivity.

Theophylline has also been shown to act as an antagonist of the adenosine receptor found on mast cells (Feoktistov, Polosa et al. 1998; Linden, Auchampach et al. 1998). In this way, mast cell degranulation and the release of inflammatory mediators can be prevented. Side effects are a problem due to the non-selective inhibition of all five PDE enzymes (Kleerup 1997). Indeed, theophylline sales have been found to correlate with asthma mortality rates in Argentina (Neffen, Baena-Cagnani et al. 2005). Sales of the drug have gone down by 63% from 1990 to 1999, which along with the increase in sales of inhaled corticosteroids, may contribute to the reduced mortality rates in this country during this time period.

1.1.3.3 Anti-Leukotrienes

Another potential target for asthma treatment is the leukotrienes. As previously mentioned, the cysteinyl leukotrienes (cLTs) are extremely potent bronchoconstrictors (Dahlen, Hedqvist et al. 1980) and also increase vascular permeability (Dahlen, Bjork et al. 1981), thus contributing to leukocyte recruitment. LTB_4 contributes further to leukocyte recruitment through increasing leukocyte adherence (Bochner 1997). In addition, elevated levels of leukotrienes have been found in the blood, urine and bronchoalveolar lavage (BAL) fluid of asthmatic patients (Zakrzewski, Barnes et al. 1985; Taylor, Taylor et al. 1989; Wenzel, Larsen et al. 1990). Therefore, anti-leukotriene treatments may be of benefit for asthmatics.

5-Lipoxygenase activating protein (FLAP) is the enzyme required to activate 5-Lipoxygenase (5-LO), thus allowing 5-LO to interact with arachidonic acid. This leads to leukotriene production as described earlier and illustrated in **figure 1.1.2.1.1.3**. Hence, treatments directed against the leukotrienes include FLAP inhibitors, 5-LO inhibitors such as zileuton and leukotriene receptor antagonists such as zafirlukast. Improved airway function and a reduced need for β–agonist or corticosteroid treatment was found following long term administration of agents such as zileuton (Israel, Rubin et al. 1993; Israel, Cohn et al. 1996) and zafirlukast (Suissa, Dennis et al. 1997) in mild to moderate asthmatics. An immediate improvement in lung function was also found in chronic asthmatics following treatment with most agents (Hui, Barnes 1991; Dahlen, Margolskee et al. 1993).

Tests carried out on zileuton have shown that for effective modulation of leukotriene formation in asthmatics, a compound should be a potent inhibitor and that tissue concentrations should be maintained for the entire treatment period. ABT-761, an N-hydroxyurea containing 5-LO inhibitor has been found to potently inhibit leukotriene formation both *in vivo* and *in vitro*. It was also found to inhibit antigen-induced bronchospasm in guinea pigs and eosinophil recruitment to the lungs in Brown Norway rats. These results further implicate the involvement of the leukotrienes in bronchospasm and eosinophilic inflammation. Therefore, anti-leukotriene treatments are a potential alternative to steroids with the advantage that they can be administered orally. In addition, ABT-761 should be of particular clinical relevance in asthma treatment since it is a more potent and longer lasting drug than even the clinically successful zileuton and zafirlukast (Bell, Harris et al. 1997).

1.1.3.4 Anti-Adhesion Molecule Agents

The influx of inflammatory cells contributes largely to the development of bronchial hyper-reactivity in both extrinsic and intrinsic asthma. As already mentioned, leukocyte recruitment requires the upregulation of a number of adhesion molecules to facilitate the rolling phase and firm adhesion phase, ultimately leading to migration of the leukocytes to the site of inflammation. ICAM-1, VCAM-1, endothelial leukocyte adhesion molecule (ELAM-1) and the selectins are examples of such adhesion molecules and are essential for lymphocyte, eosinophil and neutrophil recruitment. Pretreatment with monoclonal antibodies directed against ICAM-1 has been found to block both eosinophil recruitment and bronchial hyper-reactivity following antigen challenge in a monkey asthmatic model (Wegner, Gundel et al. 1990). Similarly,

pretreatment with a mixture of soluble antigen and antibodies directed against both ICAM-1 and its corresponding T cell adhesion molecule lymphocyte function-associated antigen (LFA-1), resulted in the inhibition of eosinophil infiltration in mice, where antigen-specific IgE production was also prevented (Freeman, Boussiotis et al. 1995). The LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions play a large role in leukocyte recruitment, which would explain why they are so important in the pathogenesis of inflammatory and autoimmune diseases. Antibodies and peptides directed against LFA-1 and VLA-4 have therefore been developed and a greater understanding of how they interact with their corresponding ligands will help in the design of selective modulators of these molecules in the future (Yusuf-Makagiansar, Anderson et al. 2002). The selectins also seem to play a crucial role in eosinophil recruitment since monoclonal antibodies directed against either L-selectin or a combination of both E-selectin and P-selectin almost completely abolished eosinophil recruitment in lipopolysaccharide (LPS)-injected mice (Henriques, Miotla et al. 1996). Therefore, agents directed against these molecules may provide another opportunity for anti-asthmatic treatment.

1.1.3.5 Disease Activated Drugs

Disease activated drugs (DAD) are pro-drugs or combinations of two covalently linked drugs that are activated in inflamed but not normal tissue, due to disease specific mechanisms such as the formation of proteolytic enzymes. Therefore, the formation of the active drug(s) should correlate with the severity of inflammation. Experiments have been carried out to test this concept using a pro-drug of an isoquinoline derivative and also combinations of this PDE IV inhibitor with two

corticosteroids, namely dexamethasone and budesonide (Charpiot, Bitsch et al. 2001). The active form of the PDE IV inhibitor was formed more readily from these new compounds in BAL fluid taken from Brown Norway rats sensitised and challenged to ovalbumin than in sensitised but non-challenged rats. Similarly, methacholine-induced bronchoconstriction was reduced by the DAD in ozone-treated (inhaled ozone induces lung inflammation) but not in ozone-untreated guinea pigs. The fact that the active form of the PDE IV inhibitor successfully reduced bronchoconstriction in both ozone-treated and untreated guinea pigs would suggest that it was formed from the DAD only in the former group of animals. From these results, it would seem that these new compounds are only effective in disease or inflammatory models and are therefore DAD.

A major advantage of this form of treatment is that side effects could be reduced and the therapeutic margin made wider due to the direct correlation between formation of the active drug(s) and disease severity. This would be of great benefit in cases such as the ones mentioned above since PDE inhibitors and corticosteroids both have severe side effects. Indeed, two of the DAD were found to reduce bronchoconstriction in the ozone-treated guinea pig model to the same extent as the active PDE4 inhibitor but without increasing blood pressure (Charpiot, Bitsch et al. 2001). Combination therapy could also allow for the use of drugs with different modes of action, which could in some cases also have a synergistic effect and therefore reduce clinically effective doses.

1.1.3.6 Other Treatments

Natural killer cells may provide another target for asthma treatment. Invariant natural killer T cells have been found to be required for the development of allergen-induced airway hyper-reactivity in mouse models of allergic asthma. Subsequent studies in humans have shown that approximately 60% of the pulmonary $CD4^+$ $CD3^+$ cells in moderate to severe persistent asthmatics are natural killer T cells (Akbari, Faul et al. 2006). These cells comprise only around 0.01% of the total immune cells in the blood of non-asthmatics. This would suggest that they contribute specifically to the pathogenesis of asthma, thus making them a suitable target for treatment. It is believed that these invariant natural killer cells may be recruited by dendritic cells although the exact mode of action is unknown. Further elucidation of the mechanisms involved in the recruitment of these rare cells to the lungs would be beneficial in finding novel treatments.

IL-4 and IL-13 are Th2 cytokines which, as discussed earlier, contribute largely to the pathogenesis of extrinsic asthma. IL-4 is the principal cytokine involved in the stimulation of the Th2 response, isotope switching of the B cells to produce IgE and subsequent mast cell degranulation, as well as contributing to the upregulation of endothelial adhesion molecules needed for leukocyte recruitment (Vercelli, Geha 1992; Herz, Bunikowski et al. 1998). IL-13 is also crucial in the Th2 response, being equally potent to IL-4 for B cell growth and differentiation. It may act to sustain IgE production following IL-4 downregulation since it is produced earlier from Th2 cells and sustained for longer than IL-4 (Robinson 1996; Bousquet, Yssel et al. 1997). Therefore, targets directed against these cytokines should be of benefit for extrinsic

asthmatics. Identification of the genes that induce both IL-4 and IL-13 should hopefully lead to the development of new treatments while several antagonists directed against both cytokines have already been developed (Izuhara, Arima et al. 2002).

Histaglobin is a known anti-allergic agent and is used to treat many allergic diseases including bronchial asthma, allergic rhinitis and atopic dermatitis. When given therapeutically in an *in vivo* mouse allergy model, it was found to reduce serum levels of IgE and IgG1 as well as reducing levels of IL-4 and TNFα, while increasing levels of IFNγ. Meanwhile, prophylactic treatment almost completely abolished responsiveness to ovalbumin and again caused an increase in serum IFNγ levels (Ayoub, Lallouette et al. 2003). These results suggest that histaglobin shifts the Th1/Th2 balance in favour of the Th1 response. It could therefore be of major benefit for extrinsic asthmatics, especially since it can easily be produced at low costs and with no known side effects.

Immunosuppressants such as CycA and tacrolimus may also be effective in asthma therapy. These are explained in **section 1.4**.

1.2 Inflammation

The inflammatory response is triggered by invading pathogens or following tissue injury or trauma. It is designed to protect the host by eliminating infection and repairing damaged tissue. Leukocyte recruitment and macrophage activation are part

of the inflammatory process and are necessary for wound healing (Ward and Lentsch 1999). However, problems arise when this response is suppressed, for example in AIDS, or when it is inappropriately deployed, either acutely as in anaphylaxis, or chronically in diseases such as asthma and rheumatoid arthritis.

Macrophage activation in the acute inflammatory response leads to the production of cytokines such as IL-1, TNFα and IFNγ. All 3 cytokines increase the activity of the COX-2 enzyme (Nakazato, Simonson et al. 1991; Vadas, Pruzanski et al. 1991; Frolich 1997), induce iNOS (Barnes and Liew 1995; Ruetten and Thiemermann 1997) and upregulate endothelial adhesion molecules such as ICAM-1 and ELAM-1 needed for leukocyte recruitment (Thorp, Southern et al. 1992). PLA_2 activity is also increased by IL-1β, thus facilitating the breakdown of the membrane phospholipids to produce AA and PAF (Vadas, Pruzanski et al. 1991). This is the same process as was explained for mast cells in **section 1.1.2.1.1.3**. The end result is the production of leukotrienes in the 5-LO pathway and prostaglandins and thromboxanes in the COX pathway. PAF and the cLTS increase vascular permeability which will facilitate migration of the leukocytes while PAF and LTB_4 increase leukocyte adherence (Bochner 1997). Meanwhile, the PGs cause vasodilatation which will allow for a greater concentration of leukocytes at the site of inflammation (Salmon and Higgs 1987; Miyake, Yamamoto et al. 1993). Thus, macrophage activation and the resulting eicosanoid production in the acute inflammatory response can contribute to leukocyte recruitment to the site of inflammation.

Leukocyte recruitment occurs through the same process as was described for asthma in **section 1.1.2.3**. This involves a rolling phase to slow down the flow rate of the

leukocytes, activation by chemokines, firm adhesion to the endothelial cells and finally migration to the site of inflammation. Non mast cell-derived cytokines that contribute to leukocyte recruitment include IL-1 and IFNγ, which are capable of upregulating endothelial adhesion molecules. IL-6, IL-10 and IL-13 have been shown to have anti-inflammatory activity (Mulligan, Warner et al. 1997). Blockade of any of these 3 cytokines has been found to cause an increase in TNFα and a 50% or greater increase in numbers of neutrophils recruited into the lung in a rat lung inflammatory model (Shanley, Schmal et al. 1995; Shanley, Foreback et al. 1997; Lentsch, Czermak et al. 1999). The elevated neutrophil levels may be explained by the upregulation of ICAM-1 by TNFα (Thorp, Southern et al. 1992).

Tissue injury occurs due to the activation of leukocytes and subsequent release of cytotoxic materials and due to the production of various oxidants from phagocytes. Eosinophil activation results in the release of toxic proteins such as EPO, ECP, MBP and eosinophil-derived neurotoxin (Robinson 1996). As previously mentioned, these cause tissue injury and may be responsible for epithelial shedding and AHR in asthma. Inflammatory cytokines such as TNFα are also produced from eosinophils along with leukotrienes, thus contributing further to inflammation. Neutrophils contribute to T cell and monocyte recruitment to the site of inflammation through the release of chemoattractants (Taub, Anver et al. 1996). Activation of neutrophils results in the release of toxic proteases which cause further tissue damage as well as eicosanoids, again amplifying the inflammatory process. Basophils also play a role, degranulating upon IgE exposure in a similar manner to mast cells and releasing histamine containing granules (Benhamou, Gutkind et al. 1990; MacGlashan, Miura et al. 2000).

As phagocytes, neutrophils, monocytes and macrophages are capable of engulfing and digesting bacteria and damaged tissue. However, they can also cause damage to healthy tissue due to one of two oxidant generating pathways. These are the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and iNOS pathways (Rosen, Pou et al. 1995; Royall, Kooy et al. 1995). In the former pathway, oxidation of NADPH allows for reduction of oxygen to form the superoxide anion (O_2^-). This is then reduced to hydrogen peroxide (H_2O_2) which can be further reduced to form the hydroxy free radical (OH^-) in the presence of a heavy metal in a transition state such as Fe^{2+}. The OH^- radical is the most active oxygen radical and can cause extensive tissue injury. H_2O_2 can also be converted to hypochlorous acid (HOCl), another highly potent and damaging oxidant. This requires the presence of MPO, released from activated neutrophils and a halide such as chloride.

In the iNOS pathway, iNOS is upregulated following cell activation and reacts with L-arginine to produce NO. As mentioned in **section 1.1.2.6**, NO can cause epithelial damage and tissue injury. It can react with and modify tyrosine containing proteins, an effect which may impair their function. Such modifications have often been found at sites of inflammation. It also has a bronchodilatory effect, acting via cGMP, which may be due to its influence on favouring the COX pathway as opposed to the 5-LO pathway. This would result in production of the PGs instead of the more potent bronchoconstrictors, the leukotrienes. NO is converted to the peroxynitrite anion ($OONO^-$), which is highly reactive with thiol groups and can cause protein degradation, lipid oxidation and DNA damage. It can be further converted to OH^-, a highly reactive radical which is also produced in the NADPH oxidase pathway. In this

case however, a heavy metal cation is not required for its production. Nitrite (NO_2^-) and nitrate (NO_3^-) are the ultimate, stable and unreactive products of $OONO^-$.

It is clear that the oxidants produced in these two pathways, including HOCl in the NADPH oxidase pathway, NO and $OONO^-$ in the iNOS pathway and OH^- in both contribute to tissue injury in inflammation. Therefore, inhibition of these pathways would be beneficial as a potential treatment. However, effective specific inhibitors of either pathway are not available. While the iNOS pathway can be inhibited using L-arginine derivatives, therefore affecting the iNOS substrate, their actions are not specific to iNOS. Systemic hypertension can result due to the non-selective inhibition of constitutive NOS, which regulates vascular smooth muscle tone (Ward and Lentsch 1999).

1.3 Th1 and Th2 responses

Following the presentation of an antigen to a naive T helper cell (Th0 cell) by an antigen presenting cell (APC), a Th1 or Th2 response can occur. Factors which determine the polarisation of the T cells include the type and dose of antigen, the type of adjuvant and genetic factors. Very low and very high antigen doses may trigger a Th2 response while moderate antigen doses may cause a Th1 response (Murray 1998). A common concept is that extracellular (e.c.) bacteria induce a Th2 response while intracellular (i.c.) bacteria induce a Th1 response (Lucey, Clerici et al. 1996; Romagnani 1997). Thus, when ovalbumin (OVA) is used in combination with aluminium hydroxide (AlOH) as an adjuvant, a Th2 response should occur whereas using Freunds complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T))

instead of AlOH as an adjuvant should stimulate a Th1 response. This is the concept from which we have attempted to replicate models of extrinsic and intrinsic asthma respectively, in our laboratory. FCA can contain either *Mycobacterium tuberculosis* or *Mycobacterium butyricum* as both are believed to induce a Th1 response (Dannenberg 1991; Kaufmann 1995; Lucey, Clerici et al. 1996; Roman and Moreno 1997; Sinha, Verma et al. 1997; Murray 1998). Meanwhile, Th2 models have been established in many animals including rats, mice and guinea pigs using OVA in combination with AlOH for sensitisation (Hatzelmann, Haefner et al. 1996; Koh, Choi et al. 2001; Wagers, Lundblad et al. 2004; McKay, Leung et al. 2004).

The Th2 or humoral immune response, commonly linked to allergic reactions (Cookson 1999) involves the production of cytokines such as IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF and TNFα. Subsequent isotope switching of the B cells and IgE production occurs, as was outlined earlier for the early phase of extrinsic asthma. The Th1 or cell-mediated immune response involves cytokines such as IL-2, IFNγ, interferon alpha (IFNα), tumour necrosis factor beta (TNFβ) and IL-12 as well as the eosinophil active cytokines (IL-3, IL-5, GM-CSF). In this case, following antigen presentation to the precursor T helper cell (Thp cell), IL-2 is produced, stimulating differentiation to the Th0 phenotype (Seder, Paul 1994; Zhai, Ghobrial et al. 1999) and IL-12 production from the APC. Th0 cells are thought to be capable of producing both IL-4 and IFNγ (Seder, Paul 1994) although their existence is controversial and they may in fact represent a mixture of Th1 and Th2 cells (Zhai, Ghobrial et al. 1999). IL-12 plays an obligatory role in the polarisation of Th0 cells to Th1 cells. It induces IFNγ production from Th0 cells, cytotoxic T cells (Tc1) and natural killer (NK) cells (Chan, Kobayashi et al. 1992; Ohshima, Delespesse 1997;

Trinchieri 2003). It also promotes the maturation and activation of the Th1 and Tc1 cells and plays a role in the proliferation of the T cells and NK cells (Gately, Carvajal et al. 1996; Stern, Magram et al. 1996). Like IL-12, IL-2 stimulates the maturation and activation of the $CD4^+$ Th1 cells and the $CD8^+$ Tc1 cells and IFNγ production. IL-2 also plays a role in macrophage activation (Chu, Field et al. 1992). IFNγ, produced from the Th0 cells will stimulate differentiation to Th1 cells. IFNγ also enhances macrophage activation and is capable of inducing iNOS, increasing COX2 activity, upregulating the endothelial adhesion molecules necessary for leukocyte recruitment as well as upregulating the MHC class I and II molecules, thus increasing antigen processing by the APCs (O'Garra 1998). Th1 cells produce more IFNγ, as well as IFNα, TNFβ, IL-2 and the eosinophil active cytokines.

The two responses (Th1 and Th2) are cross regulated. The three main cytokines involved in cross regulation are IL-4, IL-12 and IFNγ. Receptors for all three cytokines are expressed on Th0 cells. IL-4, the Th2 inducer, is capable of increasing its own expression and upregulating its own receptor in Th0 and Th2 cells. It can also downregulate IL-12 production and receptor expression, thus inhibiting the Th1 response. IL-4 may cause a Th1 to Th2 switch, possibly through activating its own receptor on Th1 cells (Breit, Steinhoff et al. 1996; Murphy 1998). IL-12 cannot directly affect IL-4 production. However, it can induce IFNγ which downregulates IL-4 production and receptor expression (Paludan 1998). Thus, cytokines produced in the Th2 response can inhibit the Th1 response and vice versa. The development of either a Th1 or Th2 response depends on the ratio of IL-4 to IFNγ and IL-12. IFNγ is produced earlier from the Th0 cells and sustained for longer than IL-4. However, IL-4

is dominant over both these cytokines, meaning that if enough IL-4 is present, a Th2 response will occur (O'Garra 1998).

Because these two responses are cross regulated, a reduced Th1 response might cause an elevated Th2 response. This would correlate with the hygiene hypothesis which suggests that Th2 mediated allergies are increasing due to a cleaner environment with reduced Th1 responses caused by fewer bacteria and viruses (Folkerts, Walzl et al. 2000; Umetsu, McIntire et al. 2002). Therefore, increasing the Th1 response with i.c. bacteria could be beneficial in treating allergic asthma. In experiments performed by Smit and co-workers, Mycobacterium vaccae was administered in a murine model of allergic asthma and was effective at reducing the Th2 response, as measured by levels of eosinophilia in the BAL fluid and airway hyper-responsiveness (AHR) to methacholine (Smit, Van Loveren et al. 2003). These effects were seen whether the Mycobacterium was administered during sensitisation or during challenging. This would suggest that the vaccae can not only suppress already established allergic asthma but may also be useful in suppressing the induction of the disease. However, it does not necessarily correlate with the hygiene hypothesis since IFNγ levels were not elevated in the BAL fluid. It may act through increasing levels of IL-10 and TGFβ (Zuany-Amorim, Sawicka et al. 2002) although its exact mode of action is unknown. In other experiments, statins, known to have anti-inflammatory activity in animal models of Th1-predominant diseases such as rheumatoid arthritis and multiple sclerosis, were found to reduce the Th2 response in a similar model of allergic asthma. In this case, simvastatin, capable of inhibiting the LFA-1/ICAM-1 interaction, reduced levels of BAL eosinophilia but also reduced IFNγ levels, as well as levels of IL-4, IL-5 and IL-6 in thoracic lymph nodes *in vitro* (McKay, Leung et al. 2004). This

again suggests that the reduced Th2 response is not caused by an elevated Th1 response. The fact that autoimmune diseases characterised by Th1 responses are, like allergic diseases, on the increase also opposes the Th1/Th2 misbalance theory of the hygiene hypothesis (Rook 2000).

1.4 Immunosuppressants

Immunosuppressants usually act in the induction phase of the immune response, inhibiting lymphocyte proliferation. They are used to treat autoimmune disease, to prevent or treat transplant rejection and are also used in asthma therapy. There are five main categories, which are distinguished based on their mode of action. They can act either by inhibiting the synthesis or action of IL-2, by inhibiting the transcription of genes for various cytokines, through cytotoxic effects, by inhibiting purine or pyrimidine synthesis or through blocking T cell signalling. Cyclosporin A (CycA) and tacrolimus (or FK506) are two immunosuppressants that have been tested in the methylated bovine serum albumin (mBSA) model, the sheep red blood cell (SRBC) model, the oxazolone contact hypersensitivity (CHS) model and the mouse bronchoalveolar lavage (BAL) model for this study. Both of these drugs act through inhibition of IL-2 synthesis. As explained in **section 1.3**, IL-2 stimulates IL-12 production from antigen presenting cells, which will lead to Th1 cell differentiation. Both of these cytokines play a crucial role in the maturation and activation of the $CD4^+$ Th1 cells and the $CD8^+$ Cytotoxic T cells (Gately, Carvajal et al. 1996; Stern, Magram et al. 1996) as well as stimulating IFNγ production (Chan, Kobayashi et al. 1992; Ohshima, Delespesse 1997; Trinchieri 2003). IL-2 also contributes to macrophage activation. Therefore, inhibition of IL-2 as caused by both of these drugs

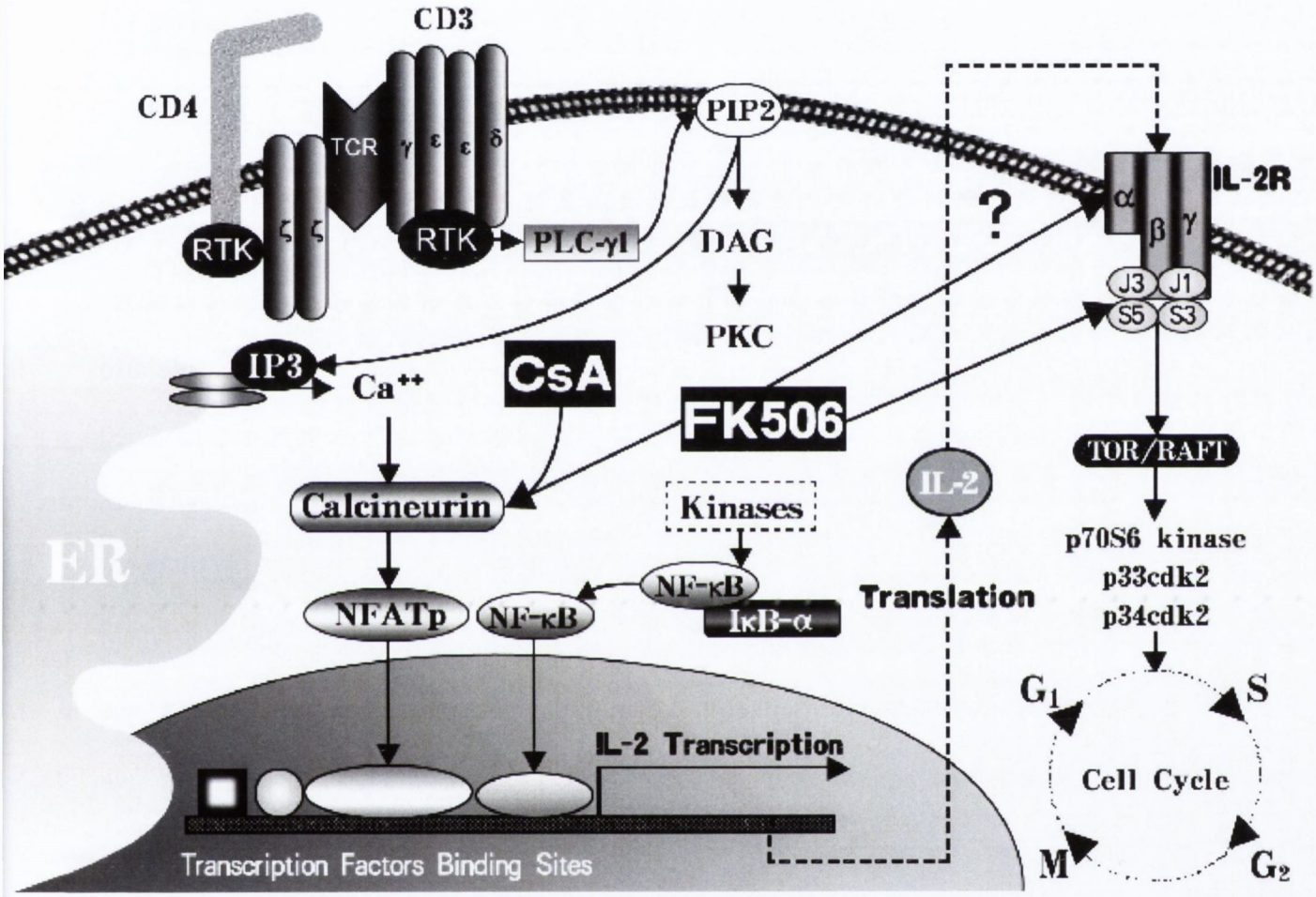
would have a large negative impact on T cell proliferation, the Th1 response and cytotoxic T cells.

The primary mode of action of CycA is the inhibition of IL-2 gene transcription. CycA binds to its corresponding immunophilin (a protein that acts as a receptor for immunosuppressants) known as cyclophilin and the resulting complex inhibits calcineurin, a phosphatase that is essential for transcription of the IL-2 gene. Nephrotoxicity, hepatotoxicity and hypertension are among the side effects of CycA (Dusting, Akita et al. 1999). Nephrotoxicity is the most common and most serious side effect and limits its suitability for immunosuppressive therapy. As mentioned, tacrolimus belongs to the same group of immunosuppressants. It is similar to CycA in that it inhibits IL-2 transcription, again by binding to and inhibiting calcineurin (Clipstone, Crabtree 1992; Clipstone, Ho et al. 1996) although the immunophilin that it binds to is FK-binding protein (FKBP) (Jiang et al. 1997).

The mode of action of both of these drugs is illustrated in **figure 1.4**. T cell activation involves binding of the T Cell Receptor-CD3 complex (T Cell Receptor; TCR) to the antigen-major histocompatibility (MHC) class 2 complex. The resulting pathway involves the activation of PLCγ which catalyses the hydrolysis of PIP_2 to IP_3 and DAG. This causes an increase in i.c. Ca^{2+} and activation of PKC, as already described for mast cell degranulation and bronchoconstriction. The calcium/calmodulin complex is then formed and is essential for calcineurin activation (Almawi and Melemedjian 2000). Calcineurin activates various transcription factors including nuclear factor of activated T cells (NF-AT), which stimulates IL-2 gene transcription. Therefore, CycA and tacrolimus can indirectly inhibit IL-2 synthesis and T cell

proliferation when the drug/immunophilin complex binds to and inhibits calcineurin (Halloran 1996).

Figure 1.4: Effects of CycA and tacrolimus on T cell activation



[Adapted from (Almawi and Melemedjian 2000)]

Jurkat T cells expressing constitutively active NF-AT and MEKK1 have been found to be resistant to the effects of both drugs while those expressing constitutively active NF-AT alone remained sensitive to both agents (Matsuda, Shibasaki et al. 2000). This would suggest that both CycA and tacrolimus act not only by inhibiting the

calcineurin-NF-AT pathway but also by inhibiting the calcineurin-independent JNK and p38 pathways. These calcineurin-independent pathways seem to be essential for cytokine production in T cell activation (Takehana, Sato et al. 1999). Tacrolimus is active at lower concentrations than CycA and has similar side effects although they are not as severe. This may be due to the fact that CycA has been found to inhibit NOS, which may cause hypertension and nephrotoxicity while tacrolimus has not (Dusting, Akita et al. 1999).

However, the actions of tacrolimus are not restricted to inhibition of calcineurin and the NF-AT pathway. It has been found to inhibit IL-2-induced IL-5 synthesis in $CD4^+$ T cells (Mori, Suko et al. 1997). This was not the case for CycA, which while also capable of inhibiting cytokine production has very little impact on cytokine stimulated T cell activation. Due to its broader range of actions, tacrolimus has been used more successfully than CycA in renal allograft patients (Rostaing, Puyoo et al. 1999; Vanrenterghem 1999). Acute and chronic rejection episodes following renal transplantation were more suppressed following tacrolimus treatment than CycA treatment and contained lower levels of TGFβ, which may contribute to progressive scarring (Miyagi, Muramatsu et al. 2002). While its exact mode of action remains unclear, it may act to inhibit the actions of IL-2 by downregulating the IL-2 receptor, as shown in **figure 1.4**.

Both immunosuppressants may be effective in asthma treatment. The inhibition of IL-2 and the Th1 response, as caused by both drugs, would suggest that they may be suitable for treating intrinsic asthma. However, the frequent and severe side effects of CycA, in particular nephrotoxicity and hypertension, would again limit its suitability.

Tacrolimus may be more suitable since, as already mentioned, it is active at lower concentrations than CycA with less severe side effects and a broader range of actions. It is thought to increase adenosine release, which acts through the cAMP pathway to dampen inflammation and may therefore be beneficial in treating non-resolving inflammatory disorders such as asthma, arthritis and bronchitis. However, continuosly high levels of adenosine have been suggested to contribute to the development of asthma and this effect of tacrolimus may therefore limit its suitability for treatment (Gilroy, Lawrence et al. 2004).

1.5 Delayed Type Hypersensitivity

DTH is a cell-mediated immune response which is protective against intracellular (i.c.) bacteria, fungi and some viruses. However when inappropriately deployed, it can cause extensive tissue damage in diseases such as rheumatoid arthritis (Henderson, Pettipher et al. 1987) and allograft rejection (Romagnani 1997). Sensitisation to a particular antigen (e.g. *Mycobacterium tuberculosis*) takes place 1 to 2 weeks after first exposure while subsequent exposure to the antigen then stimulates what is thought to be a Th1 response (Lucey, Clerici et al. 1996). This effector phase occurs within 24 hours, peaking after 2 to 3 days. The result is an influx of inflammatory cells, especially macrophages which are the main effector cells in the DTH response. Th1 cells have been proposed to be the inducer of this type of hypersensitivity since the resulting IFNγ production would activate macrophages (Mosmann, Moore 1991). However in some cases, DTH has been induced by a Th2 response (Muller, Jaunin et

al. 1993). Models of DTH are often used to assess T cell mediated immune responses *in vivo*.

Cytokines involved include IL-2, IL-3, GM-CSF, TNFα and IFNγ. The Th1 pathway which is thought to be involved in DTH was already discussed in **section 1.3**. IL-2 is crucial for the development of the Th1 response. It potently induces IFNγ which enhances macrophage activation (Chu, Field et al. 1992). Anti-IL-2 receptor antibodies administered during the sensitisation phase have been shown to suppress DTH (Kelley, Naor et al. 1986). IL-3 and GM-CSF induce localised hematopoiesis of monocytes and neutrophils while GM-CSF is chemotactic for both monocytes and granulocytes (Wang, Colella et al. 1987). IFNγ and TNFα are of particular importance in DTH. IFNγ activates macrophages and natural killer (NK) cells (Schultz, Kleinschmidt 1983) and like TNFα, it upregulates the endothelial adhesion molecules necessary for recruitment of macrophages and other leukocytes. It is the dominant cytokine in DTH. High levels of IFNγ have been found at the site of antigen injection in models of DTH (Muller, Kropfe et al. 1993). Moreover, anti-IFNγ has reduced footpad swelling induced by Th1 clones by 55% in Balb/C and CBA/J mice (Fong and Mosmann 1989). TNFα can induce a DTH reaction in humans and has been found to be essential for an effective DTH response (Hernandez-Pando and Rook 1994). These two cytokines act synergistically to upregulate the adhesion molecules and MHC molecules, thus increasing antigen processing by the APCs.

IL-12, produced from the APCs is also significant in cell-mediated immunity through the induction of IFNγ from NK cells and T cells and also enhances the cytotoxic activity of NK cells (Stern, Magram et al. 1996). Another cytokine which may be

involved is IL-16. It has been shown to upregulate the IL-2 receptor α chain as well as major histocompatibility complex class II molecules on T cells (Cruikshank, Center et al. 1994). This would facilitate the actions of IL-2 in DTH as already described and increase antigen processing by the APCs. IL-16 levels have been found in infiltrating cells and epithelial cells in DTH footpads as measured by immunohistochemical analysis as well as ELISA and western blot analysis of footpad extracts (Yoshimoto, Wang et al. 2000). Meanwhile, chemokines such as IL-8, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α) and macrophage migration inhibitory factor (MIF) have been shown to be involved in leukocyte recruitment to the DTH reaction site (Larsen, Thomsen et al. 1995; Bernhagen, Bacher et al. 1996; Rand, Warren et al. 1996; Doyle, Murphy et al. 1997).

There are four types of allergic reaction. Type I reactions involve the production of IgE in response to harmless material such as grass or pollen. Type II reactions occur due to antibody and complement mediated cytotoxicity directed against host cells. An example of this would be incompatible blood transfusions. Type three reactions involve complement activation by the antigen-antibody complex and have been implicated in lupus erythematosus. Finally, there are type IV reactions, also known as DTH responses (Gell and Coombs 1968). As already mentioned, these involve the influx of inflammatory cells caused by T cell activation (Poulter, Seymour et al. 1982). The tuberculin DTH reaction induced by intradermal (i.d.) injection of *Mycobacterium tuberculosis* primarily involves macrophages and basophils in humans and mice. In contrast, neutrophils have been found to be the most prominent infiltrate in guinea pigs in this model of DTH (Waksman 1978; Dannenberg 1991). Treatment of rats with anti-rat neutrophil antiserum has been found to reduce

subsequent development of chronic DTH, again implicating the significance of neutrophils in this response (Kudo, Yamashita et al. 1993). Through the release of chemotactic factors such as LTB_4, neutrophils are believed to contribute to the influx of inflammatory cells including monocytes and more neutrophils to the site of inflammation (Lewis, Austin 1984).

Graft versus host disease has been identified as a DTH response with a large monocyte presence, similar to the tuberculin reaction (Medawar 1944). Autoimmune diseases such as adrenalitis and pernicious anaemia are very similar and also have a large monocyte infiltrate (Waksman 1978). There are two distinct forms of leprosy; tubercoloid and lepromatous leprosy. The former is characteristic of a Th1 response with a strong DTH reaction and activated macrophages and T cells. Conversely, lepromatous leprosy is characteristic of a Th2 response with a suppressed DTH reaction and raised antibody levels (Stern, Magram et al. 1996; Singh 1998). Factors which determine a DTH reaction have been recognised as the involvement of T cells, T cell infiltration in the resulting lesion, a lack of sensitisation in thymic aplasia patients and ablation of sensitisation following anti-lymphocyte serum treatment (Waksman 1978).

1.6 History and Structures of Novel Compounds

The compounds tested in a number of animal models throughout this thesis originate from the fern *Pteridium aquilinum*. This fern, also known as bracken fern, has been shown to have carcinogenic properties, which may be due to the presence of the

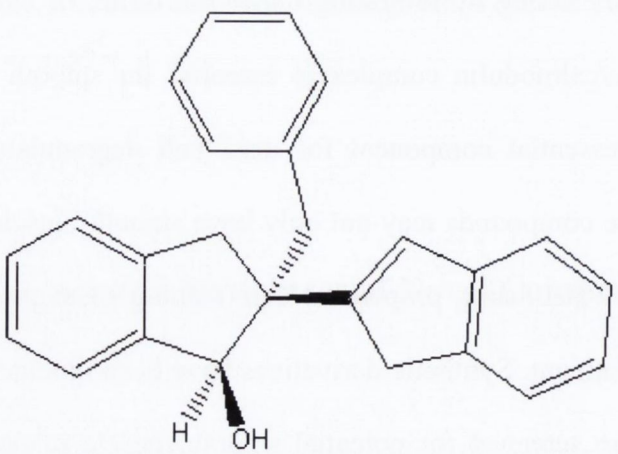
known carcinogen ptaquiloside (Potter, Baird 2000; Schmidt, Rasmussen et al. 2005). However, active glucosides have also been isolated from a toxic fraction of bracken fern by partition column chromatography on Sephadex G-25 and have been shown to enhance release of histamine from rat peritoneal mast cells (Saito, Kirihara et al. 1984; Saito, Mochizuki 1986). A number of naturally occurring products from this fern possess smooth muscle relaxant properties and these include pterosin Z and acetylpterosin Z. These natural products have previously been tested in our laboratory and have been found to inhibit calcium-induced contractions in potassium-depolarised smooth muscle from guinea pig ileum. Pterosin Z was found to be particularly potent with greater than 100 times inhibitory activity compared to other natural compounds such as onitin, onotisin and otninoside (Sheridan, Frankish et al. 1999b). An extensive range of analogues of natural pterosins have been synthesised using modified Heck coupling and several of these compounds have shown significant smooth muscle relaxant activity (Sheridan, Frankish et al. 1999a).

Although the mode of action of these compounds is not yet fully understood, it is likely that they are acting by inhibiting either the influx of calcium or calmodulin since the calcium/calmodulin complex is essential for smooth muscle contraction. This is also an essential component for mast cell degranulation. Therefore, it is possible that these compounds may not only have smooth muscle relaxant properties but also mast cell stabilising properties, thus making them potentially suitable for anti-asthmatic treatment. Synthetic derivatives have been obtained from these natural products and were screened for potential smooth muscle relaxant and/or mast cell stabilising properties. A number of these compounds have been shown to inhibit compound 48/80-stimulated histamine release from rat peritoneal mast cells

(Frankish, Farrell et al. 2004). It is from these tests that 3C8, 3C9 and other compounds have been selected for further testing in this thesis. The chemistry and structures of all novel compounds as well as those of CycA and tacrolimus are shown in **figures 1.6 (a)-(j)**. 3C8 [**figure 1.6 (a)**] and 3C9 [**figure 1.6 (b)**] are a pair of diastereoisomers.

In previous experiments in our laboratory, some of these novel compounds have shown a partial inhibitory effect against the Th1 response, through inhibiting IL-2 but were not found to inhibit TNFα. 3C8 has, for example, been found to inhibit IL-2 release from Jurkat cells (Frankish, Sheridan unpublished data). It would therefore be interesting to examine these compounds in models of DTH to further explore these effects. As mentioned earlier, there may be a link between DTH and intrinsic asthma, meaning that compounds that are effective at inhibiting DTH may also be effective against this variant of the disease.

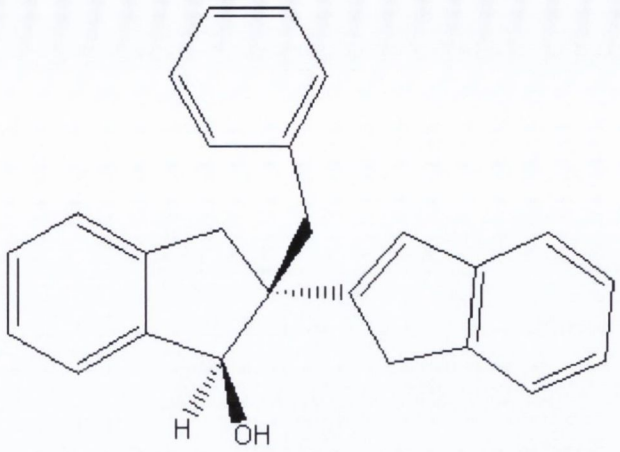
Figure 1.6 (a): The chemical structure and properties of compound 3C8



$C_{25}H_{22}O$; 2-benzyl-2,3-dihydro-1H,1'H-2,2'-biinden-1-ol.

Molecular Weight: 338.44. Melting Point: 609.19K.

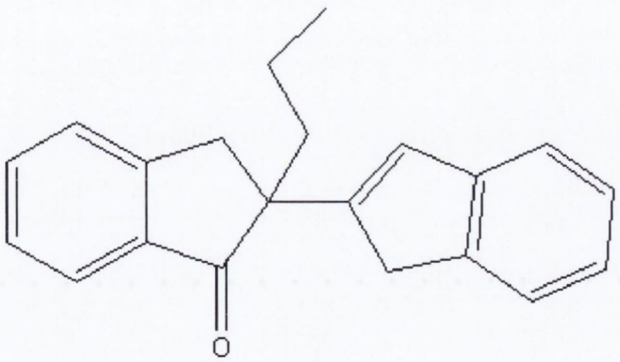
Figure 1.6 (b): The chemical structure and properties of compound 3C9



$C_{25}H_{22}O$; 2-benzyl-2,3-dihydro-1H,1'H-2,2'-biinden-1-ol.

Molecular Weight: 338.44. Melting Point: 609.19K.

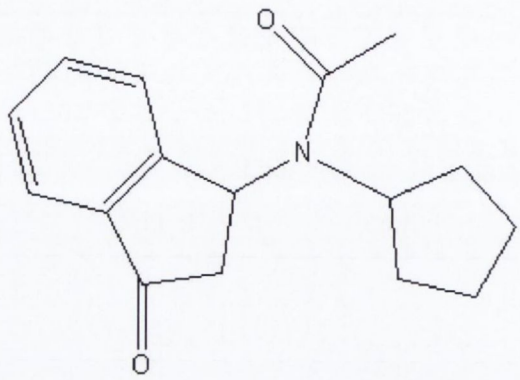
Figure 1.6 (c): The chemical structure and properties of compound 3C4



$C_{21}H_{20}O$; 2-(2-indenyl)-2-propylindan-1-one.

Molecular Weight: 288.38. Melting Point: 549.33K.

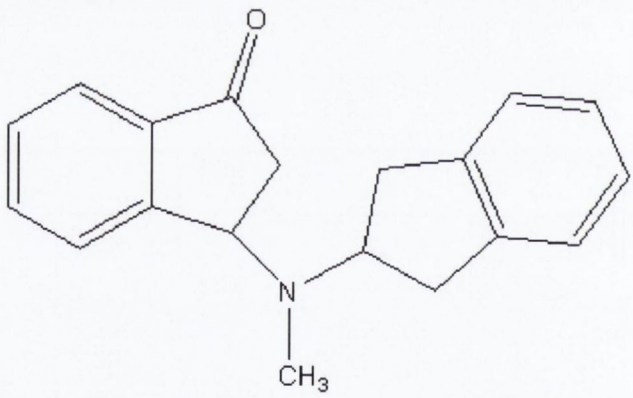
Figure 1.6 (d): The chemical structure and properties of compound 7C9



$C_{16}H_{19}NO_2$; N-cyclopentyl-N-3-indan-1-onyl ethanamide.

Molecular Weight: 257.33. Melting Point: 487.98K.

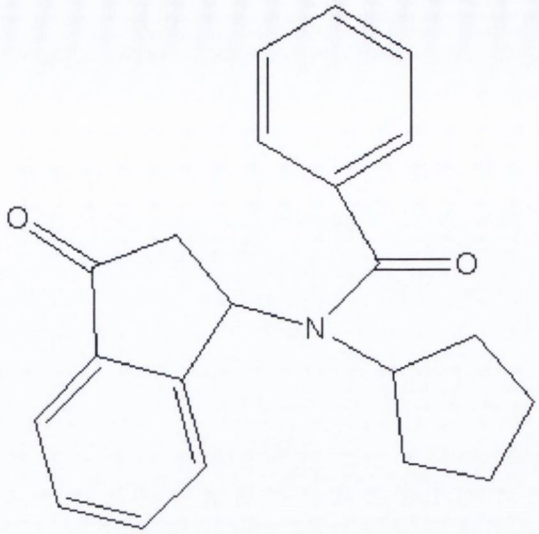
Figure 1.6 (e): The chemical structure and properties of compound 6C6



$C_{19}H_{19}NO$; 3-[2,3-dihydro-1H-inden-2-yl(methyl)amino]indane-1-one.

Molecular Weight: 277.36. Melting Point: 517.84K.

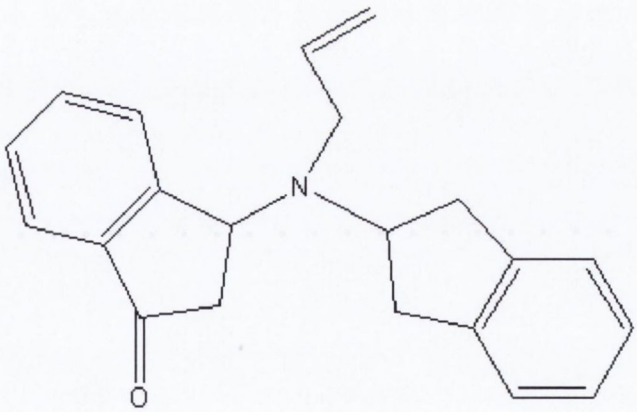
Figure 1.6 (f): The chemical structure and properties of compound 7C17



$C_{21}H_{21}NO_2$; N-Cyclopentyl-N-(3-oxo-2,3-dihydro-1H-inden-1-yl)benzamide.

Molecular Weight: 319.4. Melting Point: 570.75K.

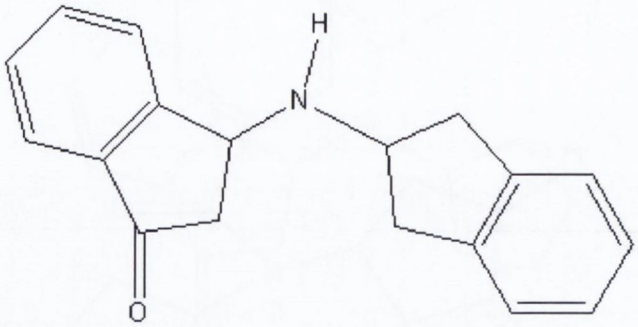
Figure 1.6 (g): The chemical structure and properties of compound 6C7



$C_{21}H_{21}NO$; 3-(N-prop-2-enyl-N-2-indanylamino)-indan-1-one.

Molecular Weight: 303.4. Melting Point: 538.62K.

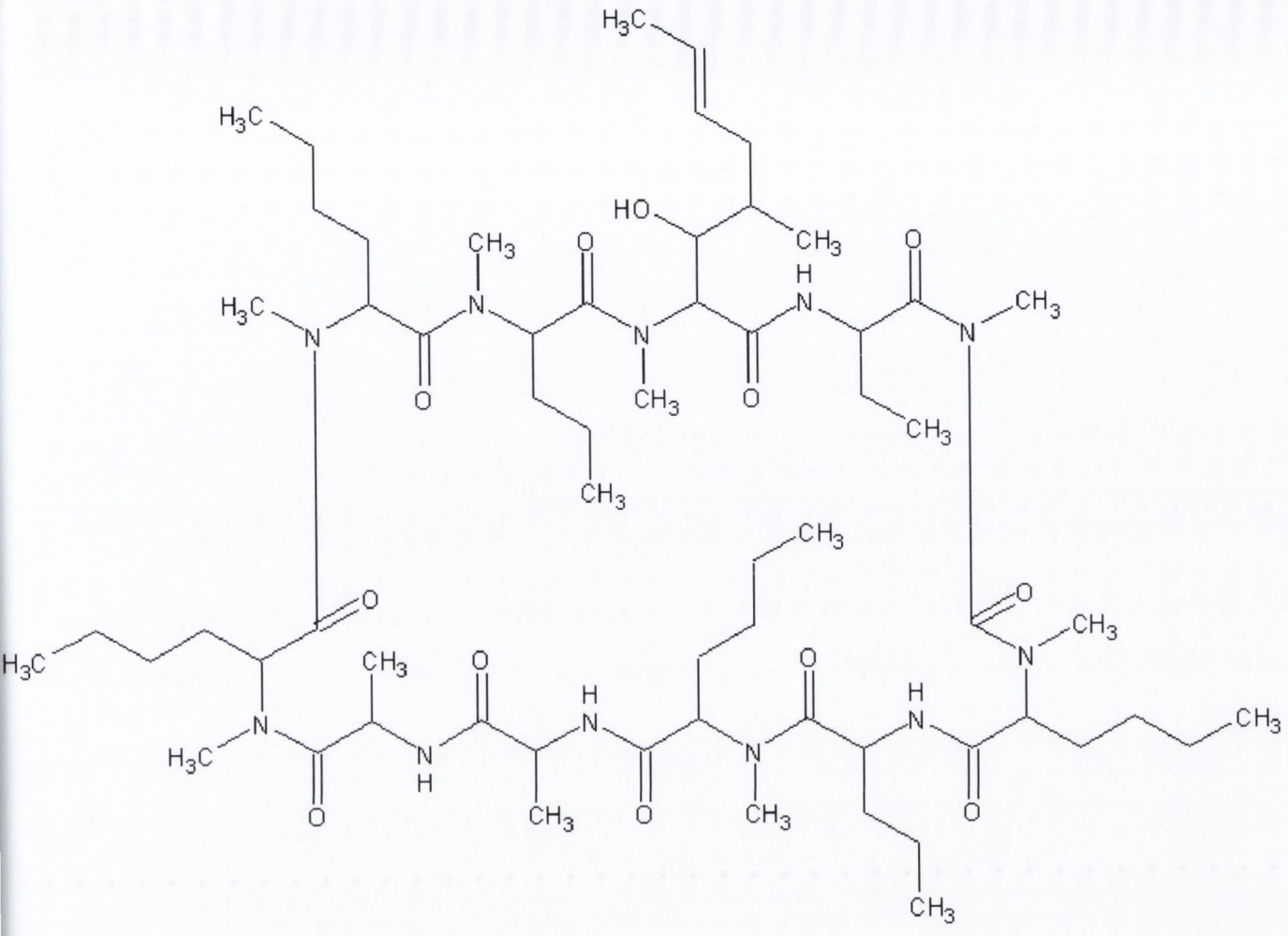
Figure 1.6 (h): The chemical structure and properties of compound 5C4



$C_{18}H_{17}NO$; 3-(N-1-indanylamino)-indan-1-ol.

Molecular Weight: 263.33. Melting Point: 526.76K.

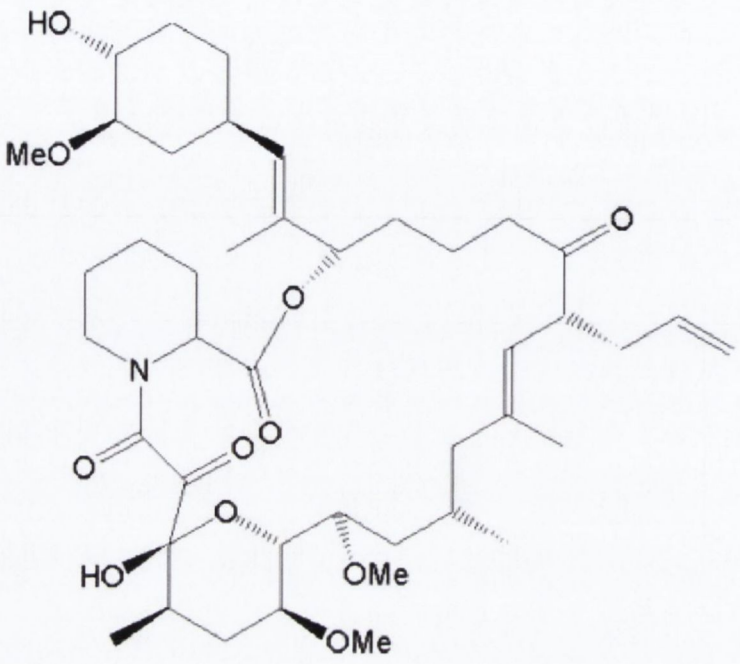
Figure 1.6 (i): The chemical structure and properties of CycA



$C_{62}H_{111}N_{11}O_{12}$; Cyclosporin A.

Molecular Weight: 1202.64. Melting Point: 148-151°C.

Figure 1.6 (j): The chemical structure and properties of tacrolimus



$C_{44}H_{69}NO_{12}H_2O$; Tacrolimus (FK506).

Molecular Weight: 822.05. Melting Point: 126-130° C.

1.7 Aims

The aims of this study were to examine the effects of various current and novel compounds on models of asthma and DTH, with particular focus on 3C8 and 3C9. Some of these novel compounds have been found to be effective in inflammatory models such as the carrageenin induced rat paw oedema model and the AA-induced mouse ear oedema model and have shown a partial anti-Th1 effect through the inhibition of IL-2 release but not TNFα (Frankish, Sheridan unpublished data). In addition, many of these compounds have been shown to inhibit compound 48/80-

stimulated histamine release from rat peritoneal mast cells (Frankish, Farrell et al. 2004). Results would hopefully be of benefit in determining the mode(s) of action of these compounds. This is important since most treatments are either relatively ineffective or have severe side effects.

The specific aims of this study were to:

- examine the effects of current and novel compounds on models of DTH.
- set up *in vivo* bronchoalveolar lavage (BAL) asthma models in rats to analyse the effects of certain compounds on rat BAL leukocyte levels, with particular focus on 3C8.
- set up similar *in vivo* BAL asthma models in mice for further analysis of the effects of 3C8 and 3C9 on mouse BAL leukocyte levels.
- examine cytokine, EPO and MPO levels in mouse BAL fluid and lung extracts for a more detailed evaluation of the effects of 3C8 and 3C9.

Chapter 2

Delayed Type Hypersensitivity

Chapter 2: Delayed Type Hypersensitivity

As previously mentioned, some novel compounds have been shown to inhibit elements of the Th1 type response, with reduced levels of IL-2 but not TNFα found. Testing of these compounds in models of delayed type hypersensitivity (DTH) may therefore confirm any such anti-Th1 effects. Due to the possible link between DTH and intrinsic asthma, compounds that are effective in these models may also be effective in treating the intrinsic form of the disease.

2.1: Methylated Bovine Serum Albumin (mBSA) model

2.1.1 Introduction

The mBSA model of DTH is very similar to the tuberculin reaction which involves i.d. injection of killed Mycobacterium. The principle behind it is that pure protein mixed with adjuvant stimulates an immune response. Ovalbumin injected into a tuberculosis tubercule was found to sensitise patients to the protein, an effect which was later replicated using killed Mycobacterium in Freund's adjuvant (Dienes, Schoenheit 1929; Uhr, Savin et al. 1957). In contrast to the tuberculin reaction, intravenous (i.v.) administration of protein prior to immunisation with the protein in adjuvant prevents sensitisation (Jones, Mote 1934). Hence, the reaction is often termed the Jones-Mote hypersensitivity reaction. This refers to DTH induced by injection of protein in adjuvant and involves a greater number of basophils than the

tuberculin reaction. $CD4^+$ and $CD8^+$ cells play a role, the $CD4^+$ cells responding to e.c. bacteria, and the $CD8^+$ cells to i.c. bacteria and viruses (Muller, Kropfe et al. 1993; Powrie, Menon et al. 1993). Both *Mycobacterium tuberculosis* and *Mycobacterium butyricum* are believed to escape macrophage killing and induce DTH in the same manner (Dannenberg 1991; Kaufmann 1995).

The aim of this model of DTH was to examine the effects of CycA, tacrolimus, 3C8, 3C9 and various other compounds on mBSA-induced paw swelling.

2.1.2 Methods

The methylated bovine serum albumin (mBSA) DTH model was set up according to a modified protocol as described by Tarayre and co-workers (Tarayre, Barbara et al. 1990). Modifications to this protocol were the strain of mice, the quantity of mBSA used on challenge and finally the method of measurement of oedema. Tarayre and co-workers used male swiss mice, whereas this study used male CD-1 mice. While the quantity of mBSA used for sensitisation was the same in this study, the quantity used for challenge was less (0.1mg/20µl as opposed to 0.25mg/25µl) and was adequate to induce a DTH response in the hind paw. In the protocol employed by Tarayre and co-workers, DTH was measured by examining changes in paw weight, whereas this study measured oedema by examining changes in paw volume, using a plethysmometer.

CD-1 mice (25-35gms) were anaesthetised with halothane and immunised intradermal (i.d.) with mBSA/Freunds complete adjuvant containing *Mycobacterium butyricum* (FCA(B)) emulsion at four sites (62.5μg/25μl at each site) on the shaved chest on day 1. The mBSA/FCA(B) is prepared by emulsifying equal volumes of mBSA and FCA(B) solutions (the mBSA solution was first prepared in sterile isotonic saline at a concentration of 5mg/mL). On days 8 and 9, mice were dosed intraperitoneal (i.p.) with either 1% carboxymethylcellulose (CMC), CycA or a novel compound in 1% CMC. The reason this route of administration was chosen for all animal models was because non-polar compounds, which are insoluble in water, were being tested. This means they could not be inhaled and they may not necessarily be orally active. 2 hours after the second i.p. dose (day 9), anaesthetised mice were challenged, by injecting mBSA in saline subcutaneous (s.c.) in the dorsal surface of the right hind paw and s.c. in the left hind paw with saline alone (20μl each injection). 24 hours later, mice were sacrificed by cervical dislocation, and the swelling of each paw was measured in triplicate with a plethysmometer.

The plethysmometer is a microcontrolled volume meter, specially designed for accurate measurement of rat and mouse paw swelling. It consists of a water filled Perspex cell into which the paw is dipped. A transducer of original design records small differences in water level, caused by volume displacement. The digital read-out shows the exact volume of the paw. Paw volume measurements (mls) were used to calculate the increase of the mBSA-challenged paw compared to the saline-injected contra lateral paw of each mouse, as follows:

Paw swelling (% difference) =

$$\frac{\{\text{mBSA injected paw volume (mls)}\} - \{\text{saline injected paw volume (mls)}\}}{\text{Saline injected paw volume (mls)}} \times 100\%$$

Paw measurements (mls) were written directly into lab books at the time of the experiments. Data was then analysed using Microsoft Excel and Graphpad Instat for statistical comparisons. All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests. Values were expressed as mean $\pm$ SEM with a P-value of less than 0.05 (compared to the positive control group) taken to be significant.

2.1.3 Materials

Sigma Aldrich, Airton Road, Tallaght, Dublin, Ireland.

mBSA, FCA(B) (contains heat killed and dried *Mycobacterium butyricum*), CMC and CycA were obtained from Sigma Aldrich.

Fujisawa Ireland Limited, Killorglin, Co. Kerry, Ireland.

Tacrolimus was obtained from Fujisawa Ireland Ltd.

Alltech Associates Ltd, 6-7 Kellet Road Industrial Estate, Carnforth, Lancs., LA5 9XP.

Microlitre syringes were obtained from Alltech Associates Ltd.

Ugo Basile, Biological Research Apparatus, Via G. Borghi 43, 21025 Comerio VA, Italy.

A Model 7140 Plethysmometer was obtained from Ugo Basile.

Bioresources Unit, TCD, Ireland.

Halothane and male CD-1 mice (25-35gms) were obtained from the Bioresources unit (BRU). All animals were housed at a room temperature of 22°C with a 12hr light/dark cycle.

2.1.4 Results

The percentage increase in paw swelling seen in CD-1 mice sensitised and challenged with mBSA was calculated for a number of test compounds at various doses. All mice were sensitised to mBSA using FCA(B) as the adjuvant, prior to challenging with mBSA. Positive control mice were untreated (given 1% CMC). All other groups were treated prior to challenging.

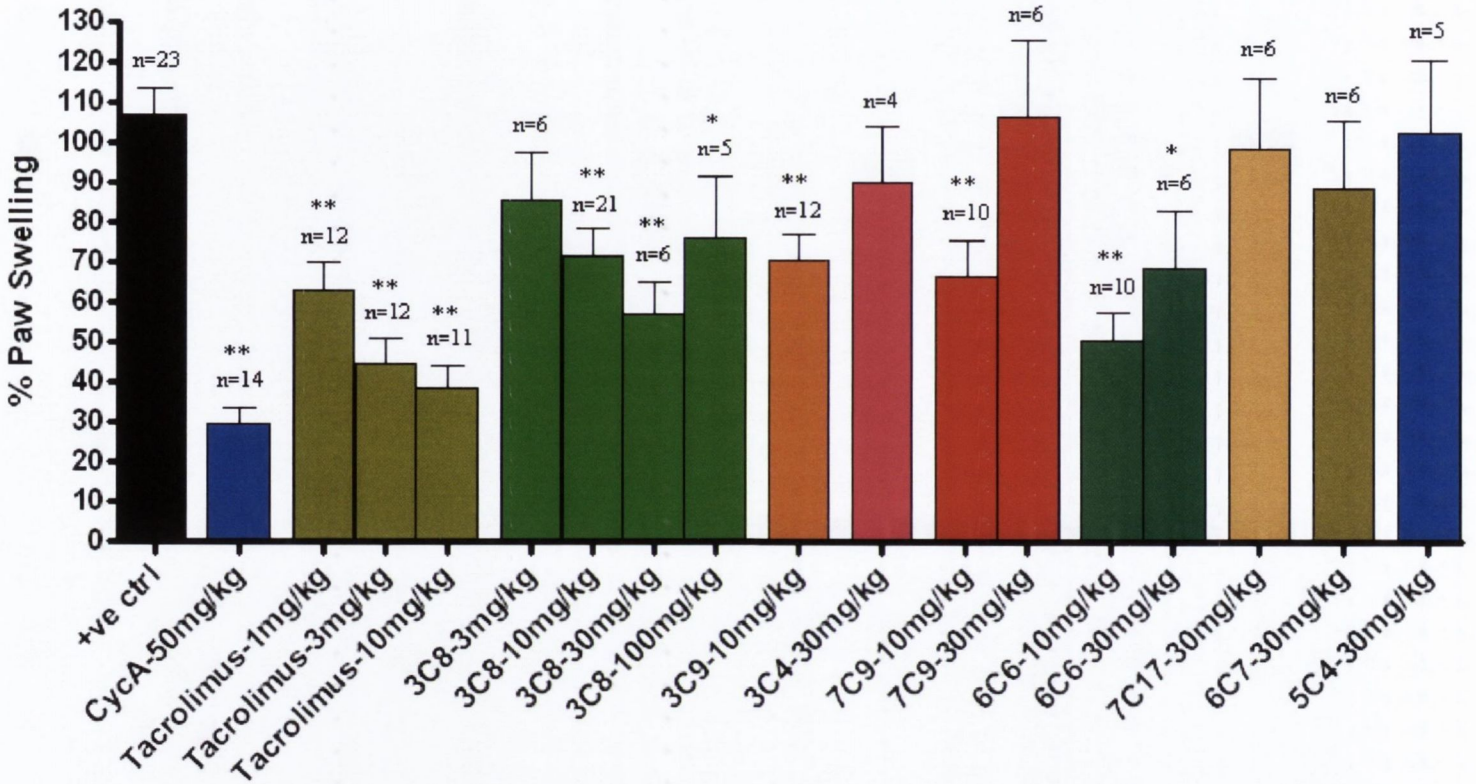
Percentage increase in inflammation for all groups is shown in **figure 2.1.4** and tabulated in **table 2.1.4 (appendix 2)**. The 18 groups shown are the positive control (+ve ctrl), CycA (50mg/kg), tacrolimus (1, 3 and 10mg/kg), 3C8 (3, 10, 30 and 100mg/kg), 3C9 (10mg/kg), 3C4 (30mg/kg), 7C9 (10 and 30mg/kg), 6C6 (10 and 30mg/kg), 7C17 (30mg/kg), 6C7 (30mg/kg) and 5C4 (30mg/kg).

The percentage increase in paw swelling seen in the positive control group was over 100%. CycA was found to reduce this positive value by more than 3.5 times, and this

was statistically significant (P<0.0001). Tacrolimus was found to significantly reduce the positive control value at 1mg/kg (P=0.0002), 3mg/kg (P<0.0001) and 10mg/kg (P<0.0001). A strong correlation was found between the dose and the reduction in paw oedema. At both 3 and 10mg/kg, paw swelling in the tacrolimus groups was reduced to well below 50%, with a reduction to almost 1/3 of the positive control value seen at 10mg/kg.

At 3mg/kg, 3C8 did not significantly reduce positive control paw oedema (P=0.119). However, 3C8 did significantly reduce this value at 10mg/kg (P=0.0002), at 30mg/kg (P<0.0001) and at 100mg/kg (P=0.045). 3C9 also significantly reduced the positive value at 10mg/kg (P=0.001). 6C6 was found to significantly reduce positive control swelling at both 10mg/kg (P<0.0001) and 30mg/kg (P=0.036). 7C9 also significantly reduced the positive value at 10mg/kg (P=0.006), to a value slightly lower than 6C6 at 30mg/kg. However, 7C9 did not seem to reduce paw swelling at all at 30mg/kg (P=0.694). Meanwhile, 6C7, 7C17, 5C4 and 3C4, all tested at 30mg/kg, were not found to significantly reduce positive control paw swelling (6C7; P=0.142, 7C17; P=0.628, 5C4; P=0.954, 3C4; P=0.531). All 4 of these test compounds showed paw swelling of at least 88%.

Figure 2.1.4: Percentage increase in mBSA induced paw oedema (DTH) in CD-1 mice



This graph represents the change in paw oedema (mean ± SEM) found in CD-1 mice sensitised and challenged to mBSA using FCA(B) as an adjuvant for sensitisation.
* indicates statistical significance with a P -value of less than 0.05 compared to the positive control
** indicates statistical significance with a P -value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two -tail Mann-Whitney tests.
Experiments were performed on 4 -6 groups at a time with n=6 per group.

2.1.5 Discussion

The mBSA model is a Th1 DTH model suitable for examining T cell mediated immune responses *in vivo*. Sensitisation to a mixture of mBSA and FCA and subsequent challenging with mBSA induces the DTH response. The adjuvant FCA can contain either *Mycobacterium tuberculosis* or *Mycobacterium butyricum*, both of which are believed to induce the DTH response (Dannenberg 1991; Kaufmann 1995; Lucey, Clerici et al. 1996; Roman and Moreno 1997; Sinha, Verma et al. 1997; Murray 1998). Therefore, it was hoped that any anti-Th1 effect of the test compounds could be examined using this model.

The positive control group showed an increase in paw swelling of 107 $\pm$ 6.4% which was even higher than expected. However, this was the mean $\pm$ SEM value for a particularly large number of 23 mice. Mean positive values of 60% and 100% have previously been obtained by different individuals in our laboratory using exactly the same protocol. It is possible that slightly different techniques may have been used, particularly for measuring oedema with the plethysmometer where individual differences could significantly alter the results.

When dosed at 50mg/kg, CycA significantly reduced the positive control value to 29.4 $\pm$ 4%, a reduction of more than 70%. Previous experiments in our laboratory resulted in a 33% reduction in paw swelling for CycA at the same dose (Frankish, Cogan unpublished data) and this was comparable to the 29% reduction found by Tarayre and co-workers (Tarayre, Aliaga et al. 1990). Tacrolimus also had a significant impact in a dose dependant manner, at 1, 3 and 10mg/kg. Swelling was

reduced by more than 50% at the two higher doses. As mentioned in **section 1.4**, both CycA and tacrolimus are immunosuppressants capable of binding to and inhibiting calcineurin, an essential phosphatase for IL-2 gene transcription (Halloran 1996). This would inhibit IL-12 production from the antigen presenting cells (APCs), thus inhibiting the Th1 response. The direct and indirect inhibition of both of these cytokines would have a negative impact on the maturation and activation of the $CD4^+$ Th1 cells and the $CD8^+$ Cytotoxic T cells (Gately, Carvajal et al. 1996; Stern, Magram et al. 1996). Both cytokines also stimulate the production of IFNγ, an essential component of the Th1 response, while IL-2 contributes to macrophage activation (Chan, Kobayashi et al. 1992; Ohshima, Delespesse 1997; Trinchieri 2003). This would explain the strong anti-Th1 effect of both drugs in this model.

3C8 was tested at 3, 10, 30 and 100mg/kg and significantly reduced oedema at all but the lowest dose. These results are in accordance with previous results for 3C8 in our laboratory, where it was also proven to be effective at 10mg/kg (Frankish, Cogan unpublished data). It is clear that this compound has a strong anti-Th1 effect, as did the immunosuppressants, CycA and tacrolimus.

Of the other compounds tested in this model, 6C6 had a significant impact at both 10 and 30mg/kg, as did 7C9 and 3C9 at 10mg/kg. Swelling was reduced to a greater extent by 6C6 at the lower dose than at the higher dose while 7C9, also tested at 30mg/kg, was not significant despite its potency at the lower dose. This may have been due to experimental error in sensitising and/or challenging or due to administration of an incomplete dose of the test compound. None of the other test

compounds significantly reduced oedema in this model. 6C7, 7C17, 5C4 and 3C4, all tested at 30mg/kg resulted in paw swelling of at least 88%.

The significant impact of 3C8, 3C9, 6C6 and 7C9 on paw oedema highlights their potential anti-Th1 effects. The exact mode(s) of action of these compounds are unknown although they could be acting in a number of ways. 3C8 has previously been tested in our laboratory and was found to inhibit $CaCl_2$-induced contraction of K^+ depolarised guinea pig ileum smooth muscle and was also found to potently inhibit rat peritoneal mast cell degranulation as induced by compound 48/80 *in vitro* (Frankish, Sheridan unpublished data). Inhibition of the latter would mean reduced formation of the PGs and LTs, which contribute to the influx of inflammatory cells to the site of inflammation. Both smooth muscle contraction and mast cell degranulation are believed to involve the calcium/calmodulin complex. It is therefore possible that 3C8 might inhibit the formation of this complex either by reducing the availability of i.c. calcium or by inhibiting calcium/calmodulin binding. Inhibition of the former could occur due to decreased release of calcium from i.c. stores or due to decreased influx of calcium from e.c. sources. It is also possible that 3C8 may have a similar mode of action to CycA and tacrolimus since these drugs bind to and inhibit calcineurin, a calcium/calmodulin-dependant phosphatase (Almawi and Melemedjian 2000). This potential inhibition of calcineurin would again result in inhibition of IL-2 gene transcription, thus inhibiting the Th1 response. However, other possible modes of action for 3C8 as well as for the other significant test compounds cannot be ruled out and may involve inhibition of a number of cytokines such as IL-2, IFNγ and IL-12, which are crucial in the Th1 response.

2.2: Sheep Red Blood Cell (SRBC) Model

2.2.1 Introduction

The sheep red blood cell model was set up following the results obtained for the mBSA model. Like the mBSA model, it can be used to generate a DTH response, therefore allowing us to hopefully confirm some of these results (from the mBSA model) in a second model of DTH. Various strains of mice, including Balb/C mice, Kumming mice and albino mice have been used to successfully generate a DTH response with sheep erythrocytes (Bekierkunst, Yarkoni et al. 1971; Nores, Courreges et al. 1997; Gan, Hua Zhang et al. 2004).

The aims of the experiments performed using the SRBC DTH model were to examine the effects of CycA, tacrolimus, 3C8 and 3C9 on paw swelling and to hopefully confirm the results already seen for these compounds in the mBSA DTH model.

2.2.2 Methods

The sheep red blood cells were first cleaned and counted as follows. 5mls of the SRBC were spun down in a centrifuge at 3000rpm for 10 minutes. The supernatant was removed and phosphate buffered saline (PBS), pH 7.4 added to the pellet. The pellet was mixed with the PBS buffer (5mls) and spun down in a centrifuge at 3000rpm for 10 minutes. The pellet was washed twice more with PBS buffer as described above. The pellet was then reconstituted in 5mls of PBS buffer following

the final wash. 10µl of a 1 in 200 dilution of the sample of reconstituted sheep red blood cells (in trypan blue stain) was used to fill the counting chamber of a haemacytometer and the number of sheep red blood cells counted.

The protocol employed was a modified version of the one used by Nores and co-workers (Nores, Courreges et al. 1997). Modifications to this protocol were the mouse strain, the volumes and concentrations of SRBC used for sensitisation and challenging, as well as the time point and method for measurement of oedema. In the protocol employed by Nores and co-workers, female Balb/C mice were sensitised with $1x10^6$ SRBC in 0.2 ml of PBS and challenged with $1x10^8$ SRBC in 50µl PBS. In contrast, male CD-1 mice were used here, with $1x10^7$ SRBC in 100µl PBS and $3x10^8$ SRBC in 20µl PBS used for sensitisation and challenging respectively, although the routes of administration (i.p for sensitising and s.c. in the hind paw for challenging) were the same in both cases. Finally, footpad swelling was measured on day 7, 2 days after challenging using a caliper in the method employed by Nores and co-workers whereas oedema was measured by calculating paw volume on day 6, 24hrs after challenging in the following protocol.

Male CD-1 mice (30-40gms) were sensitised on day zero. The negative control group was sensitised with 100µl PBS intraperitoneally (i.p.), while the positive control group and drug treated groups were sensitised with $1x10^7$ SRBC (100µl i.p.).

Animals were challenged 5 days later with SRBC. The SRBC were cleaned and counted as described above. Animals anaesthetised with halothane were challenged s.c. by injecting SRBC ($3x10^8$ cells in 20µl) into the right hind paws and PBS (20µl)

into the left hind paws. This was done for all groups including the negative control group.

Drug treated groups were dosed i.p on day 4 and 2 hours prior to challenge on day 5. The negative control and positive control groups were dosed with 1% CMC using the same dosing schedule.

The difference in paw swelling between the saline-injected and SRBC-challenged contralateral paw of each mouse was calculated by measuring paw volume (mls) using a plethysmometer. This was carried out 24 hours after challenge to SRBC with triplicate measurements made on each paw. This was done exactly as for the mBSA delayed-type hypersensitivity model:

$$\text{Paw swelling (\% difference)} = \frac{\{\text{SRBC injected paw volume (mls)}\} - \{\text{saline injected paw volume (mls)}\}}{\text{Saline injected paw volume (mls)}} \times 100\%$$

Paw measurements (mls) were written directly into lab books at the time of the experiments. Data was then analysed using Microsoft Excel and Graphpad Instat for statistical comparisons. All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests. Values were expressed as mean $\pm$ SEM with a P-value of less than 0.05 (compared to the positive control group) taken to be significant.

2.2.3 Materials

Sigma Aldrich, Airton Road, Tallaght, Dublin, Ireland.

CMC, CycA, PBS and trypan blue stain were obtained from Sigma Aldrich.

Cruinn Diagnostics, Unit 6, Phase 2, Western Industrial Estate, Knockmitten Lane, Dublin 12.

SRBC were obtained from Cruinn Diagnostics.

Fujisawa Ireland Limited, Killorglin, Co. Kerry, Ireland.

Tacrolimus was obtained from Fujisawa Ireland Ltd.

Alltech Associates Ltd, 6-7 Kellet Road Industrial Estate, Carnforth, Lancs., LA5 9XP.

Microlitre syringes were obtained from Alltech Associates Ltd.

Ugo Basile, Biological Research Apparatus, Via G. Borghi 43, 21025 Comerio VA, Italy.

A Model 7140 Plethysmometer was obtained from Ugo Basile.

Bioresources Unit, TCD, Ireland.

Halothane and male CD-1 mice (30-40gms) were obtained from the Bioresources unit (BRU). All animals were housed at a room temperature of 22°C with a 12hr light/dark cycle.

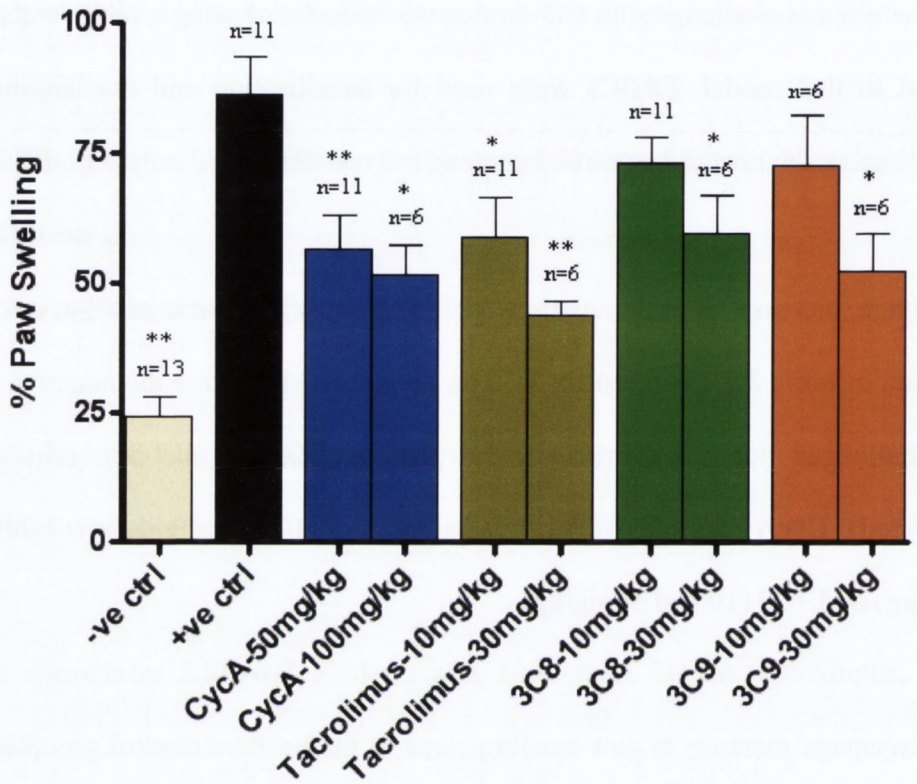
2.2.4 Results

As for the mBSA DTH model, the percentage increase in paw swelling following sensitisation and challenging in CD-1 mice was calculated using a plethysmometer. In contrast to that model, SRBCs were used for sensitisation and challenging for all groups except the negative control group, which was sensitised only with PBS.

Percentage increase in inflammation for all groups is shown in **figure 2.2.4** and tabulated in **table 2.2.4 (appendix 2).** The 10 groups shown are the negative ctrl (–ve ctrl; challenged but not sensitised), the positive control (+ve ctrl; sensitised and challenged), CycA (50 and 100mg/kg), tacrolimus (10 and 30mg/kg), 3C8 (10 and 30mg/kg) and 3C9 (10 and 30mg/kg).

The percentage increase in paw swelling seen in the positive control group was more than 3.5 times greater than swelling in the negative control group. CycA was found to significantly reduce this positive value at both 50mg/kg (P=0.008) and 100mg/kg (P=0.010). Similarly, tacrolimus significantly reduced this positive value at both tested doses. At 10mg/kg, tacrolimus produced an almost identical result to that of CycA at 50mg/kg (P=0.014) and was more effective at 30mg/kg than CycA was at 100mg/kg, reducing the positive value by almost half (P=0.001). Percentage paw swelling was very similar for 3C8 and 3C9. Neither drug significantly reduced oedema at 10mg/kg with values not far below the positive control (3C8; P=0.217, 3C9; P=0.180) while both caused a significant reduction at 30mg/kg (3C8; P=0.038, 3C9; P=0.028).

Figure 2.2.4: Percentage increase in SRBC induced paw oedema (DTH) in CD-1 mice



This graph represents the percentage increase in paw oedema (mean ± SEM) in CD-1 mice sensitised and challenged with SRBC (except for the –ve ctrl group which was sensitised only with PBS).
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 4-6 groups at a time with n=6 per group.

2.2.5 Discussion

The SRBC model is, like the mBSA model, a model of DTH. It was therefore hoped that the results obtained for 3C8 and 3C9 in the mBSA model could be repeated here, again using the known immunosuppressants CycA and tacrolimus for comparison.

Unlike the mBSA model, a negative control group was used and mice in this group were not sensitised to SRBC while all groups were challenged.

Animals were dosed i.p. with test compound 24hrs and 2hrs prior to challenge as in the mBSA model although the SRBC model had a shorter time course, with mice challenged 5 days after sensitisation. Paw volume was again measured as a marker for oedema 24hrs after challenge in exactly the same manner as was done for the mBSA model. The percentage difference in oedema between challenged and unchallenged paws increased from 24.4 $\pm$ 3.8% in the negative control group to 86.4 $\pm$ 7.2% in the positive control group. This was larger than the 56 $\pm$ 4.2% increase found 24hrs after challenge in other SRBC experiments (Neishabouri, Hassan et al. 2004). However, it should be stated that the strain of mouse as well as the methods used for sensitisation and challenging were not identical in these experiments and may account for such differences. A direct comparsion between these results and those found for the protocol on which this model was based (Nores, Courreges et al. 1997), cannot be made since results were only expressed as percentage inhibition of inflammation by Nores and co-workers, assuming the positive control group to be 100%.

CycA was not found to have a significant impact on oedema in this model in previous experiments in our laboratory although this may have been due to the fact that it was administered orally. When administered i.p. in these experiments, it was found to reduce paw oedema to 56.8 $\pm$ 6.2%, a reduction of 35% when dosed at 50mg/kg, and to 51.6 $\pm$ 6.0%, a reduction of 40% when dosed at 100mg/kg. This is further confirmation of the anti-Th1 effect of CycA, as was demonstrated in the mBSA model. Other reports have also found CycA to be effective in SRBC models (Shidani,

Milon et al. 1984; Webster and Thomson 1987; Thomson, Propper et al. 1993). Tacrolimus also had a significant impact, being almost as potent at 10mg/kg as CycA was at 50mg/kg and being even more effective at 30mg/kg than CycA was at 100mg/kg, reducing the positive value by almost 50%. This also confirms the results found for tacrolimus in the mBSA model as well as other evaluations of the drug in SRBC models (Woo, Ross et al. 1990; Thomson, Propper et al. 1993). As mentioned earlier, both CycA and tacrolimus can inhibit cytokine production by antagonising calcineurin. However, the effects of tacrolimus are not solely due to inhibition of calcineurin and the NF-AT pathway. Unlike CycA, tacrolimus is also capable of blocking cytokine receptor expression and inhibiting the effects of cytokines on target cells (Mori, Suko et al. 1997). Examples of such effects are the inhibition of IL-2 induced IL-5 production in human $CD4^+$ T cells and the inhibition of T cell proliferation stimulated by IL-2 and IL-7 (Mori, Suko et al. 1997; Almawi and Melemedjian 2000). These additional effects may explain the greater potency of the drug compared to CycA in this model. Direct comparisons between these results and others found for these immunosuppressants in SRBC models are difficult due to differing species/strains, protocols and drug doses administered.

3C8 and 3C9 produced very similar results in this model, both significantly reducing oedema when dosed at 30mg/kg, while neither were significant at 10mg/kg. These results are in slight contrast to the results found in the mBSA model of DTH, where both 3C8 and 3C9 had a significant impact at the lower dose of 10mg/kg. This would suggest that the mBSA model was more sensitive to the effects of these compounds, which could be due to a number of reasons. First of all, different antigens were used in the two models and secondly, the SRBC model used a shorter time interval

between sensitisation and challenging. It is possible that a longer interval might have exaggerated the positive control oedema, thus making the test compounds more effective. It should be noted that levels of oedema at the higher dose for both test compounds were similar to those found for CycA. As mentioned earlier, 3C8 and other compounds such as 3C9 might be acting in a similar way to CycA, through inhibition of calcineurin. They might also act to inhibit calcium/calmodulin formation, either by reducing i.c. calcium availability or by inhibiting calcium/calmodulin binding.

2.3: Oxazolone Contact Hypersensitivity (CHS) model

2.3.1 Introduction

CHS is a form of DTH which is very similar to the protein-adjuvant reaction seen in the mBSA model. It can be induced by injecting a complex of hapten carrier-bound protein. Basophilic infiltration is again prominent although it develops more slowly and peaks after 3 to 6 days (Waksman 1978). IFNγ and TNFα are thought to contribute to CHS by activating macrophages while Th2 cytokines suppress Th1 cytokine secretion, thus inhibiting this response. However, IL-4 may also contribute to CHS since levels of the cytokine were elevated in some oxazolone models (Xu, Bulfone-Paus et al. 2003). The first of three essential phases in CHS is sensitisation. Sensitisation to hapten usually occurs through the skin and involves the binding of hapten to e.c. protein. The resulting complex is antigenic and is processed by APCs. Trafficking of these cells back into the lymph node then occurs, where they will

present the antigen to the T cells. Finally, the elicitation phase is mediated by these T cells, resulting in the influx of inflammatory cells (Kripke et al. 1990; Grabbe et al. 1992). Oxazolone is a hapten which is a well known mediator of this form of DTH (Tarayre et al. 1984; Cavey et al. 1990).

The primary aim of these experiments was to examine the effects of CycA, tacrolimus, 3C8 and 3C9 on oxazolone-induced ear swelling. This would hopefully confirm results already obtained for these compounds in both the mBSA and SRBC DTH models. In addition, dexamethasone and 6C6 would be tested.

2.3.2 Methods

The following protocol is a modified version of the one used by Xu and co-workers (Xu, Bulfone-Paus et al. 2003). Differences between their protocol and the one employed here are the method of sensitisation (route of administration and concentration of oxazolone), a slightly different time interval between sensitisation and challenging and a small difference in the oxazolone concentration used for challenging. In the protocol employed by Xu and co-workers, 100µl of 3% oxazolone was applied topically to the shaved abdomen for sensitisation as opposed to the 20µl of 2% oxazolone applied to both ears in our method. 6 days later, both sides of the right ear were challenged with 15µl of 1% oxazolone in the protocol described by Xu and co-workers whereas in the method employed here, mice were challenged with 20µl of 2% oxazolone only on the right ear and this was 5 days after sensitisation. The full protocol is outlined below.

Day 1: Oxazolone sensitisation:

Female Balb/C mice (30-40gms) were sensitised with 20µl of 2% oxazolone in acetone on each ear (10µl on the inner and outer aspects of both ears).

Day 6: Oxazolone challenging and drug dosing:

All mice were challenged with 20µl of 2% oxazolone in acetone only on the right ear; again, 10µl on both the inner and outer aspects of the ear. Immediately following this, the mice were treated with test compound. The test compound was administered in exactly the same way as the oxazolone, with 10µl on each side of the right ear. All compounds were prepared in acetone at a concentration of 15mg/ml; 300µg/ear. For the positive control group, acetone was administered.

Day 7: Ear measurements by thickness and weight:

All animals were killed 24hrs later and the percentage increase in ear swelling was calculated. This was done in two ways. The thickness of the unchallenged left ear and the challenged right ear were measured using a micrometer caliper (µm). The increase in weight of both ears was also measured using a 5mm biopsy punch (mgs). The percentage increase in oedema was then calculated for both weight and thickness by expressing the difference between the unchallenged left ear and the challenged right ear as a percentage of the left ear control.

Paw measurements (weight and thickness) were written directly into lab books at the time of the experiments. Data was then analysed using Microsoft Excel and Graphpad Instat for statistical comparisons. All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests. Values were expressed as mean $\pm$ SEM with a P-value of less than 0.05 (compared to the positive control group) taken to be significant.

2.3.3 Materials

Sigma Aldrich, Airton Road, Tallaght, Dublin, Ireland.

Oxazolone, acetone, CycA, dexamethasone and trypan blue stain were obtained from Sigma Aldrich.

Fujisawa Ireland Limited, Killorglin, Co. Kerry, Ireland.

Tacrolimus was obtained from Fujisawa Ireland Ltd.

Alltech Associates Ltd, 6-7 Kellet Road Industrial Estate, Carnforth, Lancs., LA5 9XP.

Microlitre syringes were obtained from Alltech Associates Ltd.

Bioresources Unit, TCD, Ireland.

Halothane and female Balb/C mice (30-40gms) were obtained from the Bioresources unit (BRU). All animals were housed at a room temperature of 22°C with a 12hr light/dark cycle.

2.3.4 Results

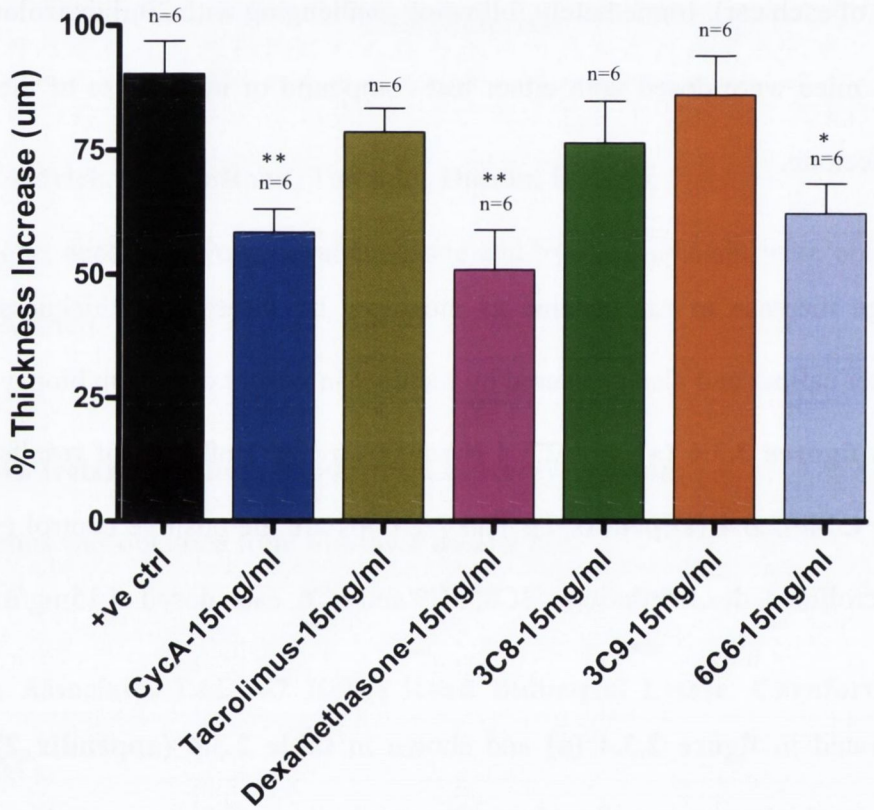
The percentage increase in ear oedema found in female Balb/C mice sensitised and challenged with oxazolone was measured using a micrometer caliper for thickness (μm) and also a 5mm biopsy punch for weight (mgs). All mice were sensitised with 20μl of 2% oxazolone in acetone on the inner and outer aspects of both ears (10μl each side of each ear). Immediately following challenging with 20μl oxazolone on the right ear, mice were dosed with either test compound or in the case of the positive control, acetone.

Percentage increase in ear oedema as measured by increase in thickness using a micrometer caliper and also measured by increase in weight of a 5mm biopsy punch is shown in **figures 2.3.4 (a) and 2.3.4 (b)** respectively. Both sets of results are also shown in **table 2.3.4 (appendix 2)**. The 7 groups are the positive control (+ve ctrl), CycA, tacrolimus, dexamethasone, 3C8, 3C9 and 6C6, each dosed at 15mg/ml.

As illustrated in **figure 2.3.4 (a)** and shown in **table 2.3.4 (appendix 2)**, a 90% increase in thickness was found in the positive control group (comparing the challenged right ear to the unchallenged left ear). CycA, dexamethasone and 6C6 were the only compounds found to significantly reduce this value. A 35% reduction was found in the group dosed with CycA, which was highly significant. The greatest reduction in swelling was seen in the dexamethasone group where approximately a 43% reduction was seen. 6C6 reduced positive control oedema by almost 31%. A smaller reduction in swelling was found in the groups dosed with tacrolimus and 3C8

but neither had a significant impact. The oedema found in the group dosed with 3C9 was almost as high as the positive control group.

Figure 2.3.4 (a): Percentage increase in ear thickness in Balb/C mice sensitised and challenged with oxazolone

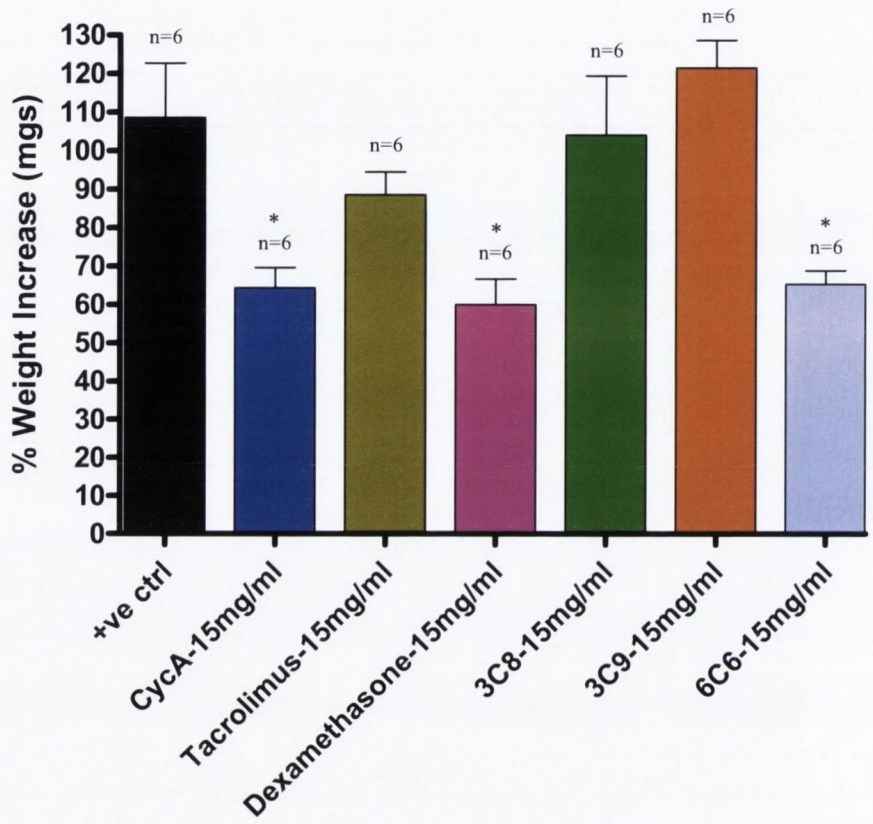


This graph represents the percentage increase in ear thickness (mean ± SEM) found in female Balb/c mice sensitised and challenged with oxazolone.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 3-4 groups at a time with n=6 per group.

Percentage increase in oedema as measured by the weight increase of a 5mm biopsy punch is illustrated in **figure 2.3.4 (b)** and again tabulated in **table 2.3.4 (appendix**

2). An increase of 108.7% was found in the positive control group (again comparing the challenged right ear to the unchallenged left ear). As found in the results measuring increase in ear thickness, CycA, dexamethasone and 6C6 were the only compounds that significantly reduced this value. A 41% reduction in swelling was found in the group dosed with CycA, slightly higher (6%) than was measured by ear thickness. Once again, dexamethasone caused the biggest reduction in ear oedema, reducing the positive value by almost 45%, just 2% more than was measured by ear thickness. 6C6 caused a 40% reduction in the positive control value, 9% more than was measured with the caliper. Tacrolimus, 3C8 and 3C9 again failed to significantly reduce positive control ear swelling despite the fact that tacrolimus caused an 18% reduction in swelling. 3C8 had almost no impact on ear swelling while the group dosed with 3C9 had a greater mean value than the positive control group.

Figure 2.3.4 (b): Percentage increase in ear weight in Balb/C mice sensitised and challenged with oxazolone



This graph represents the percentage increase in ear weight (mean ± SEM) found in female Balb/c mice sensitised and challenged with oxazolone.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 3-4 groups at a time with n=6 per group.

2.3.5 Discussion

The final DTH model used to examine the effects of our novel compounds was the oxazolone model, a model which has been used to identify potential anti-inflammatory and immune-modulating drugs. By inducing a contact hypersensitivity

(CHS) response with oxazolone, it was hoped that further confirmation of the anti-Th1 effects of 3C8 and 3C9 found in the previous 2 models of DTH, as well as for 6C6 in the mBSA model, could be demonstrated. Another advantage of using this model is that it is suitable for topical drug administration, meaning that the compounds could be evaluated using a different route of administration. CycA and tacrolimus were again tested along with dexamethasone in order to compare their potency to the potency of the novel compounds.

The protocol involved sensitising Balb/C mice to oxazolone through topical administration to both ears, with subsequent challenging to oxazolone on the right ear occurring 5 days later. This was different to the mBSA and SRBC models in that the ear was challenged instead of the paw, and both a 5mm biopsy punch and a caliper reading of the challenged right ears and unchallenged left ears were used to estimate oedema 24hrs after challenge, as opposed to the use of a plethysmometer to measure paw volume. Measurement of both ear weight and thickness are methods that have been shown to be sensitive and accurate for measuring oedema (Chapman, Ruben et al. 1986). Another difference in this method is that while animals were dosed i.p. with test compound 24hrs and 2hrs prior to challenge in the previous models of DTH, drugs were administered topically in the same manner as for sensitising and challenging here and this was done straight after challenge. Ears were allowed a brief time to dry following oxazolone challenge since simultaneous application of oxazolone and a number of drugs including CycA have been found to inhibit oedema more potently than when administered separately (van den Hoven, van den Berg et al. 1997). In this way, any possible drug-oxazolone interactions could be avoided, thus making topical testing more reliable.

The increase in oedema in the positive control group, calculated as a percentage of the left ear (control) was 90.2 $\pm$ 6.6% using ear thickness and 108.7 $\pm$ 14.1% using ear weight as markers for oedema. Topical administration of CycA at 15mg/ml significantly reduced these values by 35% and 41% respectively whereas tacrolimus did not have a significant impact on ear swelling. This is in contrast to the mBSA and SRBC results where not only was tacrolimus significant but seemed to be more potent than CycA. Both immunosuppressants have also been found to be effective in other oxazolone models of CHS (van den Hoven, van den Berg et al. 1997; Fujii, Takeuchi et al. 2002; Meingassner, Fahrngruber et al. 2003). Oral dosing of both drugs was found to significantly reduce ear weight, as well as impairing elicitation and sensitisation; tacrolimus being even more potent at 30mg/kg than CycA at higher doses (Meingassner, Fahrngruber et al. 2003). The fact that tacrolimus was significant, in contrast to the results found here, is unlikely to be due to different routes of administration since topical application has also proven to be effective (Fujii, Takeuchi et al. 2002). It is possible that the 15mg/ml dose of tacrolimus used here was too low to significantly reduce oedema since higher doses have proved to be effective in other oxazolone models (Fujii, Takeuchi et al. 2002; Meingassner, Fahrngruber et al. 2003). However, we might have expected tacrolimus to be at least as potent here as CycA since it would then correlate with the mBSA and SRBC results as well as results found in other oxazolone models, as mentioned above. Perhaps CycA acts differently to tacrolimus although this has not been reported. Tacrolimus should theoretically be more effective since it is active at lower concentrations than CycA and has a broader range of actions.

The corticosteroid dexamethasone reduced ear swelling by approximately 45%, as measured by both weight and thickness, a greater reduction than was seen in any other group. Previous studies have also shown the drug to be highly potent in similar models (Chapman, Ruben et al. 1986; Taube and Carlsten 2000). Corticosteroids seem to inhibit the effector phase of DTH, possibly by exerting their effects on sensitised memory T cells since dexamethasone has been found to significantly reduce DTH when administered 1 day prior to oxazolone challenge but not when administered 1 day prior to sensitisation (Taube and Carlsten 2000). This is in contrast to the immunosuppressants CycA and tacrolimus, which as mentioned have been found to be effective in both the sensitisation and elicitation phases (Meingassner, Fahrngruber et al. 2003).

Of the 3 novel compounds evaluated in this model, only 6C6 significantly reduced ear swelling. This may be due to the fact that it has a different structure to that of 3C8 and 3C9 and is the only one of these 3 compounds to contain nitrogen. It was very similar in potency to CycA, reducing oedema by approximately 31% and 40% according to ear thickness and weight respectively. Although found to be significant using both methods to estimate oedema, this 9% difference (31% vs 40%) is quite large and is likely due to experimental error. Overestimation of caliper readings and/or underestimation of ear weight, possibly due to incomplete punches taken from the ear could account for such differences. In any case, 6C6 had a significant effect and follows results for the compound in the mBSA model, where it was also a potent inhibitor of oedema, thus suggesting that it inhibits the Th1 response in some way. The exact mode of action is unknown and could be due to calcineurin inhibition which would inhibit IL-2 gene transcription, similar to CycA and tacrolimus. In

similar oxazolone models, infiltration of inflammatory cells consisting of monocytes, granulocytes and macrophages, but not eosinophils has been reported, while mRNA levels for IFNγ, IL-4, TNFα, IL-1β and COX-2 have also been induced (Wee, Shin et al. 2005). Therefore, perhaps 6C6 was effective because it was able to regulate COX-2, TNFα or IL-1β produced by macrophages and/or IFNγ or IL-4 produced by Th cells. Another possibility is that it may have inhibited the Fas/Fas ligand pathway since this has been shown to partially contribute to apoptosis and the development of CHS in other murine oxazolone models (Xu, Bulfone-Paus et al. 2003).

The insignificant impact of 3C8 and 3C9 was unexpected following successful results for these compounds in both the mBSA and SRBC models. It is possible that topical application of 3C8 and 3C9 is not as effective as i.p. dosing, which was used in both previous DTH models. Also, while 10mg/kg of both compounds was sufficient to significantly reduce paw swelling in the mBSA model, it was not sufficient in the SRBC model where 30mg/kg was required. Therefore, perhaps the oxazolone and SRBC models were not as sensitive to these compounds. A higher dose may have been significant since the 15mg/ml dose used here was less than the 30mg/kg top dose found to be significant in both previous models. As mentioned earlier, 6C6 has a different structure to that of 3C8 and 3C9. This might also make it more effective than the latter compounds in this model. However, other factors such as the fact that the oxazolone model uses a different antigen and a different strain of mouse compared to the other 2 models of DTH may also be responsible for such discrepancies.

As already stated, the oxazolone model is a Th1 model of CHS. Inflammatory cell infiltration and elevated protein and mRNA levels for IFNγ have been found in a

chronic oxazolone-induced dermatitis model in rats with minimal changes in levels of the Th2 cytokine IL-4 (Fujii, Takeuchi et al. 2002). Therefore, perhaps compounds found to be effective in this model such as 6C6 act by inhibiting the influx of inflammatory cells or by inhibiting IFNγ. It is possible that even compounds such as 3C8 and 3C9, which did not successfully reduce oedema here, might also have had this effect if higher doses had been used since tacrolimus was similarly insignificant here but successfully reduced ear swelling in the model used by Fujii and co-workers as well as reducing levels of IFNγ. It is thought that IFNγ and TNFα contribute to CHS by activating macrophages while Th2 cytokines suppress Th1 cytokine secretion, thus inhibiting this response. However, IL-4 levels were elevated in some oxazolone models, suggesting that it may also contribute to CHS (Xu, Bulfone-Paus et al. 2003). Analysis of the levels of IL-4, IFNγ and other cytokines in this model could help to improve understanding of this process as well as the actions of the novel compounds.

Chapter 3

Bronchoalveolar Lavage

Chapter 3: Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) is a commonly used technique for taking fluid samples from the epithelial lining of the respiratory tract for analysis. It has been used to study cystic fibrosis, interstitial lung diseases and asthma (Connett 2000). BAL fluid can be analysed by enzyme-linked immunosorbent assay (ELISA) to measure levels of specific cytokines. It can also be used to generate total cell counts and to determine the percentage of each type of leukocyte present in a sample. These methods are commonly employed to measure levels of Th1 and Th2 cytokines, eosinophil and neutrophil derived mediators such as EPO and MPO, and to estimate levels of eosinophilia as well as the infiltration of other inflammatory cells in models of asthma (Schneider and Issekutz 1996; Koh, Choi et al. 2001; Itami, Latinne et al. 2003; Konduri, Nandedkar et al. 2003; McKay, Leung et al. 2004).

3.1: Rat BAL and Cell Counts

3.1.1 Introduction

Many models of asthma have previously been set up in various rat strains. The idea of this is to mimic the human form of the disease as closely as possible and to examine the symptoms as well as to test potential new drugs for treating the disease. Inbred Brown Norway (BN) rats are particularly popular because they are genetically Th2-predisposed. The BN rat model of allergic asthma mimics the human form of the

disease in many ways, with high levels of IgE produced following immunisation (Waserman, Olivenstein et al. 1992) and both early and late phase characteristics evident following allergen challenge (Pauwels, Bazin et al. 1979; Martin, Zu et al. 1993; Renzi, Olivenstein et al. 1993).

The BN model has been compared to the same allergic model set up in outbred Sprague Dawley (SD) rats which are not genetically predisposed. While an allergic response was found in the SD model with significantly increased eosinophil levels and higher IL-5 mRNA levels found in the lung compared to controls, it was not as pronounced as the response in the BN model, where both these parameters were increased to an even greater extent (Renzi, al Assaad et al. 1996; Hylkema, Hoekstra et al. 2002). Since a Th2 response was seen in the strain that is not Th2 predisposed, it suggests that there are other factors apart from genetics that contribute to allergic disease. SD rats are therefore not completely unsuitable and have been used in models of allergic asthma and lung injury (Koh, Choi et al. 2001; Savani, Godinez et al. 2001). However, the BN model seems to be more suitable to mimic human allergic asthma and has been used by many authors (Tominaga, Watanabe et al. 1995; Hatzelmann, Haefner et al. 1996; Huang, Adcock et al. 2001; Tigani, Hannon et al. 2003). An asthma-like phenotype, characterised by reversible airway obstruction, airway hyper-responsiveness and airway remodelling has also been developed in BN rats infected with parainfluenza type 1 (Sendai) virus, but not in non-atopic Fischer 344 rats (Uhl, Castleman et al. 1996; Sorkness, Castleman et al. 1999). This would again suggest that BN rats would be the most suitable choice to mimic the human form of the disease. However, both BN rats and Wistar rats have successfully been used in our laboratory to establish a respiratory asthma model in previous unpublished

results, the latter being even more effective. Because of this and the fact that SD rats have as mentioned, been used in models of allergic asthma and lung injury, all 3 strains were used in the following asthmatic models.

The aims of these experiments involving the rat asthmatic models were to compare the percentage of each type of leukocyte, particularly eosinophils, in the BAL fluid of rats sensitised to ovalbumin (OVA) using aluminium hydroxide (AlOH), Freunds complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T)) or both as adjuvants and to examine the effects of CycA and 3C8 on each of these models. Sensitisation to OVA using AlOH as an adjuvant should result in a Th2 response (Hatzelmann, Haefner et al. 1996; Koh, Choi et al. 2001; Wagers, Lundblad et al. 2004; McKay, Leung et al. 2004) and mimic extrinsic asthma while using FCA(T) as an adjuvant should result in a Th1 response (Dannenberg 1991; Kaufmann 1995; Lucey, Clerici et al. 1996; Roman and Moreno 1997; Sinha, Verma et al. 1997; Murray 1998) and therefore mimic intrinsic asthma. Meanwhile, the third model using both adjuvants together may result in a mixed Th1/Th2 response.

3.1.2 Methods

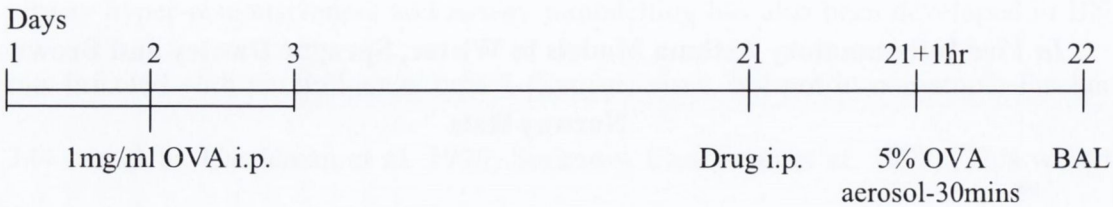
In Vivo Inflammatory Asthma Models in Wistar, Sprague Dawley and Brown Norway Rats

Three asthmatic models were used to test our compounds. These were based on the protocol used by Renzetti and co-workers (Renzetti, Paciorek et al. 1996). There were two main differences in the methods employed to do this. Firstly, the method of

sensitisation to ovalbumin (OVA) was different, and secondly, a different time interval between OVA sensitisation and OVA challenge was used. Animals were sensitised to OVA using the adjuvants aluminium hydroxide (AlOH; Th-2 inducer), Freunds complete adjuvant containing *Mycobacterium tuberculosis* (FCA(T); Th-1 inducer), or the two together (three asthmatic models). A 3-week time interval was used between OVA sensitisation and final OVA challenge. A respiratory chamber and nebuliser were used for the challenging procedure. 24 hours after OVA challenge, the animals were sacrificed and BAL was carried out. Differential cell counts were performed, mainly to examine leukocyte numbers. An increased leukocyte number was used as a marker for increased asthmatic inflammation.

As illustrated in **figure 3.1.2**, male Wistar, Sprague Dawley and Brown Norway rats (250-350gms) were sensitised intraperitoneally (i.p.) to OVA on days 1, 2 and 3 of the experiment, using either AlOH, FCA(T) or the two together. On day 21, the rats were treated i.p. with either vehicle (1% CMC; carboxymethylcellulose) or test compound. One hour later, they were challenged with either vehicle (distilled water; dH20) or 5% OVA by nebuliser. 24 hours after challenge, BAL was carried out.

Figure 3.1.2: Schematic representation of protocol for rat asthma models



This figure represents the timepoints at which animals were sensitised and challenged with OVA and dosed with test compound in rat asthma models.

3.1.2.1: Sensitisation of rats to OVA using AlOH as an adjuvant

Rats were sensitised on days 1, 2 and 3 as described in **table 3.1.2.1 (a)** (appendix 1), using AlOH (Th-2 inducer) as an adjuvant. The vehicle/drug treatment and OVA challenging procedures carried out on day 21 are described in **table 3.1.2.1 (b)** (appendix 1). 24 hours after OVA challenge, BAL was carried out.

3.1.2.2: Sensitisation of rats to OVA using FCA(T) as an adjuvant

Rats were sensitised on days 1, 2 and 3 as described in **table 3.1.2.2 (a)** (appendix 1), using FCA(T) (Th-1 inducer) as an adjuvant. The vehicle/drug treatment and OVA challenging procedures carried out on day 21 are described in **table 3.1.2.2 (b)** (appendix 1). 24 hours after OVA challenge, BAL was carried out.

3.1.2.3: Sensitisation of rats to OVA using AlOH and FCA(T) as adjuvants

Rats were sensitised on days 1, 2 and 3 as described in **table 3.1.2.3 (a)** (appendix 1), using AlOH (Th-2 inducer) and FCA(T) (Th-1 inducer) as adjuvants. The vehicle/drug treatment and OVA challenging procedures carried out on day 21 are described **in table 3.1.2.3 (b)** (appendix 1). 24 hours after OVA challenge, BAL was carried out.

Bronchoalveolar lavage procedure and cell counts

24 hours after OVA challenge, BAL was carried out. In order to eliminate blood from the preparation, the animals were first anaesthetised with halothane, opened by midline incision, and bled by cutting the *vena cava*. Following this procedure, which would kill the animals, the diaphragm was cut in order to deflate the lungs. Next, the trachea was exposed and cannulated. 1.5mls of phosphate buffered saline (PBS) [137mM sodium chloride (NaCl), 2.7mM potassium chloride (KCl), 8.1mM disodium hydrogen phosphate (Na_2HPO_4), 1.5mM potassium dihydrogen phosphate (KH_2PO_4)] was used to lavage the lungs (inserted using syringe). After gently massaging the chest for 30 seconds, the fluid was withdrawn by syringe, reinserted, and finally collected, by allowing it to drip from the cannula into a vial. When this was done, the sample was immediately placed on ice. All samples were then centrifuged at 1000rpm for 6 minutes at room temperature, using a Sigma 204 centrifuge. The pellet was resuspended in 1.5mls PBS for slide preparation.

100µl of the resuspended pellet of each sample was then spun onto a slide at 500rpm for 4 minutes at room temperature, using a Thermoshandon cytospin. The slides were allowed to dry before staining with Leishmans stain. 1ml of stain was pipetted onto each slide, and 2mls distilled water was added 20 seconds later. This mixture was left for 35 minutes, before being washed off with distilled water. Once the slides were dry, they were mounted with a drop of DPX Mountant, and a coverslip. This improves the refractive index and forms a reasonably permanent mount. A total of 300 to 400 cells were counted for each slide, and differential cell counts were carried out under a 100X magnification. The percentage eosinophils, neutrophils, basophils, monocytes

and lymphocytes were calculated for each animal in each of the three asthmatic models. This method, although time consuming, has been successfully used by many authors for determining BAL leukocyte levels (Tominaga, Watanabe et al. 1995; Hatzelmann, Haefner et al. 1996; Renzetti, Paciorek et al. 1996). Increased BAL leukocyte levels, in particular eosinophils, would act as a marker for asthmatic inflammation.

BAL leukocyte counts were written directly into lab books at the time of the experiments. Data was then analysed using Microsoft Excel and Graphpad Instat for statistical comparisons. All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests. Values were expressed as mean $\pm$ SEM with a P-value of less than 0.05 (compared to the positive control group) taken to be significant.

3.1.3 Materials

Sigma Aldrich, Airton Road, Tallaght, Dublin, Ireland.

OVA (Grade 5 chicken egg albumin), AlOH, FCA(T) (contains heat killed and dried *Mycobacterium tuberculosis*), PBS, CycA, CMC and DPX mountant were obtained from Sigma Aldrich.

Lennox Chemicals Ltd, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.

Leishmans stain was supplied to Lennox Chemicals Ltd. by BDH, Laboratory Supplies, Poole, BH15 1TD, England.

Bioresources Unit, TCD, Ireland.

Halothane, male Wistar rats, male Sprague Dawley rats and male Brown Norway rats (250-350gms) were obtained from the Bioresources unit (BRU). Male Brown Norway rats were supplied to BRU by Harlan, U.K. All animals were housed at a room temperature of 22°C with a 12hr light/dark cycle.

3.1.4 Results

Despite successful attempts by other authors to use SD rats for models of allergic asthma and lung injury (Koh, Choi et al. 2001; Savani, Godinez et al. 2001) as well as the successful use of Wistar rats in unpublished results for a respiratory asthma model in our laboratory, neither strain was effective in any of the 3 asthmatic models detailed in **section 3.1.2**. This was seen in the BAL samples from each strain, in which no significant difference in leukocyte levels was found following OVA challenge. Brown Norway rats did however prove effective in each of these models and were therefore chosen to examine the effects of the test compounds as outlined below.

3.1.4.1 Brown Norway rats sensitised to OVA using AlOH as an adjuvant

The leukocyte numbers (mean $\pm$ SEM of % leukocytes in total cell count) seen in BAL fluid taken from BN rats sensitised to OVA using AlOH as an adjuvant are shown in **table 3.1.4.1 (appendix 2)**, and illustrated in **figures 3.1.4.1 (a) and 3.1.4.1 (b)**. A magnified photograph of a slide prepared from the BAL fluid of one of these BN rats is shown in **figure 3.1.4.1 (c)**. The 4 groups are the negative control (–ve ctrl;

untreated and unchallenged), the positive control (+ve ctrl; untreated and OVA challenged), CycA (10mg/kg) and 3C8 (10mg/kg).

As shown in **table 3.1.4.1 (appendix 2)** and **figure 3.1.4.1 (a),** the percentage neutrophils found in the +ve ctrl group was more than 10 times greater than in the –ve ctrl group, and this was statistically significant ($P<0.0001$). CycA reduced this value greatly, to a number closer to the –ve ctrl value, and was also found to be statistically significant ($P<0.0001$). 3C8 was almost as effective as CycA at reducing neutrophil levels ($P=0.0001$).

A statistically significant increase was also seen in the percentage eosinophils (+ve ctrl group). A greater than 20-fold increase was seen compared to the –ve ctrl group ($P<0.0001$). Following CycA treatment, eosinophil levels were more than 4 times smaller than the positive control value ($P<0.0001$), while 3C8 also significantly reduced positive control eosinophils ($P=0.002$).

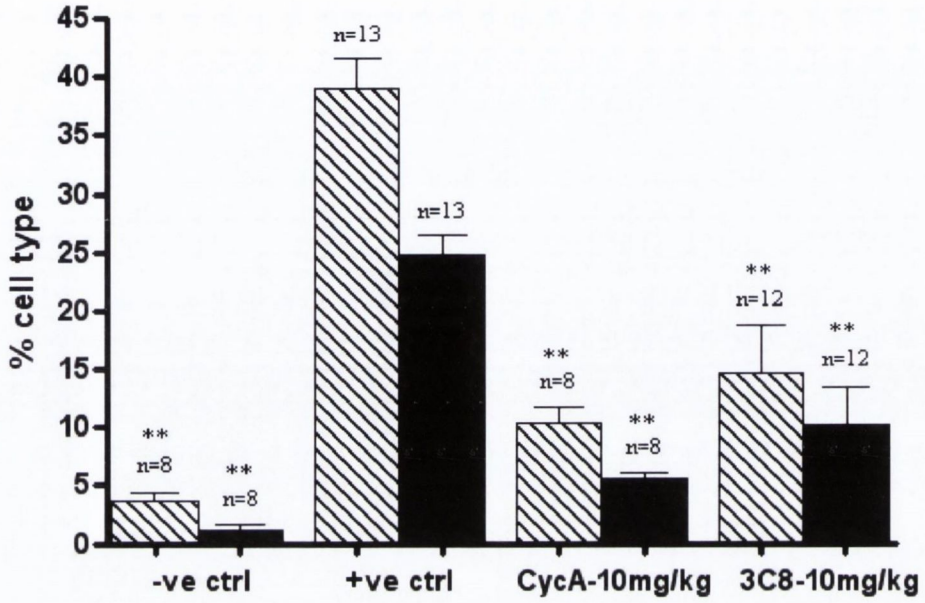
In summary {**table 3.1.4.1 (appendix 2)** and **figure 3.1.4.1 (a)**}, a statistically significant increase in both neutrophil and eosinophil levels was seen upon challenge with OVA, following sensitisation with OVA using AlOH as an adjuvant. The levels of both leukocytes were found to be significantly reduced by both CycA and 3C8 (10mg/kg) compared to the positive control. All were statistically significant ($P<0.05$).

The levels of small lymphocytes, large lymphocytes and monocytes are illustrated in **figure 3.1.4.1 (b)** and again shown in **table 3.1.4.1 (appendix 2)**. Small lymphocyte

levels in the -ve ctrl group were about twice as big as in the +ve ctrl group ($P< 0.0001$). This was also the case for both the CycA group ($P=0.003$) and 3C8 group ($P=0.001$). Large lymphocyte levels were similar in the –ve and +ve ctrl groups ($P=0.414$). However, a significant difference was seen in both the CycA group ($P=0.005$) and 3C8 group ($P=0.001$), compared to the +ve ctrls. The percentage monocytes was significantly different in the –ve ctrl group ($P<0.0001$), CycA group ($P<0.0001$) and 3C8 group ($P=0.001$).

To summarise the results for these 3 leukocytes {**table 3.1.4.1 (appendix 2)** and **figure 3.1.4.1 (b)**}, a statistically significant difference was seen in all 3 leukocyte levels in both the CycA and 3C8 groups, compared to the +ve ctrls. This was also the case for the –ve ctrl group for all 3 leukocytes, except for the large lymphocytes.

Figure 3.1.4.1 (a): Percentage neutrophils and eosinophils in BAL fluid taken from Brown Norway rats sensitised to OVA using AlOH as an adjuvant



This graph represents the percentage of neutrophils and eosinophils (mean ± SEM) found in the BAL fluid taken from BN rats sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.

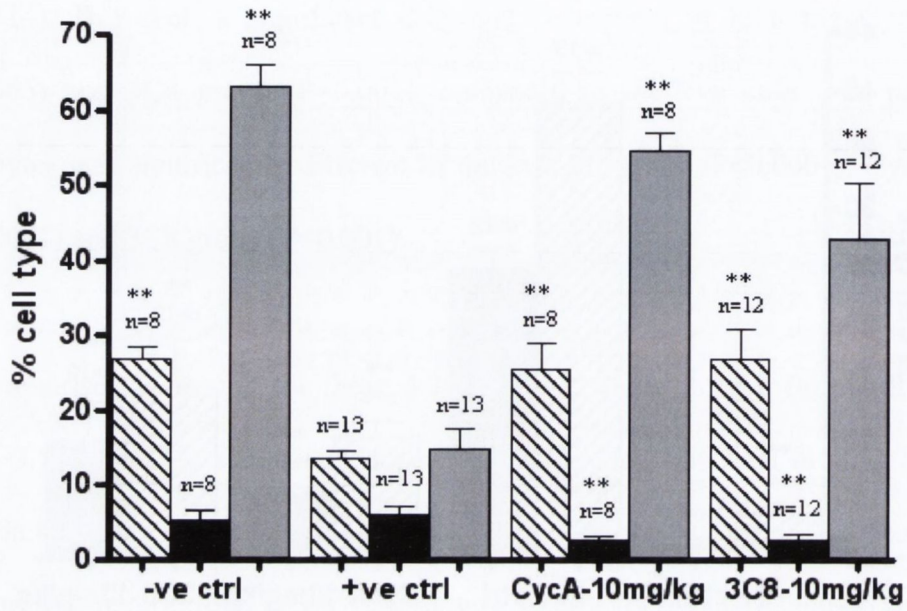
▨ = neutrophils; ■ = eosinophils

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-4 groups at a time with n=6 per group.

Figure 3.1.4.1 (b): Percentage lymphocytes and monocytes in BAL fluid taken from Brown Norway rats sensitised to OVA using AlOH as an adjuvant



This graph represents the percentage of small lymphocytes, large lymphocytes and monocytes (mean ± SEM) found in the BAL fluid taken from BN rats sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.

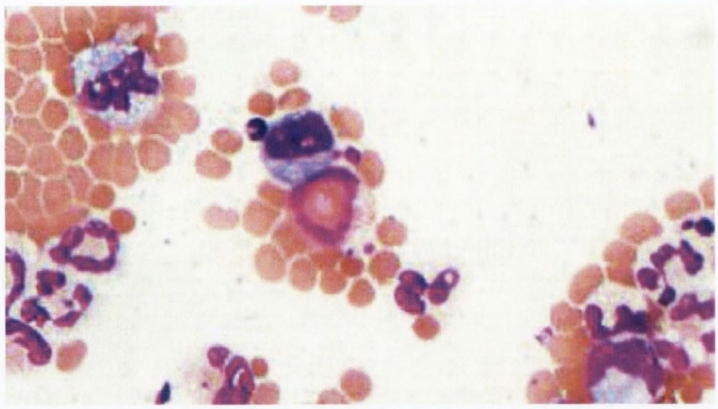
▨ = small lymphocytes; ■ = large lymphocytes; ▭ = monocytes

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-4 groups at a time with n=6 per group.

Figure 3.1.4.1 (c): Eosinophils and neutrophils in the BAL fluid of a BN rat sensitised to OVA using AlOH as an adjuvant



An eosinophil at the centre of the picture is characterised by a purple ring-shaped nucleus and red granules. Neutrophils at the left and right of the picture are similar in size and structure, with a multi-lobed nucleus but without the red granules in the cytoplasm.

3.1.4.2 Brown Norway rats sensitised to OVA using FCA(T) as an adjuvant

The leukocyte numbers (mean $\pm$ SEM of % leukocytes in total cell count) found in BAL fluid taken from BN rats sensitised to OVA using FCA(T) as an adjuvant are shown in **table 3.1.4.2 (appendix 2)**, and illustrated in **figures 3.1.4.2 (a) and 3.1.4.2 (b)**. The experiment was performed in exactly the same way as for section **3.1.4.1,** except that FCA(T) was used as the adjuvant for OVA sensitisation, instead of AlOH. The 4 groups are the negative control (–ve ctrl; untreated and unchallenged), the positive control (+ve ctrl; untreated and OVA challenged), CycA (10mg/kg) and 3C8 (10mg/kg).

In **table 3.1.4.2 (appendix 2)** and **figure 3.1.4.2 (a)**, we can see that there was a statistically significant increase in neutrophils in the +ve ctrl group compared to the -ve ctrl group ($P<0.0001$). CycA reduced this positive control value by more than half, and was also found to be statistically significant ($P=0.001$). 3C8 was very nearly as effective as CycA at reducing neutrophil levels ($P=0.0003$). Eosinophil levels in all groups were far lower than neutrophil levels. However, a statistically significant increase was seen in the +ve ctrl group ($P<0.0001$). Eosinophil levels in the CycA group were almost identical to the positive control value ($P>0.999$), while values in the 3C8 group were even higher than the positive control ($P=0.905$). No statistically significant difference was found in comparing eosinophil levels of positive controls to either CycA or 3C8.

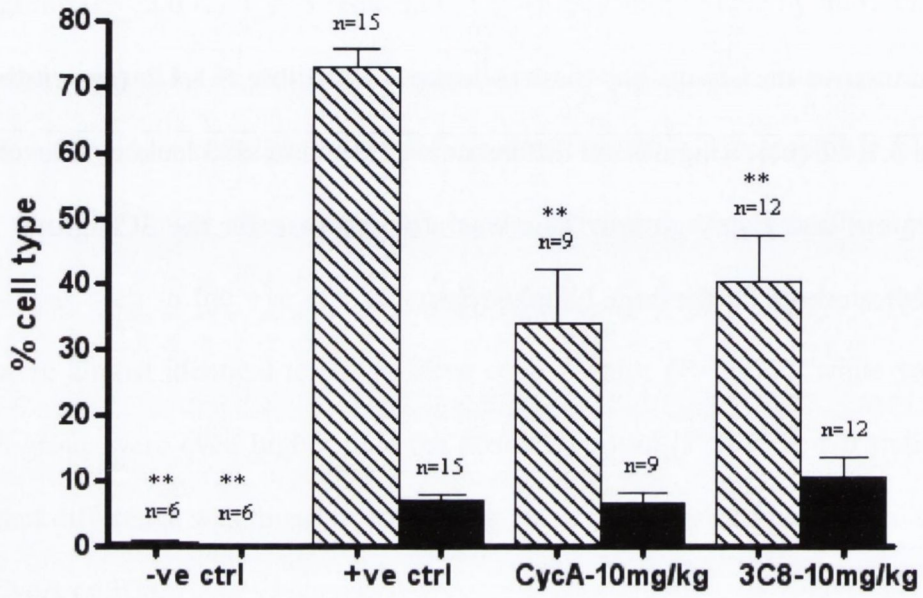
In summary {**table 3.1.4.2 (appendix 2)** and **figure 3.1.4.2 (a)**}, a statistically significant increase in both neutrophil and eosinophil levels was seen upon challenge with OVA, following sensitisation with OVA using FCA(T) as an adjuvant. Neutrophil levels were found to be greatly reduced by both CycA and 3C8 (10mg/kg) compared to the positive control, and both were statistically significant. Neither CycA nor 3C8 significantly reduced positive control eosinophil levels.

The levels of small lymphocytes, large lymphocytes and monocytes are shown in **table 3.1.4.2 (appendix 2)** and illustrated in **figure 3.1.4.2 (b)**. Small lymphocyte levels were significantly lower in the -ve ctrl group compared to the +ve ctrl group ($P=0.045$). Levels were significantly higher in both the CycA ($P=0.007$) and 3C8 groups ($P=0.007$). Large lymphocytes were significantly smaller in number in both

the –ve ctrl (P=0.007) and CycA groups (P=0.043), although not in the 3C8 group (P=0.113). Monocyte levels were significantly greater in the –ve ctrl (P<0.0001), CycA (P=0.003) and 3C8 groups (P<0.0001).

To summarise the results for these 3 leukocytes {**table 3.1.4.2 (appendix 2)** and **figure 3.1.4.2 (b)**}, a significant difference was found in all 3 leukocyte levels in both the -ve ctrl and CycA groups. This was also the case for the 3C8 group for all 3 leukocytes except for the large lymphocytes.

Figure 3.1.4.2 (a): Percentage neutrophils and eosinophils in BAL fluid taken from Brown Norway rats sensitised to OVA using FCA(T) as an adjuvant



This graph represents the percentage of neutrophils and eosinophils (mean ± SEM) found in the BAL fluid taken from BN rats sensitised and challenged to OVA, using FCA(T) as an adjuvant for sensitisation.

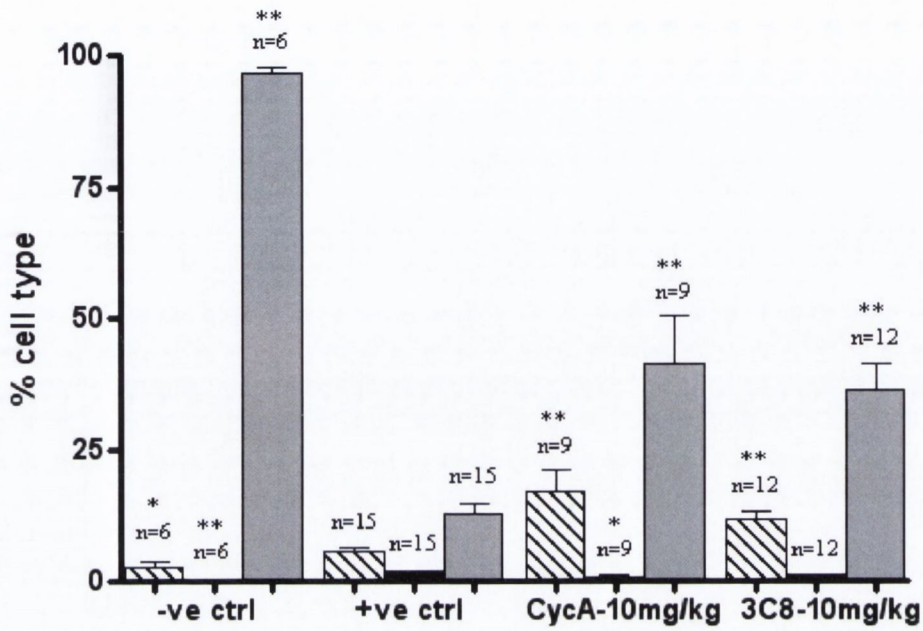
▨ = neutrophils; ■ = eosinophils

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-4 groups at a time with n=6 per group.

Figure 3.1.4.2 (b): Percentage lymphocytes and monocytes in BAL fluid taken from Brown Norway rats sensitised to OVA using FCA(T) as an adjuvant



This graph represents the percentage of small lymphocytes, large lymphocytes and monocytes (mean ± SEM) found in the BAL fluid taken from BN rats sensitised and challenged to OVA, using FCA(T) as an adjuvant for sensitisation.

▨ = small lymphocytes; ■ = large lymphocytes; ▭ = monocytes

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-4 groups at a time with n=6 per group.

3.1.4.3 Brown Norway rats sensitised to OVA using both AlOH and FCA(T) as adjuvants

The leukocyte numbers (mean ± SEM of % leukocytes in total cell count) found in BAL fluid taken from BN rats sensitised to OVA using both AlOH and FCA(T) as adjuvants are shown in **table 3.1.4.3 (appendix 2)**, and illustrated in **figures 3.1.4.3 (a) and 3.1.4.3 (b)**. The experiment was performed in exactly the same way as for

sections **3.1.4.1** and **3.1.4.2,** except that AlOH and FCA(T) were used in combination as the adjuvants for OVA sensitisation. The 4 groups are the negative control (–ve ctrl; untreated and unchallenged), the positive control (+ve ctrl; untreated and OVA challenged), CycA (10mg/kg) and 3C8 (10mg/kg).

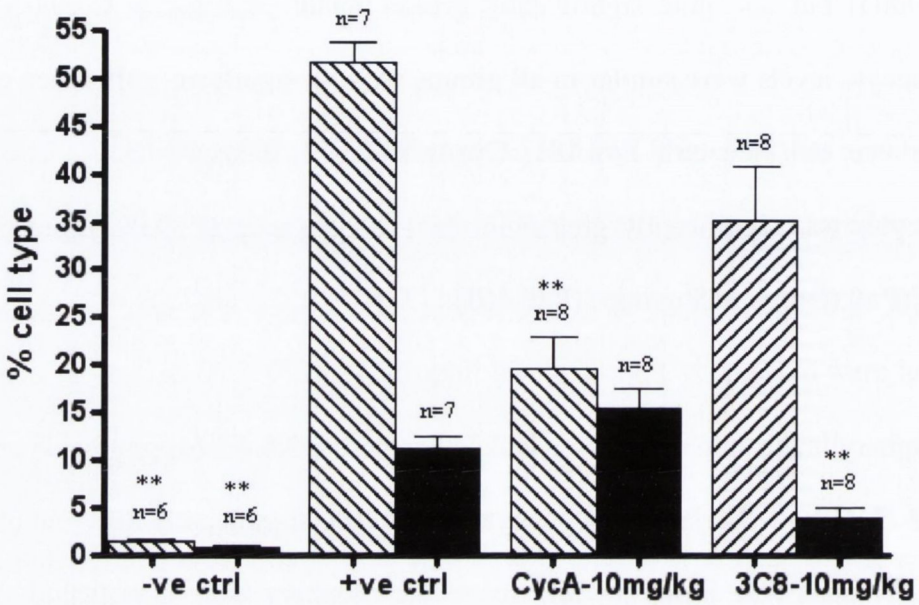
From **table 3.1.4.3 (appendix 2)** and **figure 3.1.4.3 (a),** it is clear that a statistically significant increase in neutrophils was seen in the +ve ctrl group compared to the –ve ctrl group, as was the case when FCA(T) and AlOH were used separately as adjuvants for OVA sensitisation (P=0.001). Neutrophil levels in the CycA group were less than 2.5 times the positive control value and this was also statistically significant (P=0.0003). 3C8 did not significantly reduce neutrophil levels (P=0.054). Eosinophil levels were also significantly increased in the +ve ctrl group (P=0.001). CycA did not reduce this positive control value at all (P=0.072). However, eosinophil levels in the 3C8 group were almost 3 times smaller than in the positive control group, and this was found to be statistically significant (P=0.001).

In summary {**table 3.1.4.3 (appendix 2)** and **figure 3.1.4.3 (a)**}, a statistically significant increase in both neutrophil and eosinophil levels was seen upon challenge with OVA, following sensitisation with OVA using both AlOH and FCA(T) as adjuvants. Neutrophil levels were found to be greatly reduced by CycA compared to the positive control, and this was statistically significant, while 3C8 did not significantly reduce the positive control value. The opposite was the case in comparing eosinophil levels. CycA did not significantly reduce eosinophil levels compared to the positive control, whereas 3C8 did significantly reduce this value.

The levels of small lymphocytes, large lymphocytes and monocytes are shown in **table 3.1.4.3 (appendix 2)** and illustrated in **figure 3.1.4.3 (b)**. Small lymphocyte numbers were significantly higher in both the –ve ctrl (P=0.001) and CycA groups (P=0.0003) but not quite significantly greater in the 3C8 group (P=0.054). Large lymphocyte levels were similar in all groups with no significant difference compared to the +ve ctrl (-ve ctrl; P=0.181, CycA; P=0.094, 3C8; P=0.336). The level of monocytes was significantly greater in the –ve ctrl group (P=0.001), but not in the CycA (P=0.094) or 3C8 groups (P=0.463).

In summary, the results for these 3 leukocytes {**table 3.1.4.3 (appendix 2)** and **figure 3.1.4.3 (b)**} show that small lymphocyte levels were significantly different only in the –ve ctrl and CycA groups, while monocyte numbers were significantly different solely in the –ve ctrl group. Large lymphocyte levels were very similar in all groups.

Figure 3.1.4.3 (a): Percentage neutrophils and eosinophils in BAL fluid taken from Brown Norway rats sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the percentage of neutrophils and eosinophils (mean ± SEM) found in the BAL fluid taken from BN rats sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.

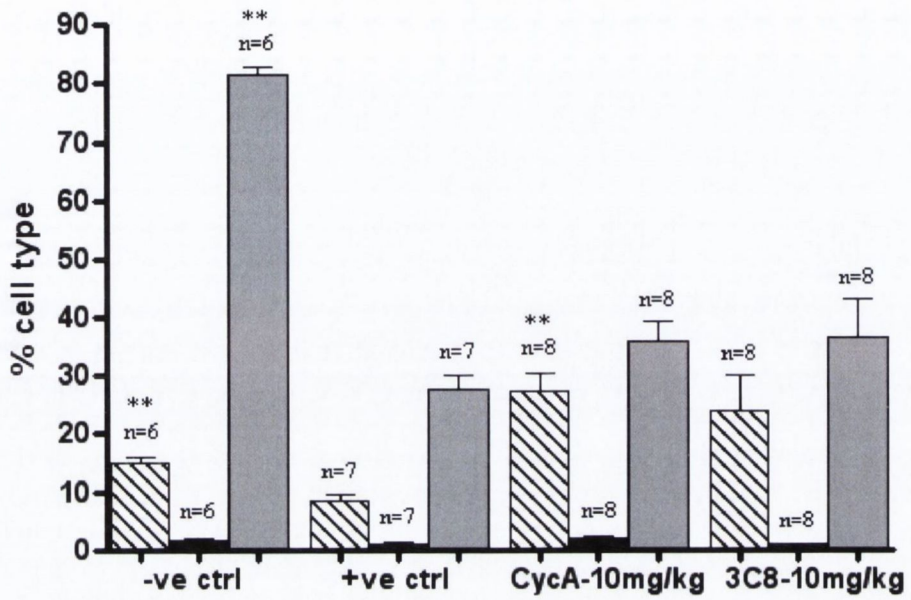
▨ = neutrophils; ■ = eosinophils

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-4 groups at a time with n=6 per group.

Figure 3.1.4.3 (b): Percentage lymphocytes and monocytes in BAL fluid taken from Brown Norway rats sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the percentage of small lymphocytes, large lymphocytes and monocytes (mean ± SEM) found in the BAL fluid taken from BN rats sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.
▨ = small lymphocytes; ■ = large lymphocytes; ▭ = monocytes
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 2-4 groups at a time with n=6 per group.

3.1.5 Discussion

In vivo models of asthma were set up in Wistar, Sprague Dawley (SD) and Brown Norway (BN) rats. Rats were sensitised to OVA using AlOH, FCA(T) or both as adjuvants. Sensitisation to OVA using AlOH as an adjuvant is believed to induce a Th2 response whereas using FCA(T) as an adjuvant is believed to induce a Th1 response (Hatzelmann, Haefner et al. 1996; Roman and Moreno 1997; Sinha, Verma et al. 1997; Murray 1998; Wagers, Lundblad et al. 2004). It was therefore hoped that a

Th2 model of extrinsic asthma, a Th1 model of intrinsic asthma and a mixed Th1/Th2 model (sensitisation to OVA using both adjuvants) could be established. Since 3C8 was already found to be effective in 2 previous Th1 models of delayed type hypersensitivity (the mBSA and SRBC models), further examination of this compound along with CycA in each of these asthmatic models would hopefully be of benefit in determining its exact mode(s) of action.

Despite positive results for Wistar rats in previous unpublished results for a respiratory asthma model and the successful use of SD rats in models of allergic asthma and lung injury (Koh, Choi et al. 2001; Savani, Godinez et al. 2001), neither strain proved effective here, with no increase in leukocyte levels found in any of the 3 asthmatic models. It is impossible to say for sure why this was although it could be explained by variations in experimental technique. Different time intervals between sensitisation and challenging or differences in the concentration of OVA or adjuvant used could account for these results. In contrast, leukocyte levels were significantly increased in all 3 asthmatic models in BN rats. Due to this success, this strain was chosen to examine the effects of our test compounds.

3.1.5.1 Brown Norway rats sensitised to OVA using AlOH as an adjuvant (Th2 model of extrinsic asthma)

Neutrophil levels in the positive control or challenged group were more than 10 times higher than in the negative control or unchallenged group, reaching 39 $\pm$ 2.7% of the total BAL cell count. Meanwhile, eosinophil levels reached 24.7 $\pm$ 1.8% of the total cell count, a greater than 20-fold increase over the unchallenged group. In contrast,

Renzetti and co-workers found that neutrophil and eosinophil levels reached 20% and 40% respectively of the total BAL cell count upon OVA challenge (Renzetti, Paciorek et al. 1996). Such differences may be due to experimental variations such as the different time interval they used between sensitisation and challenging or the method used for OVA challenge by aerosol, which was not fully described by the authors. The levels of both leukocytes were significantly reduced by both CycA and 3C8. Approximately a 4-fold reduction in both neutrophils and eosinophils was found following CycA treatment. 3C8 was nearly as effective at reducing neutrophil levels while eosinophil levels were twice as high as in the CycA group although still significantly reduced.

As previously mentioned, CycA and tacrolimus bind to and inhibit calcineurin, an essential component for IL-2 gene transcription, thus inhibiting the Th1 response. However, this anti-Th1 effect would not explain the results found in this, a Th2 model. The potency of the immunosuppressant in this model may be explained by the fact that both CycA and tacrolimus have been found to inhibit the release of mediators and cytokines from inflammatory cells such as monocytes, eosinophils and mast cells (De Paulis, Stellato et al. 1992; Hatfield and Roehm 1992; Sperr, Agis et al. 1996). The fact that CycA may inhibit mast cell activity is of particular relevance to this model due to the role that mast cells play in the Th2 response and the late phase of extrinsic asthma. Platelet activating factor (PAF) and the cysteinyl leukotrienes (cLTS) increase vascular permeability (Dahlen, Bjork et al. 1981), allowing for migration of the leukocytes while PAF and leukotriene B_4 (LTB_4), also produced following mast cell degranulation, increase leukocyte adherance (Bochner 1997), therefore contributing further to leukocyte recruitment. The prostaglandins (PGs)

cause vasodilation, which will allow for a greater concentration of leukocytes at the site of inflammation (Salmon and Higgs 1987; Miyake, Yamamoto et al. 1993). In addition, many cytokines are produced from mast cells and these include the 3 main eosinophil-active cytokines, IL-3, IL-5 and GM-CSF. IL-5 acts specifically on mature eosinophils, contributing to their chemotaxis and degranulation (Meng, Ying et al. 1997). IL-4, IL-6 and TNFα are also produced from mast cells. IL-4 is essential for the Th2 response and is also capable of upregulating vascular cell adhesion molecule (VCAM1), the endothelial adhesion molecule necessary for eosinophil recruitment (Krishna, Chauhan et al. 1996) while IL-6 has positive feedback on eosinophils and mast cells. Meanwhile, TNFα upregulates a number of endothelial adhesion molecules, including VCAM-1 for eosinophils and endothelial leukocyte adhesion molecule (ELAM-1) for neutrophils, thus making it crucial for leukocyte recruitment and may also contribute directly to the late phase through assisting in tissue remodelling (Krishna, Chauhan et al. 1996; Kumar, Dhawan et al. 1998).

It is therefore clear that inhibition of mast cell activity, as was found for both CycA and tacrolimus (De Paulis, Stellato et al. 1992; Hatfield and Roehm 1992; Sperr, Agis et al. 1996) would inhibit the Th2 response and leukocyte recruitment in the late phase. This would explain the significant reduction in both neutrophils and eosinophils found following CycA treatment in this Th2 model. This effect may be related to inhibition of calcineurin since it has been identified as a necessary target for the inhibition of mast cell degranulation as caused by both drugs (Toyota, Hashimoto et al. 1996; Hultsch, Brand et al. 1998).

As mentioned in **section 1.4**, immunosuppressants usually act in the induction phase of the immune response, inhibiting lymphocyte proliferation. In a similar eosinophil model in BN rats, CycA has been found to reduce eosinophil levels in BAL fluid when given during the induction phase but was not effective when given in the effector phase (Tominaga, Watanabe et al. 1995). CycA was also effective in other OVA sensitised and challenged BN rats, inhibiting eosinophil and lymphocyte influx but without affecting bronchial responsiveness to inhaled acetylcholine, in contrast to dexamethasone which did both (Elwood, Lotvall et al. 1992). This would suggest that inhibition of T-lymphocyte activation and eosinophil recruitment in this model is not sufficient to inhibit the induction of airway hyper-responsiveness (AHR). Dexamethasone was more effective, probably due to the wider range of actions of such corticosteroids on inflammatory cells, meaning that such drugs may be more potent in airway diseases such as asthma. Tigani and co-workers found that in a similar OVA/AlOH model, AHR to adenosine was reduced by the corticosteroid budesonide and by IMM125 and MLD987, analogues of CycA and tacrolimus respectively (Tigani, Hannon et al. 2003). It is likely that these analogues act in a similar way to CycA and tacrolimus through mast cell inhibition since hypersensitivity to adenosine develops following 5-hydroxytryptamine (5-HT) production from mast cells. Of these 3 drugs, only budesonide reduced levels of eosinophils, neutrophils and neutrophil myeloperoxidase (MPO), providing further evidence of the greater range of effects and therefore suitability of corticosteroids for asthma treatment.

3C8, found to have an anti-Th1 effect in the mBSA and SRBC models of DTH also had a significant impact here, significantly reducing both eosinophil and neutrophil

levels. Since the calcium/calmodulin complex seems to be essential for both smooth muscle contraction (as described in **section 1.1.2.1.1.5**) and even more significantly with respect to these results, for mast cell degranulation (described in **section 1.1.2.1.1.2**), a reduction in the availability of i.c. calcium or inhibition of calcium/calmodulin binding by 3C8 could explain the reduction in these leukocytes found in the BAL fluid. The resulting inhibition of mast cell degranulation would inhibit production of the cytokines, PGs and LTs that contribute to leukocyte recruitment as described above. Certain cytokines produced from mast cells may be released in part from preformed stores (for example TNFα), some of which may be associated with the cytoplasmic granules of the cell, while others may be newly synthesised (Li, Li et al. 1998; Williams and Galli 2000). Mast cell degranulation is associated with the activation of G proteins that cause actin polymerisation and relocalisation (Abraham, Thankavel et al. 1997; Church, Levi-Schaffer 1997; Kalesnikoff, Huber et al. 2001). Since these events are also accompanied by the transcription of cytokine genes, inhibition of degranulation would not only result in the reduced release of preformed cytokines, but also in a reduced production of newly synthesised cytokines.

3C8 may even act in a similar manner to CycA and tacrolimus through inhibition of calcineurin. As previously mentioned, calcineurin is a calcium/calmodulin-dependant phosphatase, which is not only essential for the Th1 response but is also a necessary target for the inhibition of mast cell degranulation as caused by both of these immunosuppressants (Almawi and Melemedjian 2000). Therefore, this potential mode of action could explain the potency of 3C8 in both the Th1 models of DTH and this Th2 model of extrinsic asthma.

Meanwhile, the levels of small lymphocytes and monocytes were significantly increased in the CycA and 3C8 treated groups, compared to the positive control group. This could be explained by the significant reduction in levels of eosinophils and neutrophils, thereby increasing the percentage of other leukocytes in the BAL fluid. Levels of large lymphocytes were significantly decreased in both groups although they were still small in number and were generally erratic.

3.1.5.2 Brown Norway rats sensitised to OVA using FCA(T) as an adjuvant (Th1 model of intrinsic asthma)

Rats were treated in the same manner as for the previous protocol, using FCA(T) instead of AlOH as an adjuvant for sensitisation. In this way, a theoretical model of intrinsic asthma was set up since FCA(T) is believed to induce a Th1 response (Dannenberg 1991; Kaufmann 1995; Lucey, Clerici et al. 1996; Roman and Moreno 1997; Sinha, Verma et al. 1997; Murray 1998). Therefore, we might expect compounds that were effective in the Th1 models of DTH to be effective here and such compounds may be effective in treating intrinsic asthma.

As for the Th2 model, both neutrophil and eosinophil levels were significantly increased in the positive control group. However, neutrophil levels reached 73.1 $\pm$ 2.6% of the total leukocyte count, almost double what was found in the previous model. Conversely, eosinophil levels only reached 6.8 $\pm$ 1.1%, only one quarter of the levels found in the Th2 model. Both CycA and 3C8 significantly reduced neutrophil levels with no significant impact on eosinophil levels.

Inhibition of calcineurin by CycA, as mentioned in **section 3.1.5.1** would inhibit the Th1 response as well as mast cell degranulation and we might therefore have expected the drug to be effective in both Th1 and Th2 models. This was confirmed by the greater than 50% reduction in neutrophils although eosinophils were unaffected. The fact that 3C8 had a very similar impact on both of these leukocytes may mean that it is acting in the same manner as CycA as previously described. This is further evidence of the anti-Th1 effect of the drug as was already found in the mBSA and SRBC models of DTH. The insignificant impact of either drug on eosinophil levels could be due to the low levels of the leukocyte found in the positive control. A higher value would have been expected here since eosinophils accumulate in both extrinsic and intrinsic asthma and are even more prominent in the latter, which should theoretically be represented in this Th1 model. It is possible that some eosinophils may not have been clearly stained, meaning that the red granules might not have been visible and some could therefore have been mistaken for neutrophils. If this was the case, the neutrophil levels would be raised and the eosinophil levels reduced, as was seen here. However, it should be stressed that this model may not necessarily be an accurate representation of intrinsic asthma and has not been confirmed as such.

Small lymphocyte and monocyte levels were significantly increased following treatment with both CycA and 3C8, compared to the positive control group, as was found in the Th2 model. This could again be due to the decreased levels of other leukocytes, in this case neutrophils, therefore raising the proportion of other leukocytes in the BAL fluid. Meanwhile, large lymphocytes were significantly reduced by CycA and not by 3C8 although levels were again low in all groups and were quite erratic.

3.1.5.3 Brown Norway rats sensitised to OVA using both AlOH and FCA(T) as adjuvants (Th1/Th2 mixed model)

The final rat model set up to examine the effects of CycA and 3C8 on leukocyte levels in the BAL fluid involved the use of both AlOH and FCA(T) as adjuvants for sensitisation. Since these are thought to induce Th2 and Th1 responses respectively, it was hoped that this model would represent a mixed Th1/Th2 response. In this way, the mode(s) of action of these compounds could be examined further in a third model.

Neutrophil and eosinophil levels were again significantly increased in the positive control group, reaching 51.7 $\pm$ 2.2% and 11.2 $\pm$ 1.3% of the total BAL cell count respectively. Interestingly, these values were between those found for the previous 2 models. It is possible that a mixed Th1/Th2 response has indeed occurred, resulting in leukocyte levels between those found in the Th1 and Th2 models. A 2.5-fold decrease in neutrophils was found following CycA treatment without any significant change in eosinophil levels. Conversely, a highly significant 3-fold decrease in eosinophil levels was found in the 3C8 treated group without any significant change in neutrophil levels.

It is interesting that only 3C8 was effective at reducing eosinophils here, giving further confirmation of its potential suitability for asthmatic treatment. As illustrated in **figure 1.1.2.4**, eosinophils contribute largely to the pathogenesis of both forms of the disease. Upon degranulation, cytotoxic proteins such as eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin are released and are believed to contribute to the development of AHR (Robinson 1996).

Elevated levels of such proteins have been found in the BAL fluid of asthmatic patients (Durham, Kay 1985; Diaz, Gonzalez et al. 1989). Meanwhile, eosinophil-derived cytokines also contribute to the pathogenesis of asthma. TNFα may contribute directly to AHR by causing tissue remodelling and subepithelial fibrosis, while also contributing to leukocyte recruitment by upregulating endothelial adhesion molecules (Krishna, Chauhan et al. 1996; Kumar, Dhawan et al. 1998). Leukotrienes, also produced upon eosinophil degranulation, contribute further to the development of AHR. Therefore, agents that are effective at inhibiting eosinophil recruitment, as 3C8 seems to be, may be of great benefit for asthma treatment.

Despite reducing eosinophil levels, 3C8 did not significantly reduce neutrophil levels. One possible explanation for this could be the specific inhibition of the VCAM1-very late antigen 4 (VLA4) interaction required for eosinophil recruitment which would have no effect on ELAM1, the endothelial adhesion molecule needed for neutrophil recruitment. However, neutrophil levels were reduced from 51.7 $\pm$ 2.2% to 35.1 $\pm$ 5.9% and this was only marginally not significant (P=0.054). In addition, levels of this leukocyte were significantly reduced in both previous asthmatic models. Despite elevated levels found in the BAL fluid of asthmatics, the role that neutrophils play in asthma is unknown and there are conflicting reports regarding the presence of MPO, an indicator of neutrophil activation, in the BAL fluid of asthmatics and in animal models (DuBuske 1995; Schneider and Issekutz 1996). Neutrophils cause tissue damage in many inflammatory diseases through the release of oxygen metabolites, proteases and cationic materials and have been found to contribute to T cell and monocyte recruitment to the site of inflammation (Taub, Anver et al. 1996). They can also undergo the nitric oxide synthase (NOS) and nicotinamide adenine dinucleotide

phosphate (NADPH) oxidase pathways, which lead to further tissue damage as described in **section 1.1.2.5**. Eicosanoids produced upon neutrophil degranulation (Lewis, Austin 1984) contribute to leukocyte recruitment as mentioned earlier. A significant decrease in neutrophil levels, as was found for 3C8 in almost all 3 asthmatic models might therefore make it even more suitable for the treatment of asthma, combined with the reduction in eosinophils as described above.

Of the 3 asthmatic models set up in BN rats, this was the only one in which the effects of CycA and 3C8 on neutrophil and eosinophil levels did not match. CycA significantly reduced neutrophil levels whereas 3C8 did not. This could be due to a more specific inhibition of eosinophil recruitment by 3C8 as mentioned above, with less impact if any on neutrophils. However, a reduction in neutrophils was seen for 3C8 and was almost significant. It is surprising that CycA was ineffective at reducing levels of eosinophils given its potent effects against both Th1 and Th2 responses through calcineurin inhibition. It should be noted however that although the response generated here is theoretically a mixed Th1/Th2 response, this cannot be assumed and the true response generated is unknown. Analysis of the levels of Th1 and Th2 cytokines in the BAL fluid and/or lung extracts after OVA challenge could be useful in determining the response(s) generated.

Once again, small lymphocyte and monocyte levels were elevated in both drug treatment groups, compared to the challenged, positive control group (although not always significantly) and this may be due to reduced neutrophil and/or eosinophil levels. Large lymphocyte levels were low with no significant change found in any group.

3.2: Mouse BAL

3.2.1: Mouse BAL and Cell Counts and lung extract preparation

3.2.1.1 Introduction

Following the successful attempts to elicit a response in each of the asthmatic models set up in BN rats, the decision was made to try to replicate these results in similar mouse models. In this way, the results found for CycA and 3C8 in the BN model would hopefully be repeated and confirmed in a second species. If successful, these compounds would then be tested at a second higher dose and other compounds would also be tested. Another reason for moving to mouse models to examine these compounds was the greater availability of mouse cytokine kits compared to rat kits, meaning that a more detailed analysis of the responses evoked in the asthmatic models as well as the effects of the compounds on these models could be obtained. With consistent results hopefully found in the mouse models, levels of eosinophil peroxidase (EPO) and myeloperoxidase (MPO) in lung extracts as well as levels of various cytokines in both BAL fluid and lung extracts would be measured.

Balb/C mice have been used successfully in models of allergic asthma by many authors using concentrations of OVA ranging from 10μg (Zuany-Amorim, Sawicka et al. 2002) to 100 μg (Finotto, De Sanctis et al. 2001; Kudlacz, Whitney et al. 2002; McKay, Leung et al. 2004) for sensitisation with various doses used for challenging and different time intervals employed. Other strains, such as the NC/Nga mice and

C57Black 6 mice may also be suitable. NC/Nga mice, used to replicate human atopic dermatitis, were found to successfully mimic human allergic asthma with increased plasma levels of OVA-specific IgE, eosinophil infiltration and AHR to acetylcholine found (Iwasaki, Tanaka et al. 2000). C57Black 6 mice showed increased levels of serum IgE as well as elevated levels of eosinophils as measured by EPO in BAL fluid (Konduri, Nandedkar et al. 2003). Therefore, either of these strains could potentially be used for models of allergic asthma.

However, Balb/C mice were chosen to try and consolidate the results found in the BN rats. They were chosen because they are the most commonly used strain in mouse models of allergic asthma. A scaled down version of the BN model was employed for sensitisation and challenging, using a modified version of the protocol used by Kudlacz and co-workers (Kudlacz, Whitney et al. 2002).

The aims of the asthmatic models set up in Balb/C mice were to hopefully repeat the results found for CycA and 3C8 in the Th2 model (using OVA/AlOH for sensitisation) and mixed Th1/Th2 model [using OVA/AlOH/FCA(T) for sensitisation] in BN rats, to then examine these compounds at higher doses and to examine the effects of 3C9 and tacrolimus on leukocyte numbers in the BAL fluid.

3.2.1.2 Methods

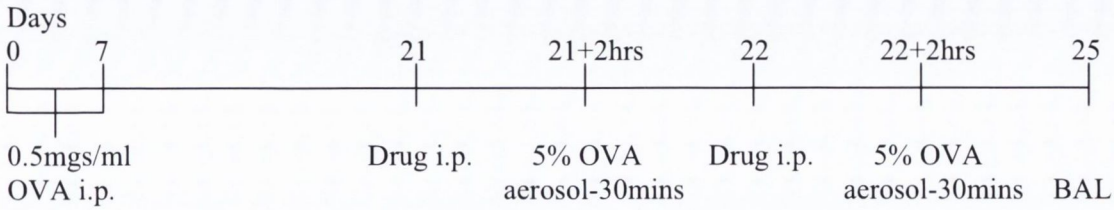
In Vivo Inflammatory Asthma Models in Balb/C mice

Female Balb/c mice were sensitised and challenged to OVA using two different asthmatic models. Animals were sensitised to OVA using either AlOH (Th2 inducer) or both AlOH and FCA(T) (Th1 inducer) as adjuvants, as was done for the Brown Norway rats. However, the concentrations of OVA and the adjuvants as well as the scheduling of sensitisation, dosing and challenging were different. The third model used for the Brown Norway rats, which involved sensitisation to OVA using FCA(T) as an adjuvant was not used for the Balb/C mice. All mice were housed in individually ventilated and filtered cages under positive pressure in specific pathogen-free (SPF) conditions.

The protocol used was a modified version of the one used by Kudlacz and co-workers (Kudlacz, Whitney et al. 2002). The only differences between the protocol used here and the one used by Kudlacz and co-workers were the concentration of OVA used for challenging and the time interval between drug dosing and OVA challenging. They used 3% OVA as an aerosol administered by a nebuliser 45 minutes after drug dosing whereas 5% OVA was used here and administered 2 hours after dosing.

As illustrated in **figure 3.2.1.2**, female Balb/C mice (30-40gms) were sensitised i.p. to OVA on days 0 and 7 of the experiment, using either AlOH or both AlOH and FCA(T) as adjuvants. The concentration of OVA and the adjuvants used for each of these two methods are shown in **tables 3.2.1.2 (a) and 3.2.1.2 (b)** (appendix 1). On days 21 and 22, mice were treated i.p. with either vehicle (0.5% CMC) or test compound. Two hours later, they were challenged with either vehicle (dH_20) or 5% OVA by nebuliser for 30 minutes. On day 25, BAL was carried out.

Figure 3.2.1.2: Schematic representation of protocol for mouse asthma models



This figure represents the timepoints at which animals were sensitised and challenged with OVA and dosed with test compound in mouse asthma models.

Mouse BAL Procedure and Cell Counts

On day 25, BAL was carried out. In order to eliminate blood from the preparation, the animals were first anaesthetised with halothane, opened by midline incision, and bled by cutting the *vena cava*. Following this procedure, which would kill the animals, the diaphragm was cut in order to deflate the lungs. Next, the trachea was exposed and cannulated. 1ml of PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4) was used to lavage the lungs (inserted using syringe). The PBS used contained a protease inhibitor tablet in 50mls, which would inhibit a broad range of proteases and facilitate the ELISA. After gently massaging the chest for 30 seconds, the fluid was withdrawn by syringe, reinserted, and finally collected, by allowing it to drip from the cannula into a vial. This was repeated so that 2mls PBS was used in total. When this was done, the pooled sample was immediately placed on ice. All samples were then centrifuged at 1000rpm for 6 minutes at room temperature, using a Sigma 204 centrifuge. The supernatant was decanted and frozen at –20°C until further analysis by ELISA. The pellet was resuspended in 1ml PBS for slide preparation.

100µl of the resuspended pellet of each sample was then spun onto a slide at 500rpm for 4 minutes at room temperature, using a Thermoshandon cytospin. The slides were

allowed to dry before staining with Leishmans stain. 1ml of stain was pipetted onto each slide, and 2mls distilled water was added 20 seconds later. This mixture was left for 35 minutes, before being washed off with distilled water. Once the slides were dry, they were mounted with a drop of DPX Mountant and a coverslip. This improves the refractive index and forms a reasonably permanent mount. A total of 300 to 400 cells were counted for each slide, and differential cell counts were carried out under a 100X magnification. The percentage eosinophils, neutrophils, basophils, monocytes and lymphocytes were calculated for each animal in each of the asthmatic models.

BAL leukocyte counts were written directly into lab books at the time of the experiments. Data was then analysed using Microsoft Excel and Graphpad Instat for statistical comparisons. All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests. Values were expressed as mean $\pm$ SEM with a P-value of less than 0.05 (compared to the positive control group) taken to be significant.

Mouse lung extract preparation

Lung extracts were prepared according to a slightly modified version of the protocol described by Schneider and co-workers (Schneider and Issekutz 1996). After BAL was performed, the lungs were removed and weighed. Samples of parenchyma from each lobe, approximating 10-20% of the total lung wet weight, were pooled and stored at –20°C until being freeze-dried. For enzyme extraction, lyophilised samples were weighed, homogenised in 50mM N-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid (Hepes), pH 8.0 at 0.5% dry w/v, spun at 10,000xg for 30 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in

0.5% cetyltrimethylammonium chloride (CTAC) solution in distilled water to the original volume, rehomogenised and spun again as before. This resulted in a pellet with a clear supernatant and a thin lipid layer on the top. An aliquot of the clear supernatant, referred to as lung extract, was taken for analysis of EPO and MPO activity. Extracts could either be analysed on the day of extraction or could be stored at –20°C without detectable loss in EPO or MPO activity over several months even after repeated freeze-thawing. The only modification to the protocol described by Schneider and co-workers was the temperature that the parenchyma samples were stored at. In their protocol, samples were stored at –70°C until being freeze-dried whereas here, samples were stored at –20°C (Schneider and Issekutz 1996).

3.2.1.3 Materials

Sigma Aldrich, Airton Road, Tallaght, Dublin, Ireland.

OVA (Grade 5 chicken egg albumin), AlOH, FCA(T) (contains heat killed and dried *Mycobacterium tuberculosis*), PBS, CycA, CMC, protease inhibitor tablets, DPX mountant and CTAC were obtained from Sigma Aldrich.

Fujisawa Ireland Limited, Killorglin, Co. Kerry, Ireland.

Tacrolimus was obtained from Fujisawa Ireland Ltd.

Lennox Chemicals Ltd, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.

Leishmans stain was supplied to Lennox Chemicals Ltd. by BDH, Laboratory Supplies, Poole, BH15 1TD, England.

HEPES was supplied to Lennox Chemicals Ltd. by Merck, KgaA, 64271 Darnstedt, Germany.

Bioresources Unit, TCD, Ireland.

Halothane and female Balb/C mice (30-40gms) were obtained from the Bioresources unit (BRU). All animals were housed at a room temperature of 22°C with a 12hr light/dark cycle.

3.2.1.4 Results

3.2.1.4.1 Balb/c mice sensitised to OVA using AlOH as an adjuvant

The leukocyte numbers (mean ± SEM of % leukocytes in total cell count) seen in BAL fluid taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant are shown in **table 3.2.1.4.1 (appendix 2)**, and illustrated in **figures 3.2.1.4.1 (a) and 3.2.1.4.1 (b)**. The 9 groups are the negative control (–ve ctrl; untreated and unchallenged), the positive control (+ve ctrl; untreated and OVA challenged), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

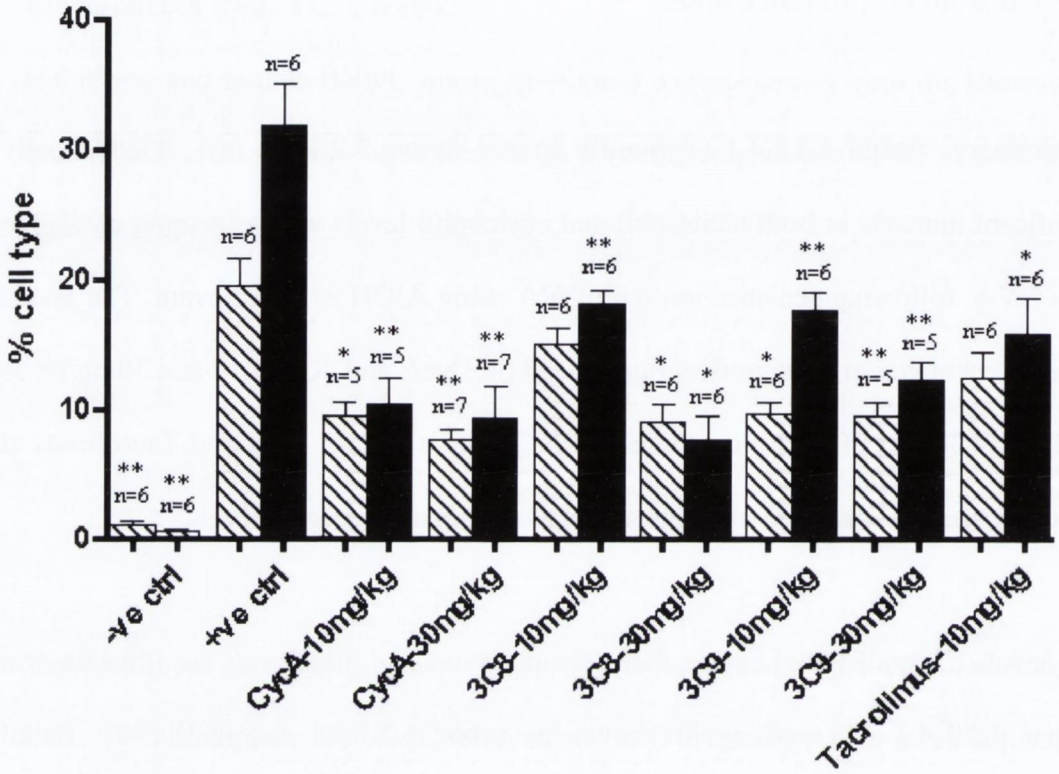
As shown in **table 3.2.1.4.1 (appendix 2)** and **figure 3.2.1.4.1 (a)**, neutrophil levels were significantly greater in the positive control group compared to the negative control group with almost a 20-fold increase seen (P=0.002). A significant reduction in this positive value was seen with CycA and 3C9 at both 10 and 30mg/kg as well as with 3C8 at 30mg/kg. All reduced the positive control value by half or more. 3C8 and

tacrolimus at 10mg/kg were not statistically significant at reducing this value. A very significant 60-fold increase in eosinophil levels was also seen in the positive control group (P=0.002). CycA, 3C8, 3C9 and tacrolimus were all found to significantly reduce this value at all tested doses.

In summary, {**table 3.2.1.4.1 (appendix 2)** and **figure 3.2.1.4.1 (a)**}, a statistically significant increase in both neutrophil and eosinophil levels was seen upon challenge with OVA, following sensitisation with OVA using AlOH as an adjuvant. The levels of both leukocytes were significantly reduced by CycA and 3C9 at 10 and 30mg/kg as well as by 3C8 at 30mg/kg compared to the positive control. 3C8 and Tacrolimus at 10mg/kg were only statistically significant at reducing eosinophil levels.

The levels of small lymphocytes, large lymphocytes and monocytes are illustrated in **figure 3.2.1.4.1 (b)** and again shown in **table 3.2.1.4.1 (appendix 2)**. Small lymphocyte levels were significantly different only in the CycA and 3C9 treated groups at 10mg/kg. There was no statistically significant difference in large lymphocyte levels in the negative control group or any drug treatment group. Conversely, a significant difference was seen in monocyte levels in all groups.

Figure 3.2.1.4.1 (a): Percentage neutrophils and eosinophils in BAL fluid taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the percentage of neutrophils and eosinophils (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.

▨ = neutrophils; ■ = eosinophils

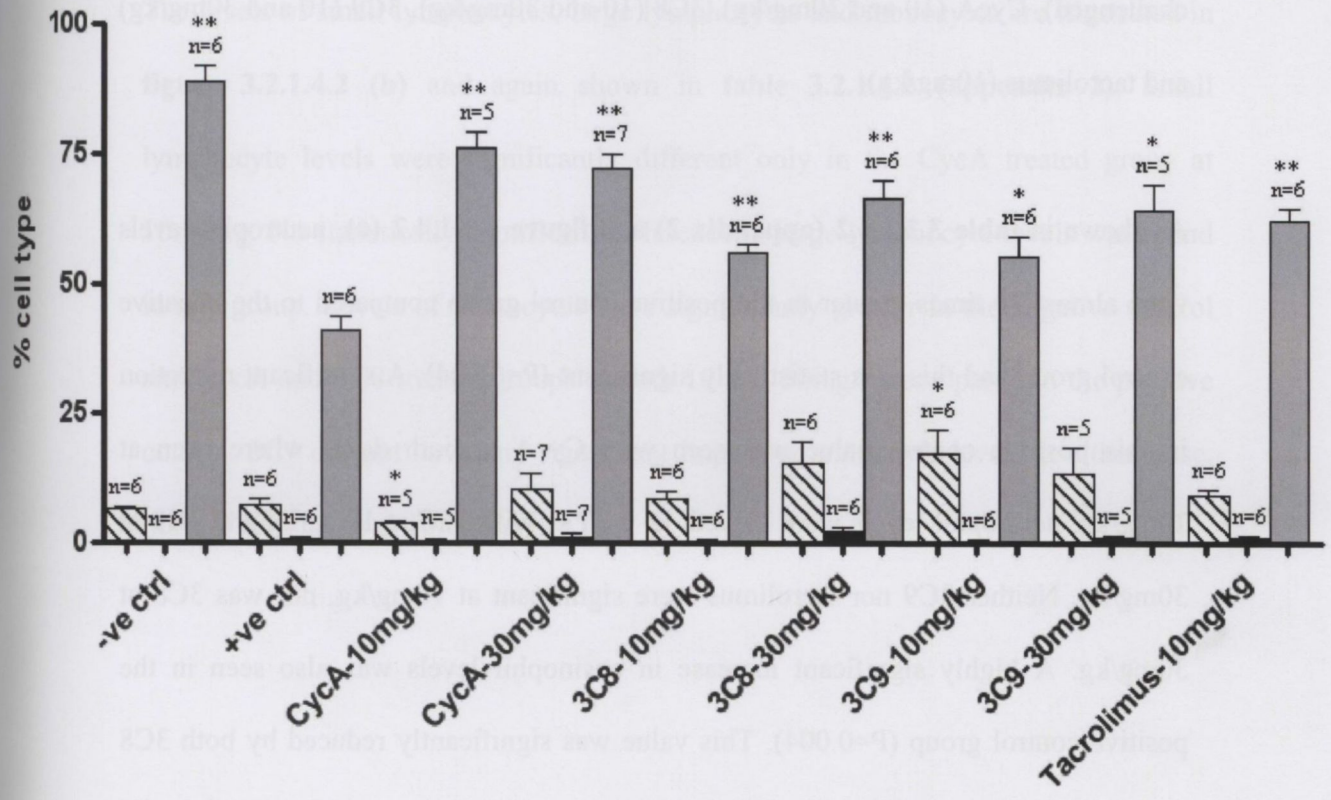
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-5 groups at a time with n=6 per group.

Figure 3.2.1.4.1 (b): Percentage lymphocytes and monocytes in BAL fluid taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the percentage of small lymphocytes, large lymphocytes and monocytes (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.

▨ = small lymphocytes; ■ = large lymphocytes; ▭ = monocytes

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-5 groups at a time with n=6 per group.

3.2.1.4.2 Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

The leukocyte numbers (mean ± SEM of % leukocytes in total cell count) seen in BAL fluid taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T)

as adjuvants are shown in **table 3.2.1.4.2 (appendix 2)**, and illustrated in **figures 3.2.1.4.2 (a) and 3.2.1.4.2 (b)**. The 9 groups are the negative control (–ve ctrl; untreated and unchallenged), the positive control (+ve ctrl; untreated and OVA challenged), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

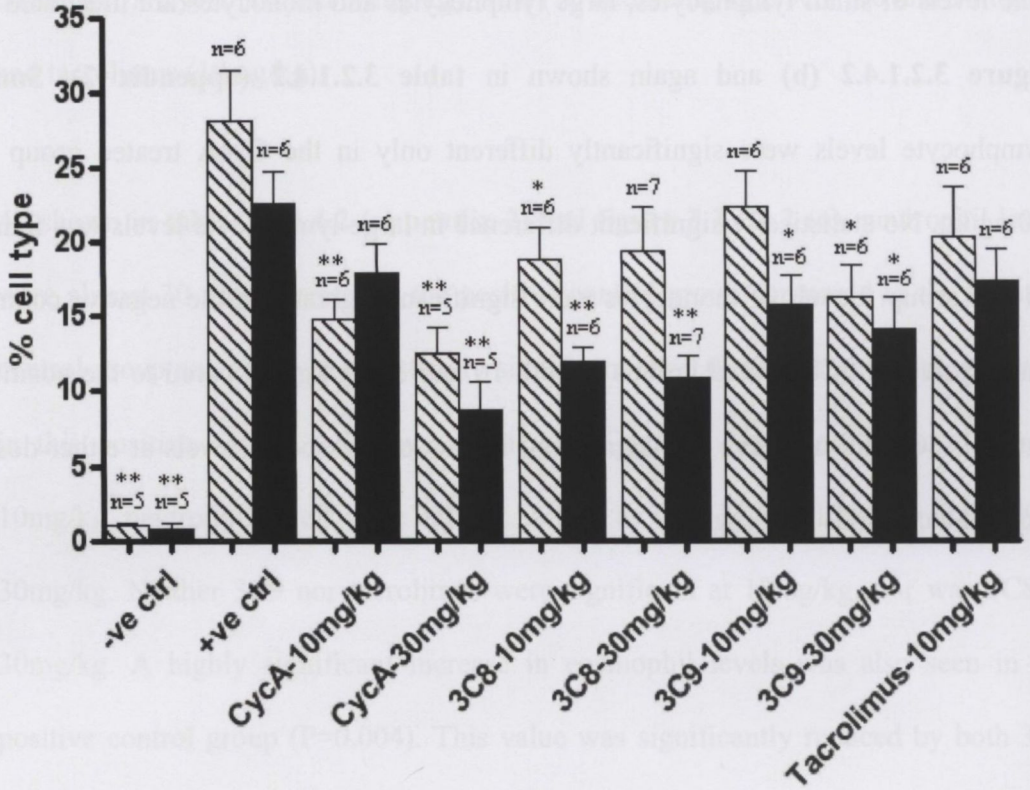
As shown in **table 3.2.1.4.2 (appendix 2)** and **figure 3.2.1.4.2 (a)**, neutrophil levels were almost 30 times greater in the positive control group compared to the negative control group and this was statistically significant (P=0.004). A significant reduction in this positive control value was seen with CycA at both doses where even at 10mg/kg, neutrophil levels were halved, as well as with 3C8 at 10mg/kg and 3C9 at 30mg/kg. Neither 3C9 nor tacrolimus were significant at 10mg/kg, nor was 3C8 at 30mg/kg. A highly significant increase in eosinophil levels was also seen in the positive control group (P=0.004). This value was significantly reduced by both 3C8 and 3C9 at 10 and 30mg/kg. Neither CycA nor tacrolimus had a statistically significant impact at 10mg/kg although CycA was significant at reducing eosinophil levels at 30mg/kg.

From **table 3.2.1.4.2 (appendix 2)** and **figure 3.2.1.4.2 (a)**, we see that a statistically significant increase in both neutrophil and eosinophil levels was seen upon challenge with OVA, following sensitisation with OVA using both AlOH and FCA(T) as adjuvants. The only compounds that were successful at significantly reducing both neutrophil and eosinophil levels were CycA and 3C9 at 30mg/kg as well as 3C8 at 10mg/kg. Tacrolimus did not significantly reduce the levels of either of these leukocytes. 3C8 at 30mg/kg and 3C9 at 10mg/kg were similar in that they both

significantly reduced eosinophil levels but not neutrophil levels. CycA at 10mg/kg was the opposite, significantly reducing levels of neutrophils but not eosinophils.

The levels of small lymphocytes, large lymphocytes and monocytes are illustrated in **figure 3.2.1.4.2 (b)** and again shown in **table 3.2.1.4.2 (appendix 2)**. Small lymphocyte levels were significantly different only in the CycA treated group at 10mg/kg. No statistically significant difference in large lymphocyte levels was found in any group. Levels of monocytes were significantly greater in the negative control and CycA and 3C8 treated groups at both 10 and 30mg/kg compared to the positive control. 3C9 did not have any significant impact on monocyte levels at either dose, nor did tacrolimus at 10mg/kg.

Figure 3.2.1.4.2 (a): Percentage neutrophils and eosinophils in BAL fluid taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the percentage of neutrophils and eosinophils (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.

▨ = neutrophils; ■ = eosinophils

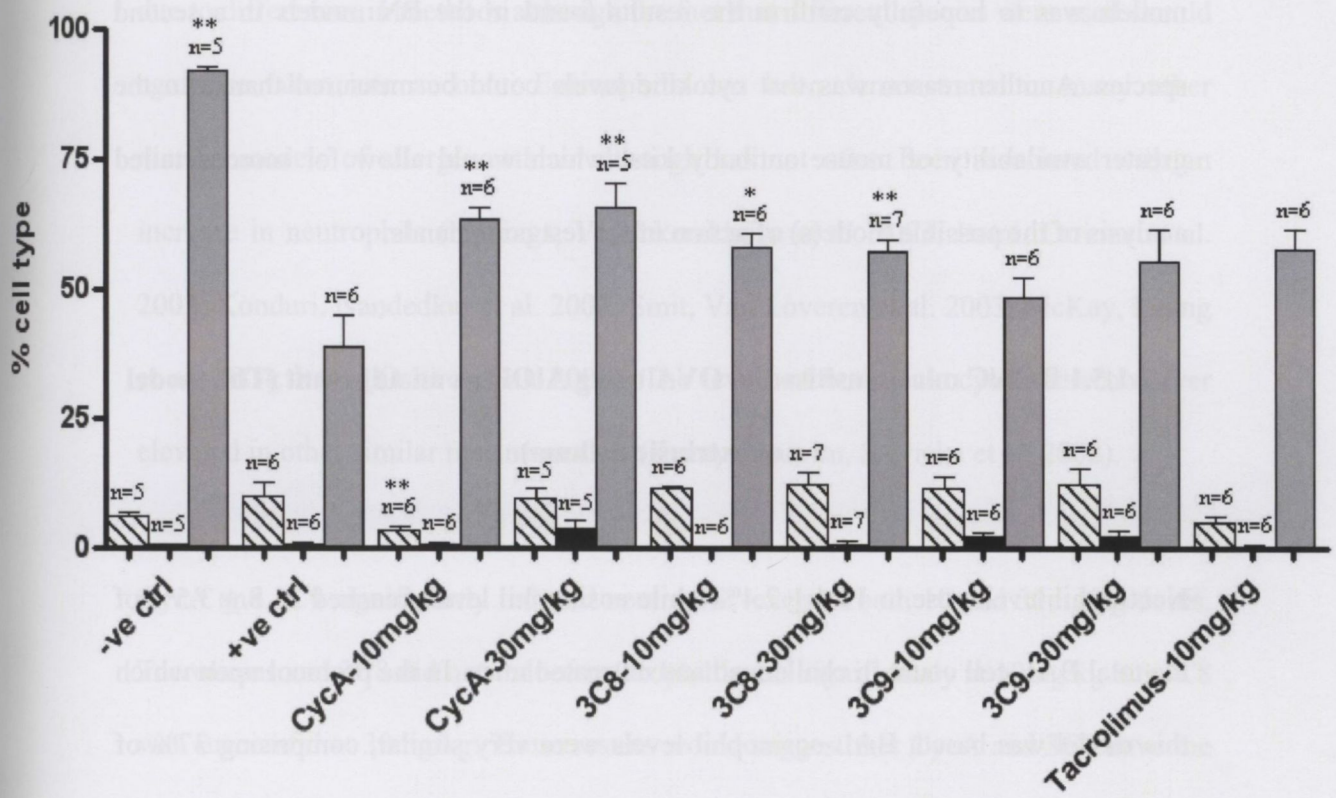
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-5 groups at a time with n=6 per group.

Figure 3.2.1.4.2 (b): Percentage lymphocytes and monocytes in BAL fluid taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the percentage of small lymphocytes, large lymphocytes and monocytes (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.

▨ = small lymphocytes; ■ = large lymphocytes; ▭ = monocytes

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Experiments were performed on 2-5 groups at a time with n=6 per group.

3.2.1.5 Discussion

2 *in vivo* models of asthma were set up in Balb/C mice. These involved sensitising mice to OVA using either AlOH or a combination of AlOH and FCA(T) as adjuvants,

as was done for BN rats. Sensitisation to OVA using AlOH as an adjuvant should generate a Th2 response while the combination of adjuvants may trigger a mixed Th1/Th2 response as described in **section 3.1.5**. One reason for setting up these models was to hopefully confirm the results found in the BN models in a second species. Another reason was that cytokine levels could be measured thanks to the greater availability of mouse antibody kits, which would allow for more detailed analysis of the possible mode(s) of action of the test compounds.

3.2.1.5.1 Balb/C mice sensitised to OVA using AlOH as an adjuvant (Th2 model of extrinsic asthma)

Neutrophil levels rose to 19.4 $\pm$ 2.4% while eosinophil levels reached 31.8 $\pm$ 3.5% of the total BAL cell count in challenged and untreated mice. In the protocol upon which this model was based, BAL eosinophil levels were very similar, comprising 37% of the white blood cell count although neutrophil levels were minimal (Kudlacz, Whitney et al. 2002). Such differences may be due to modifications to this protocol. In the method described here, 5% OVA was used for challenge and administered 2 hours after drug dosing whereas Kudlacz and co-workers used 3% OVA administered 45 mins after dosing. Perhaps the increased OVA concentration and longer time interval between dosing and challenging used here favoured neutrophil recruitment. This was the same time interval and OVA concentration used in the equivalent BN model (**section 3.1.5.1**) in which neutrophils were also significantly raised, which would support this theory. However, such differences may be due to change of species. It is interesting that neutrophils were higher than in the protocol used by Kudlacz and co-workers but lower than was found in the equivalent model in BN rats

while the reverse was found for eosinophils. All of this would suggest that the BN model was more effective for raising neutrophils whereas eosinophil levels may be more significantly elevated in the mouse model used by Kudlacz et al. This may be due to differences in methods although the fact that different species were used should again be taken into account. Eosinophilia has been demonstrated in many other murine models of allergic asthma although has not often been associated with an increase in neutrophils (Hellings, Vandekerckhove et al. 2003; Itami, Latinne et al. 2003; Konduri, Nandedkar et al. 2003; Smit, Van Loveren et al. 2003; McKay, Leung et al. 2004; Itami, Oshiro et al. 2005). The levels of both leukocytes were however elevated in other similar murine models (Zuany-Amorim, Sawicka et al. 2002).

CycA and 3C9 significantly reduced neutrophil levels at both 10 and 30mg/kg doses. Tacrolimus and 3C8 did not reduce neutrophil levels significantly at 10mg/kg but 3C8 was successful at 30mg/kg. These results would suggest that CycA and 3C9 have the greatest potency for reducing neutrophil levels in this model, with levels of the leukocyte reduced by at least 50% at both tested doses. In the equivalent BN model (Th2 model), CycA also had a significant impact on neutrophil levels when dosed at 10mg/kg. However, 3C8 was also quite potent at this dose in the rat model which was not the case in this mouse model. This may be due to the different species used and/or differences in experimental methods. As mentioned in **section 3.1.5.3**, neutrophils cause tissue damage in many inflammatory diseases but the role that they may play in asthma remains unknown. Eicosanoid production from neutrophils could potentially contribute to leukocyte recruitment, which occurs in both forms of the disease. Therefore, reducing the levels of this leukocyte may be of benefit in treating asthma.

Meanwhile, eosinophil levels were significantly reduced by all drugs at all tested doses. This correlates well with the results found in the Th2 model in BN rats, in which CycA and 3C8 also significantly reduced eosinophil levels at 10mg/kg. As previously mentioned, 3C8 may be acting through the inhibition of calcium/calmodulin formation since this complex is required for mast cell degranulation, which occurs in the early phase of extrinsic asthma. 3C8 may have this effect either by reducing the availability of i.c. calcium or by inhibiting calcium/calmodulin binding. This could also be the case for 3C9 which was also effective here. These compounds might even act in a similar manner to CycA and tacrolimus through inhibition of calcineurin since it is apparently a necessary target for the inhibition of mast cell degranulation as caused by both of these immunosuppressants (Toyota, Hashimoto et al. 1996; Hultsch, Brand et al. 1998). CycA and tacrolimus have been found to reduce antigen-induced airway inflammation (including airway eosinophil levels) and airway hyperresponsiveness in rats and mice (Elwood, Lotval et al. 1992; Nagai, Yamaguchi et al. 1995; Eum, Zuany-Amorim et al. 1997). Unlike neutrophils, the role of eosinophils in the pathogenesis of asthma is well documented. They are believed to contribute to the development of AHR through the release of cytotoxic proteins and leukotrienes while cytokines released include TNFα which causes tissue remodelling and is important in leukocyte recruitment. Therefore, the fact that 3C8 and 3C9 were, like CycA and tacrolimus, potent at reducing levels of this leukocyte in this Th2 model, suggests that they may be effective in treating allergic asthma.

As was found in the rat Th2 asthmatic model, monocyte levels were significantly elevated in all groups compared to the positive control, probably due to the reduced

levels of neutrophils and eosinophils found in each case. BAL levels of small lymphocytes also increased (although not always significantly) following each treatment with the exception of CycA which significantly reduced levels of this leukocyte at 10mg/kg. This is the opposite of what was found for CycA in the rat model. However, when dosed at 30mg/kg, levels of this leukocyte were higher than in the positive control group and results were generally consistent with the equivalent rat model. Large lymphocytes were rare with no significant change found in any group.

3.2.1.5.2 Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants (Th1/Th2 mixed model)

Using exactly the same protocol as for the above Th2 model, mice were sensitised to OVA using both adjuvants. This would theoretically generate a mixed Th1/Th2 response as was done for BN rats and explained in **section 3.1.5.3**. In this way, the results already obtained in the equivalent rat model would hopefully be confirmed.

Neutrophil and eosinophil levels in the positive control group reached 29 $\pm$ 4.4% and 22.6 $\pm$ 2.8% of the total BAL cell count respectively. Interestingly, neutrophil counts were lower and eosinophil counts higher than in the equivalent rat model, as was found when comparing the Th2 rat and mouse models. This would again suggest that the mouse model is more suited for elevating BAL levels of eosinophils whereas neutrophils may be raised more significantly in the BN model.

CycA, found to significantly reduce BAL neutrophils at 10mg/kg in the rat Th1/Th2 model, also had a significant impact here at both 10 and 30mg/kg. Tacrolimus had no

significant impact at 10mg/kg although a higher dose may have been significant since a 30% reduction was seen. Meanwhile, 3C8 caused a significant reduction in neutrophils at 10mg/kg and not at 30mg/kg. This could have been due to an error in the preparation of 3C8 although once again, approximately a 30% reduction was seen at the higher dose. This compound did not significantly reduce neutrophils in the rat mixed model although a reduction was seen. 3C9 also significantly reduced levels of this leukocyte at this higher dose. These results show that 3C8 and 3C9 are effective at inhibiting neutrophil recruitment to the lungs in an asthmatic model which may represent a mixed Th1/Th2 response.

Eosinophil levels in the BAL fluid were significantly reduced in all groups except for those that were treated with CycA or tacrolimus at 10mg/kg. CycA was also unable to reduce BAL eosinophils in the equivalent rat model and in the Th1 rat model. These results are surprising given the anti-Th1 and anti-Th2 effects of both CycA and tacrolimus through the inhibition of calcineurin. However, it should again be stressed that these mixed Th1/Th2 and Th1-specific models are not proven as such and the exact responses generated in each are unknown. It should also be pointed out that while a 10mg/kg dose of CycA was ineffective at reducing BAL eosinophils in the 3 asthmatic models in which FCA(T) was used (the rat and mouse mixed Th1/Th2 models and the rat Th1 model), a significant 60% reduction in eosinophils was seen when dosed at 30mg/kg in this mouse Th1/Th2 model. Therefore, perhaps a higher dose may also have been effective in the rat model(s). As mentioned, a 10mg/kg dose of tacrolimus was also insignificant here but did reduce eosinophils to almost exactly the same extent as CycA at the same dose. It is possible that a higher dose may therefore have been effective as was found for CycA.

Results from these 5 asthmatic models (in rats and mice) would suggest that CycA is more potent at reducing eosinophils in a Th2 model and higher doses may be required to be effective in a Th1 or mixed response model. This might also be the case for tacrolimus which, while not tested in the rat models, significantly reduced eosinophils in the Th2 mouse model only. Interestingly, both 3C8 and 3C9 significantly reduced eosinophils at both 10 and 30mg/kg. Therefore, it would seem that 3C8 and 3C9 are more effective than the established immunosuppressants in this model. This confirms what was found for 3C8 in the rat model where a significant reduction in eosinophils was seen at 10mg/kg whereas the same dose of CycA failed to reduce levels of this leukocyte. The exact mechanisms of action of these drugs are of course unknown. They may still be acting in a similar manner to CycA and tacrolimus through inhibiting calcineurin but with a more potent effect.

Monocyte levels were once again raised in each group, although not always significantly. Small lymphocyte levels were generally higher than the positive control group although they were significantly reduced by CycA at 10mg/kg. This is the opposite of what was found in the equivalent rat model, in which a significant increase was seen. However, this result (in the mouse model) seems to be erratic since levels of the leukocyte were a lot higher in the group dosed with CycA at 30mg/kg. Large lymphocyte levels were very low with no significant difference found in any group.

3.2.2: Enzyme-Linked Immunosorbent Assay (ELISA) on Mouse BAL fluid and Lung Extracts

3.2.2.1 Introduction

In order to get a better understanding of the results found in **section 3.2.1**, the levels of certain cytokines found in the BAL fluid and lung extracts were measured by ELISA. In this way, the effects of the test compounds on both leukocyte levels and cytokine levels could be evaluated in both mouse asthmatic models, thus hopefully clarifying their modes of action. IL-4, IL-5, IL-10 and IFNγ were the cytokines evaluated.

IL-4 is a Th2 cytokine produced from mast cells and eosinophils. As previously mentioned, it is crucial for the development of the Th2 response and may inhibit the Th1 response (Vercelli, Geha 1992; Herz, Bunikowski et al. 1998). It also contributes to eosinophil recruitment through upregulation of VCAM-1 (Krishna, Chauhan et al. 1996). IL-5 is also produced from mast cells and eosinophils and contributes to the differentiation, activation and survival of the eosinophils as well as inducing their degranulation and chemotaxis (Clutterbuck, Hirst et al. 1989; Sanderson 1992). It has an additive effect on IgE production in the Th2 response but is not a Th2 specific cytokine (Pene, Rousset et al. 1988). IL-10 is another Th2 cytokine which, like IL-4, may inhibit the Th1 response. It has been found to inhibit production of the Th1 cytokines IL-2, IFNγ and TNFα as well as reducing footpad swelling in a mouse model of DTH (Li, Elliott et al. 1994). Furthermore, the anti-inflammatory potency of IL-10 has been rated equal to IL-13 and higher than IL-4, IL-6 and IL-12 in an

immune complex-induced model of alveolitis in rats (Mulligan, Warner et al. 1997). Finally, IFNγ is crucial in the development of the Th1 response and negatively affects the Th2 response by inhibiting IL-4 secretion and receptor expression (Paludan 1998). It also contributes to leukocyte recruitment through the upregulation of endothelial adhesion molecules (Doukas and Pober 1990). Therefore, evaluation of levels of these cytokines would be important since all contribute to either the Th1 or Th2 response and/or leukocyte recruitment.

The aim of the ELISA experiments was to evaluate and compare cytokine levels in both the Th2 and mixed Th1/Th2 asthmatic models to leukocyte numbers already calculated in **section 3.2.1**. In this way, further information regarding the modes of action of the novel compounds would hopefully be obtained.

3.2.2.2 Methods

The levels of four different cytokines, namely IL-4, IL-5, IL-10 and IFNγ were measured in both the BAL fluid and lung extracts taken from Balb/C mice. The mice were first sensitised to OVA using either AlOH or both AlOH and FCA(T) as adjuvants, before being challenged to OVA, as in **section 3.2.1.2.**

The protocol followed was the exact protocol that was supplied with all the ELISA antibodies from RnD. It was exactly the same for each of the four cytokines being measured except for the concentration of the capture, detection and standard antibodies used in each case. The following protocol was used in all ELISA

experiments. The concentration of each antibody is shown in **table 3.2.2.2** (appendix 1).

General ELISA protocol

The concentration of each cytokine found in both the BAL fluid and lung extracts taken from Balb/C mice was determined by immunoassay using the antibodies described in **table 3.2.2.2** (appendix 1). 96 well microtitre plates were coated with 100µl/well of the specific capture antibody for the cytokine (except for the a1 well which is left untreated throughout the experiment as a blank). Plates were sealed with tinfoil and left overnight at room temperature. The next day, plates were inverted to remove the unbound capture antibody and then washed three times by adding 400µl wash buffer to each well with a multi-channel pipette. Following each wash, plates were inverted and shaken to remove as much liquid as possible, which is essential for good performance. After the final wash, plates were blotted on a paper towel. Next, 300µl block buffer was added to each well to block non-specific binding sites and plates were incubated at room temperature for a minimum of one hour. Plates were again washed, and dilution of standards and test samples carried out with diluent. Serial dilutions of the cytokine-specific standard were made starting with the top working concentration as in **table 3.2.2.2** (appendix 1), to construct a seven point standard curve. 100µl of sample or standard was added to each well. Each sample and standard was added in triplicate so that the mean value from 3 different wells could be used to estimate cytokine levels for each sample. Plates were tapped gently for 1 minute to ensure proper mixing, and were again covered with tinfoil and either incubated for 2 hours at room temperature or else left overnight in the fridge. The

plates were washed three times again and 100µl of biotinylated detection antibody added to each well. Plates were covered with tinfoil and incubated for 2 hours at room temperature. After washing three times, 100µl streptavidin horse radish peroxidase (HRP) (diluted 1/200 in diluent) was added to each well. Plates were covered with tinfoil and incubated for 20 minutes at room temperature. Finally, after washing three more times, plates were incubated with 100µl per well soluble tetramethylbenzidine (TMB) substrate solution and covered in tinfoil. After being allowed to develop in the dark for up to 20 minutes at room temperature (until the blue colour has fully developed), the reaction was stopped with the addition of 50µl stop solution (1M sulphuric acid; H_2SO_4) to each well. Plates were then tapped to ensure thorough mixing. The optical density (O.D.) of each test sample and cytokine standard in each well was read using a Labsystems Multiscan plate reader set to 450nm. The concentration of the given cytokine in each test sample was then calculated after reference to the standard curve, prepared from recombinant cytokines of known concentration and potency.

Values (O.D. at 450nm) were written directly into lab books at the time of the experiments. Data was then analysed using Microsoft Excel and Graphpad Instat for statistical comparisons. All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests. Values were expressed as mean $\pm$ SEM with a P-value of less than 0.05 (compared to the positive control group) taken to be significant.

3.2.2.3 Materials

Sigma Aldrich, Airton Road, Tallaght, Dublin, Ireland.

H_2SO_4 (95-97%), tween 20 and BSA were obtained from Sigma Aldrich.

Lennox Chemicals Ltd, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.

Sucrose and sodium azide were supplied to Lennox Chemicals Ltd. by BDH, Laboratory Supplies, Poole, BH15 1TD, England.

TMB (soluble), NaCl, KCl, Na_2HPO_4 and KH_2PO_4 were supplied to Lennox Chemicals Ltd. by Merck, KgaA, 64271 Darnstedt, Germany.

R&D Systems Europe LTD, 19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, U.K.

Streptavidin HRP and mouse monoclonal (capture), biotinylated (detection) and recombinant (standard) antibodies for IL-4, IL-5, IL-10 and IFNγ were obtained from R&D Systems Europe Ltd.

3.2.2.3.1 ELISA solutions

ELISA solutions were prepared as follows:

PBS: 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in distilled water; adjusted to pH 7.0.

Diluent: 1% (w/v) BSA in PBS; adjusted to pH 7.4.

Substrate solution: TMB, soluble.

Wash Buffer: 0.05% (v/v) Tween 20 in PBS; adjusted to pH 7.4.

Stop Solution: 1M H_2SO_4.

Block Buffer: 1% (w/v) BSA, 5% (w/v) sucrose, 0.05% (w/v) NaN_3 in PBS.

3.2.2.4 Results

3.2.2.4.1 Interleukin-4 (IL-4) ELISA

3.2.2.4.1.1 IL-4 levels measured by ELISA in BAL fluid and lung extracts of Balb/C mice sensitised to OVA using AlOH as an adjuvant

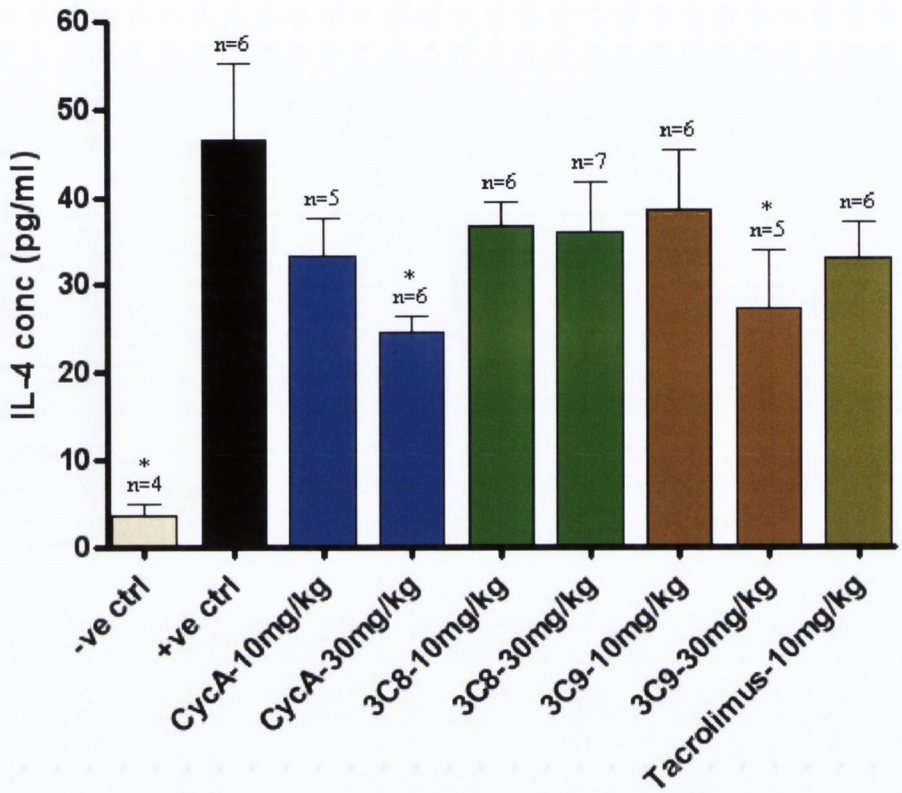
The IL-4 levels (pg/ml) measured in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant are shown in **table 3.2.2.4.1.1 (appendix 2)**, and illustrated in **figures 3.2.2.4.1.1 (a) and 3.2.2.4.1.1 (b)**. The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

IL-4 levels (pg/ml) in the BAL fluid are shown in **table 3.2.2.4.1.1 (appendix 2)** and illustrated in **figure 3.2.2.4.1.1 (a)**. A highly significant 13-fold increase in IL-4 levels was found in the positive control group compared to the negative controls ($P = 0.01$). CycA and 3C9 both significantly reduced the positive control value at 30mg/kg to approximately half, but neither was significant at 10mg/kg. 3C8 had no significant impact at either dose, nor did tacrolimus at 10mg/kg.

IL-4 levels in the lung extracts are again shown in **table 3.2.2.4.1.1 (appendix 2)** and illustrated in **figure 3.2.2.4.1.1 (b)**. A 16-fold increase was found in the positive control group compared to the negative controls, which was highly significant ($P = 0.004$). Whereas CycA only had a significant impact at 30mg/kg in the BAL fluid, it

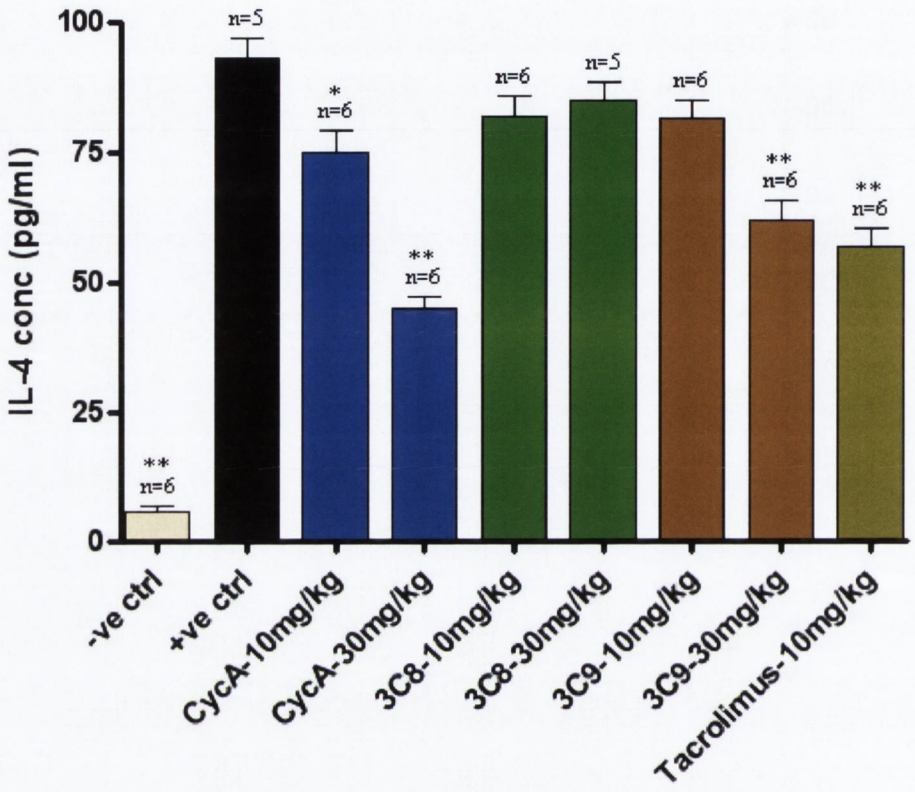
significantly reduced the positive control value at both tested doses in the lung extracts, more than halving this value when dosing was at 30mg/kg. 3C8 had no significant impact at either dose while 3C9 was only significant at 30mg/kg, the same as was found in the BAL fluid. Tacrolimus, which did not significantly affect IL-4 levels in the BAL fluid, had a significant impact at 10mg/kg in the lung extracts, causing a greater reduction in IL-4 levels than CycA at the same dose or 3C9 at 30mg/kg.

Figure 3.2.2.4.1.1 (a): IL-4 levels in BAL fluid taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the IL-4 levels (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using AlOH an adjuvant for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Figure 3.2.2.4.1.1 (b): IL-4 levels in lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the IL-4 levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, using AlOH an adjuvant for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

3.2.2.4.1.2 IL-4 levels measured by ELISA in BAL fluid and lung extracts of Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

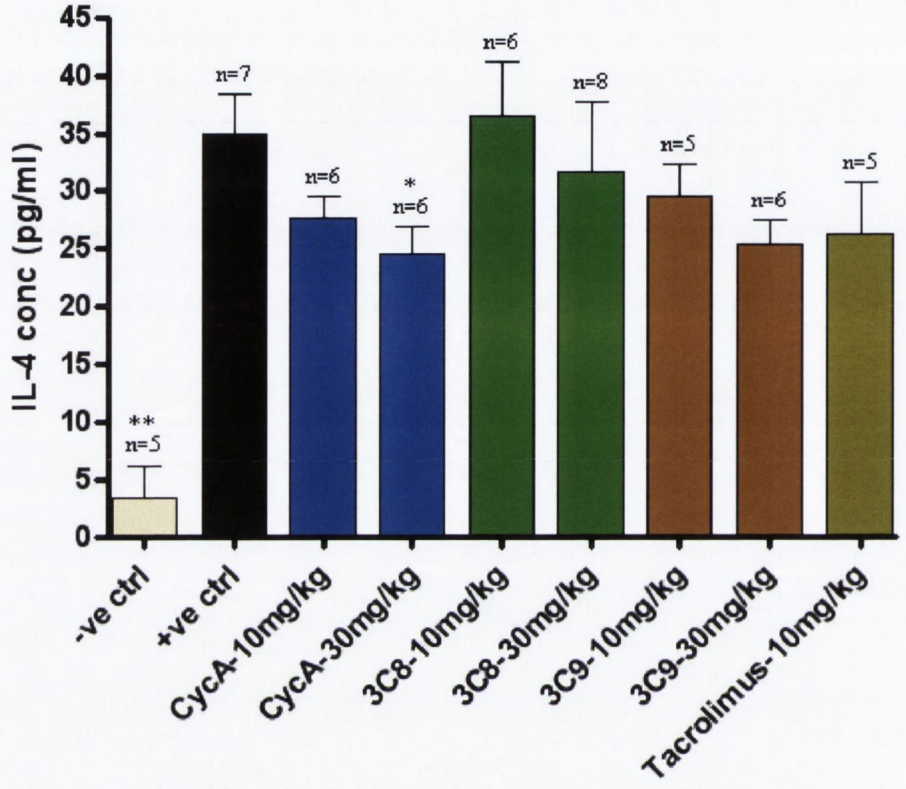
The IL-4 levels (pg/ml) measured in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants are shown in

table 3.2.2.4.1.2 (appendix 2), and illustrated in **figures 3.2.2.4.1.2 (a) and 3.2.2.4.1.2 (b)**. The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

IL-4 levels (pg/ml) in the BAL fluid are shown in **table 3.2.2.4.1.2 (appendix 2)** and illustrated in **figure 3.2.2.4.1.2 (a)**. A statistically significant 10-fold increase in IL-4 levels was found in the positive control group compared to the negative controls ($P = 0.003$). Neither 3C8 nor 3C9 had a significant impact on IL-4 levels at either dose, nor did tacrolimus at 10mg/kg. The only drug that successfully significantly reduced the positive control value was CycA, when dosing was at 30mg/kg, but not at 10mg/kg. A 30% reduction in IL-4 levels was seen at 30mg/kg.

IL-4 levels in the lung extracts are also shown in **table 3.2.2.4.1.2 (appendix 2)** and illustrated in **figure 3.2.2.4.1.2 (b)**. A 16-fold increase was found in the positive control group compared to the negative controls, and this was highly significant ($P = 0.002$). None of the four tested compounds significantly reduced IL-4 levels when dosing was at 10mg/kg. However, the three compounds that were also tested at 30mg/kg (CycA, 3C8 and 3C9) all significantly reduced this positive control value. All three were similarly effective with a 35-40% reduction in IL-4 levels seen in each case. The only conflict between these results and the results found in the BAL fluid is that 3C8 and 3C9 had a significant impact at 30mg/kg in the lung extracts, which was not found in the BAL fluid.

Figure 3.2.2.4.1.2 (a): IL-4 levels in BAL fluid taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the IL-4 (mean ± SEM) levels found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.

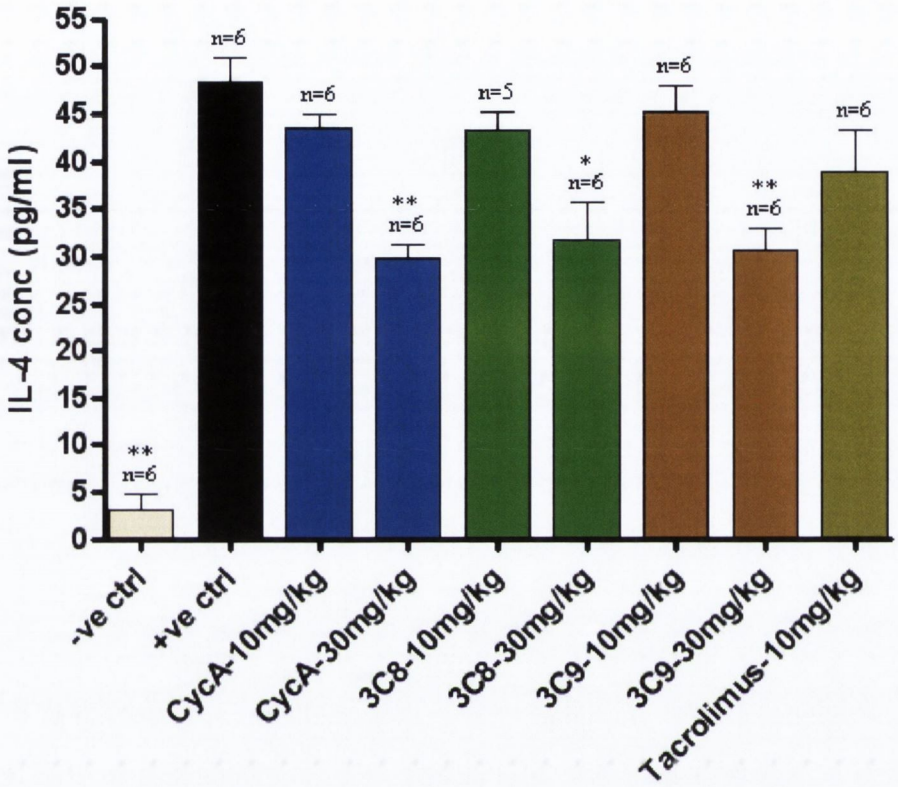
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Results are from 3 ELISA experiments.

Figure 3.2.2.4.1.2 (b): IL-4 levels in lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the IL-4 levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, both AlOH and FCA(T) as adjuvants for sensitisation.

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Results are from 3 ELISA experiments.

3.2.2.4.2 Interleukin-5 (IL-5) ELISA

3.2.2.4.2.1 IL-5 levels measured by ELISA in BAL fluid and lung extracts of Balb/C mice sensitised to OVA using AlOH as an adjuvant

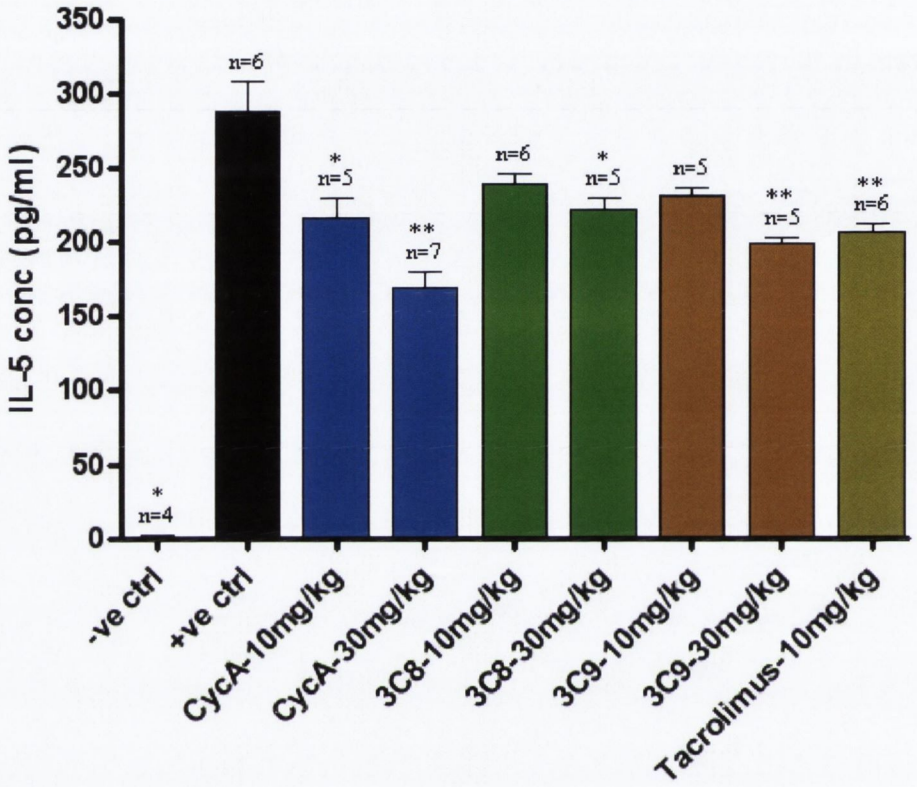
The IL-5 levels (pg/ml) measured in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant are shown in **table 3.2.2.4.2.1 (appendix 2)**, and illustrated in **figures 3.2.2.4.2.1 (a) and 3.2.2.4.2.1 (b)**. The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

IL-5 levels (pg/ml) in the BAL fluid are shown in **table 3.2.2.4.2.1 (appendix 2)** and illustrated in **figure 3.2.2.4.2.1 (a)**. Almost a 250-fold increase in IL-5 levels was found in the positive control group compared to the negative controls, which was statistically significant ($P = 0.024$). CycA significantly reduced IL-5 levels at both 10 and 30mg/kg, as did tacrolimus at 10mg/kg. At 30mg/kg, CycA reduced the positive control value by approximately 40%, which was the greatest reduction seen in any treatment group. Both 3C8 and 3C9 had a significant impact on IL-5 levels when dosing was at 30mg/kg but neither was significant at 10mg/kg.

IL-5 levels in the lung extracts are again shown in **table 3.2.2.4.2.1 (appendix 2)** and illustrated in **figure 3.2.2.4.2.1 (b)**. A significant and almost 80-fold increase was found in the positive control group compared to the negative controls ($P = 0.01$). Of the four test compounds, only tacrolimus significantly reduced the positive control

value when dosing was at 10mg/kg. CycA, 3C8 and 3C9 had no significant impact at this dose. However, all three were significant at 30mg/kg. The only contrast between these results and the results for the BAL fluid is that CycA had a significant effect on IL-5 levels at 10mg/kg, only in the BAL fluid. The greatest reduction in IL-5 levels was seen in the group dosed with 3C9 at 30mg/kg, where approximately a 42% reduction was seen. This was the only group that reduced the positive control value more than CycA at 30mg/kg.

Figure 3.2.2.4.2.1 (a): IL-5 levels in BAL fluid taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the IL-5 levels (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.

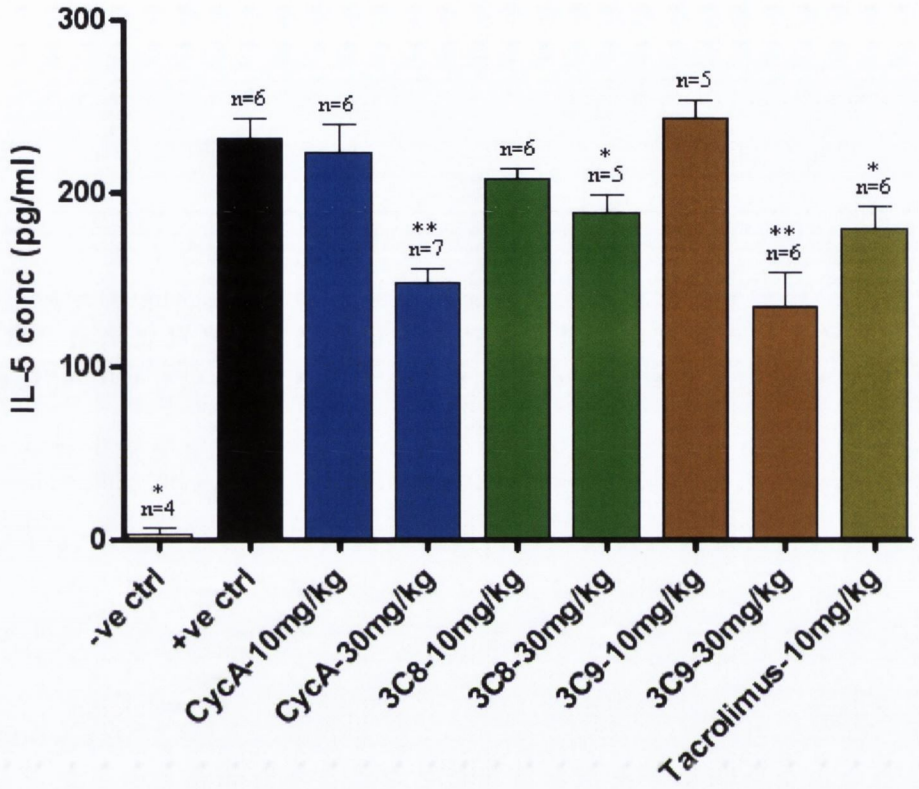
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Results are from 3 ELISA experiments.

Figure 3.2.2.4.2.1 (b): IL-5 levels in lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the IL-5 levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, using AlOH an adjuvant for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

3.2.2.4.2.2 IL-5 levels measured by ELISA in BAL fluid and lung extracts of Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

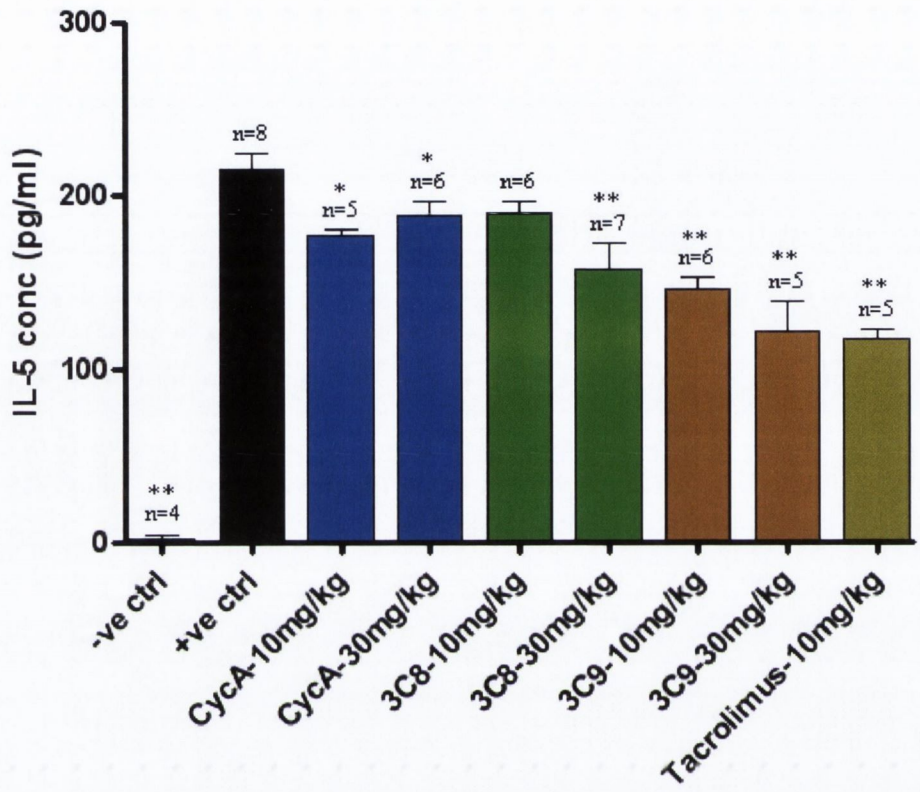
The IL-5 levels (pg/ml) measured in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants are shown in

table 3.2.2.4.2.2 (appendix 2), and illustrated in **figures 3.2.2.4.2.2 (a) and 3.2.2.4.2.2 (b)**. The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

IL-5 levels (pg/ml) in the BAL fluid are shown in **table 3.2.2.4.2.2 (appendix 2)** and illustrated in **figure 3.2.2.4.2.2 (a)**. A highly significant 115-fold increase in IL-5 levels was found in the positive control group compared to the negative controls ($P = 0.004$). CycA and 3C9 both significantly reduced the positive control value at both 10 and 30mg/kg. 3C8 had a significant impact only at 30mg/kg. Tacrolimus also significantly reduced the positive control value and although only dosed at 10mg/kg, it caused the greatest reduction in IL-5 levels of approximately 45%.

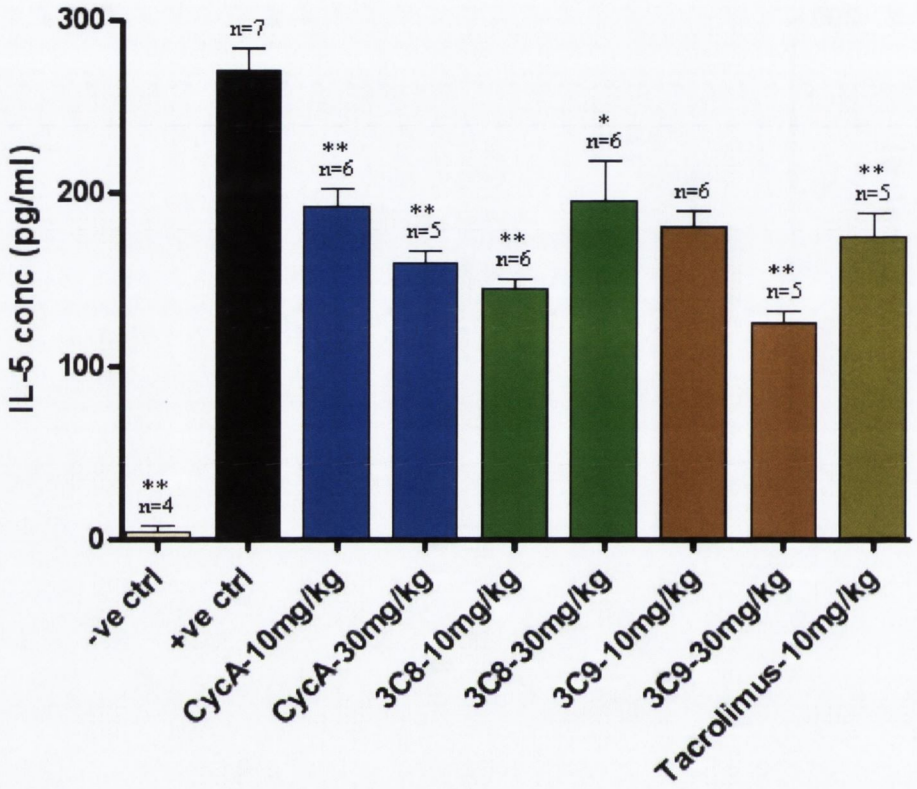
IL-5 levels in the lung extracts are also shown in **table 3.2.2.4.2.2 (appendix 2)** and illustrated in **figure 3.2.2.4.2.2 (b)**. Approximately a 65-fold increase was found in the positive control group compared to the negative controls, and this was highly significant ($P = 0.006$). CycA again significantly reduced IL-5 levels at both tested doses. However, whereas 3C9 also had a significant effect at both doses in the BAL fluid, it was found to be significant only at 30mg/kg in the lung extracts. However, this dose of 3C9 caused the greatest reduction in IL-5 levels of any treatment group, reducing the positive control value by almost 55%. Conversely, whereas 3C8 only significantly reduced IL-5 levels at 30mg/kg in the BAL fluid, it was found to be significant at both tested doses in the lung extracts. Tacrolimus was again found to be significant at 10mg/kg.

Figure 3.2.2.4.2.2 (a): IL-5 levels in BAL fluid taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the IL-5 levels (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Figure 3.2.2.4.2.2 (b): IL-5 levels in lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the IL-5 levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, both AlOH and FCA(T) as adjuvants for sensitisation.

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Results are from 3 ELISA experiments.

3.2.2.4.3 Interleukin-10 (IL-10) ELISA

3.2.2.4.3.1 IL-10 levels measured by ELISA in BAL fluid and lung extracts of Balb/C mice sensitised to OVA using AlOH as an adjuvant

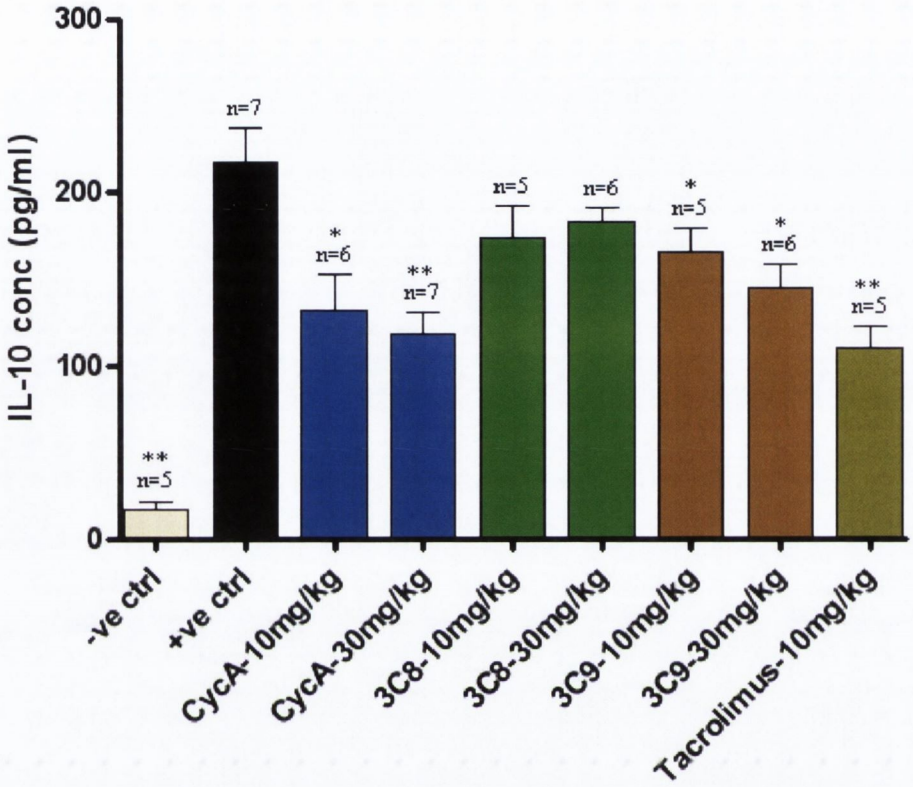
The IL-10 levels (pg/ml) measured in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant are shown in **table 3.2.2.4.3.1 (appendix 2)**, and illustrated in **figures 3.2.2.4.3.1 (a) and 3.2.2.4.3.1 (b)**. The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

IL-10 levels (pg/ml) in the BAL fluid are shown in **table 3.2.2.4.3.1 (appendix 2)** and illustrated in **figure 3.2.2.4.3.1 (a)**. A very significant 13-fold increase in IL-10 levels was found in the positive control group compared to the negative controls ($P = 0.003$). CycA and 3C9 were both found to significantly reduce this value when dosing was at either 10 or 30mg/kg. 3C8 however, had no significant impact at either dose. At 10mg/kg, tacrolimus almost halved IL-10 levels. This was highly significant and was the greatest reduction in the positive control value seen with any group.

IL-10 levels in the lung extracts are again shown in **table 3.2.2.4.3.1 (appendix 2)** and illustrated in **figure 3.2.2.4.3.1 (b)**. A greater than 70-fold increase was seen in the positive control group compared to the negative controls and this was highly significant ($P = 0.004$). CycA and 3C9 both caused a significant reduction in this positive control value when dosing was at 30mg/kg, but neither was significant at

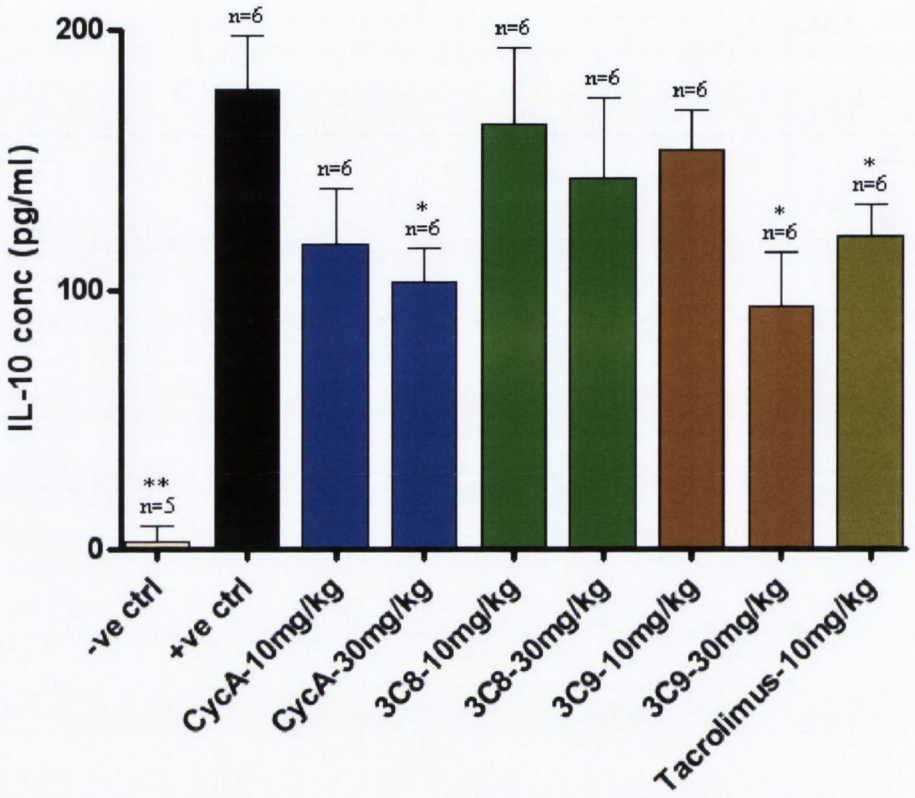
10mg/kg. This is in contrast to the BAL IL-10 levels where both drugs had a significant impact at both doses. 3C8 was again found not to have any significant impact at either dose, while tacrolimus was again successful at significantly reducing IL-10 levels at 10mg/kg. The greatest reduction in IL-10 levels in the lung extracts was seen in the group dosed with 3C9 at 30mg/kg where almost a 50% reduction was seen.

Figure 3.2.2.4.3.1 (a): IL-10 levels in BAL fluid taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the IL-10 levels (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Figure 3.2.2.4.3.1 (b): IL-10 levels in lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the IL-10 levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, using AlOH an adjuvant for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

3.2.2.4.3.2 IL-10 levels measured by ELISA in BAL fluid and lung extracts of Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

The IL-10 levels (pg/ml) measured in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants are shown in

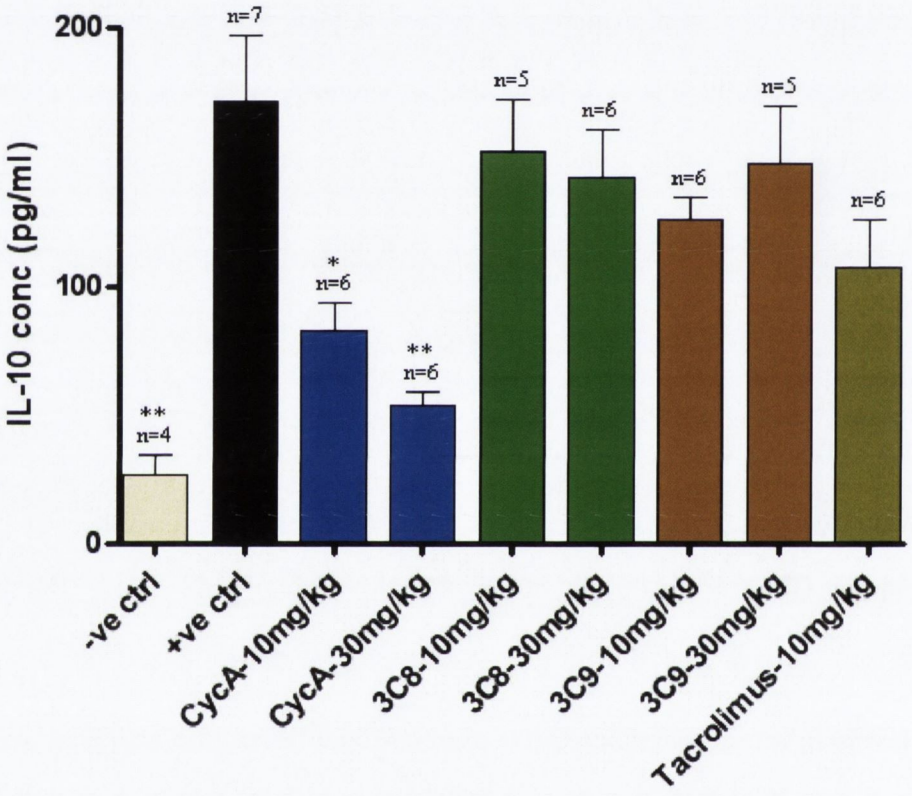
table 3.2.2.4.3.2 (appendix 2), and illustrated in **figures 3.2.2.4.3.2 (a) and 3.2.2.4.3.2 (b)**. The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

IL-10 levels (pg/ml) in the BAL fluid are shown in **table 3.2.2.4.3.2 (appendix 2)** and illustrated in **figure 3.2.2.4.3.2 (a)**. A statistically significant 6-fold increase in IL-10 levels was found in the positive control group compared to the negative controls ($P = 0.006$). CycA significantly reduced the positive control value at both doses. At 10mg/kg, CycA more than halved IL-10 levels and at 30mg/kg, this value was reduced by almost 70%. This was the greatest reduction seen in any group. 3C8 and 3C9 failed to significantly reduce IL-10 levels whether dosing was at 10 or 30mg/kg. Tacrolimus, dosed at 10mg/kg, also failed to have a significant impact although IL-10 levels were reduced by more than 35%; a greater reduction than was seen with 3C8 or 3C9 at either dose.

IL-10 levels in the lung extracts are again shown in **table 3.2.2.4.3.2 (appendix 2)** and illustrated in **figure 3.2.2.4.3.2 (b)**. A highly significant 13-fold increase was found in the positive control group compared to the negative controls ($P = 0.004$). CycA was again found to significantly reduce levels of IL-10 at both doses. It also caused the biggest reduction in the positive control value of any drug treatment group when dosing was at 30mg/kg, the same as was found in the BAL fluid. In this case, approximately a 45% reduction was seen. 3C9 was again found not to have any significant impact at either dose. However, while 3C8 and tacrolimus had no

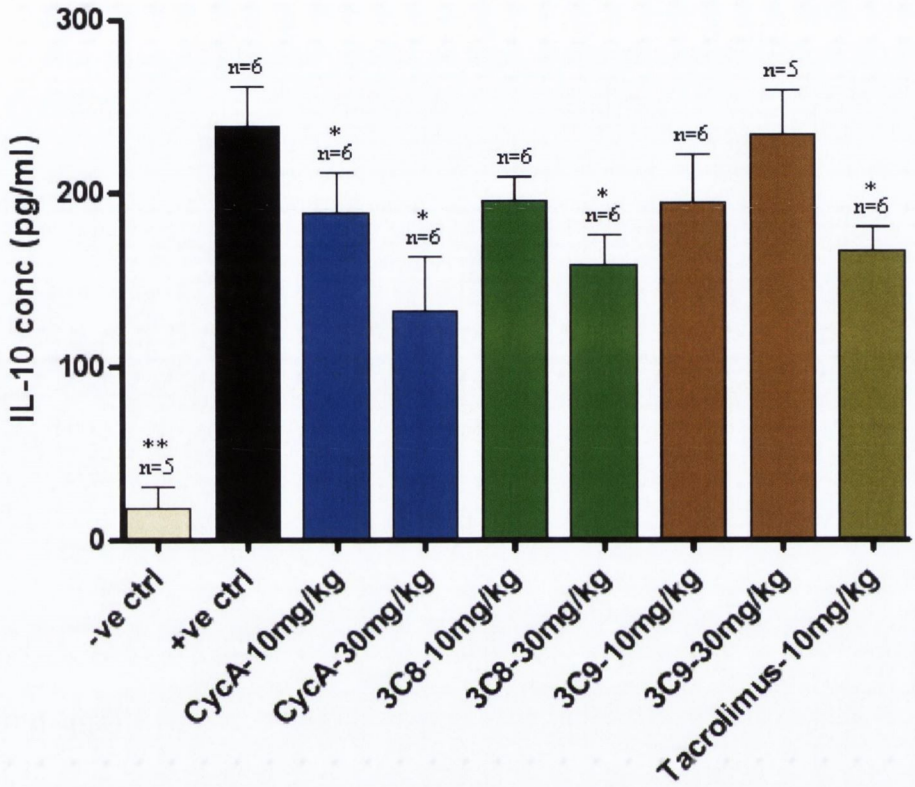
significant effect in the BAL fluid, 3C8 was found to be significant at 30mg/kg, as was tacrolimus at 10mg/kg in the lung extracts.

Figure 3.2.2.4.3.2 (a): IL-10 levels in BAL fluid taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the IL-10 levels (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Figure 3.2.2.4.3.2 (b): IL-10 levels in lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the IL-10 levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, both AlOH and FCA(T) as adjuvants for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

3.2.2.4.4 Interferon-gamma (IFNγ) ELISA

3.2.2.4.4.1 IFNγ levels measured by ELISA in BAL fluid and lung extracts of Balb/C mice sensitised to OVA using AlOH as an adjuvant

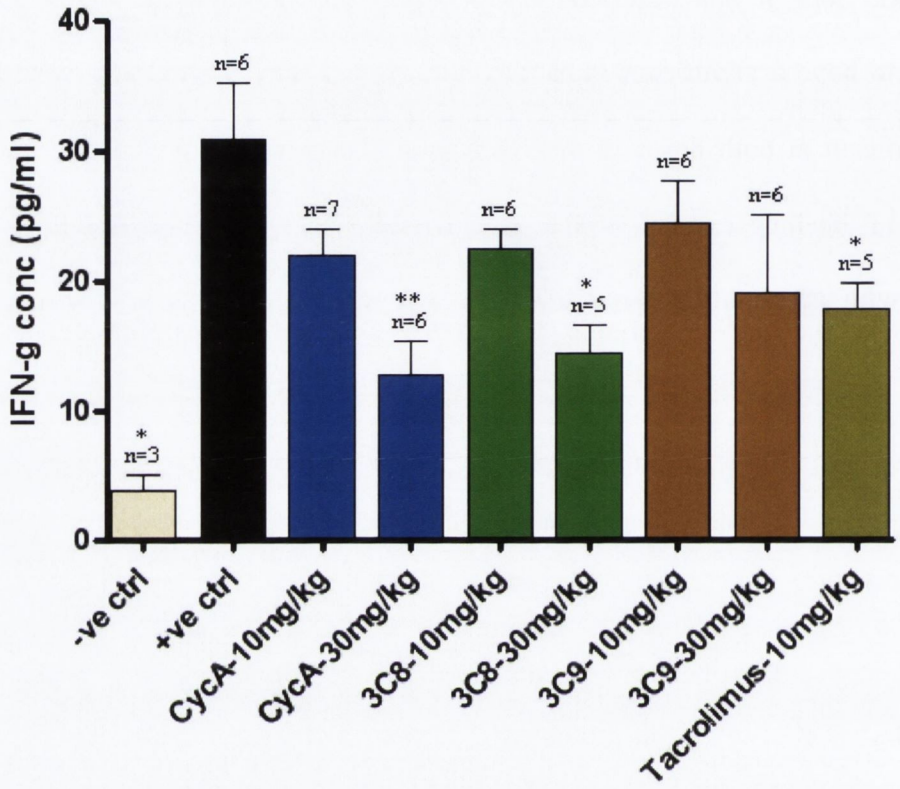
The IFNγ levels (pg/ml) measured in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant are shown in **table 3.2.2.4.4.1 (appendix 2)**, and illustrated in **figures 3.2.2.4.4.1 (a) and 3.2.2.4.4.1 (b)**. The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

IFNγ levels (pg/ml) in the BAL fluid are shown in **table 3.2.2.4.4.1 (appendix 2)** and illustrated in **figure 3.2.2.4.4.1 (a)**. A significant 8-fold increase in IFNγ levels was found in the positive control group compared to the negative controls ($P = 0.024$). CycA and 3C8 both significantly reduced IFNγ levels at 30mg/kg but neither was significant at 10mg/kg. 3C9 failed to have any significant impact at either dose. However, tacrolimus significantly reduced the positive control value at 10mg/kg. The greatest reduction was seen in the group dosed with CycA at 30mg/kg where a 60% reduction in IFNγ levels was found.

IFNγ levels (pg/ml) in the lung extracts are shown in **table 3.2.2.4.4.1 (appendix 2)** and illustrated in **figure 3.2.2.4.4.1 (b)**. Approximately a 14-fold increase was found in the positive control group compared to the negative controls, which was highly

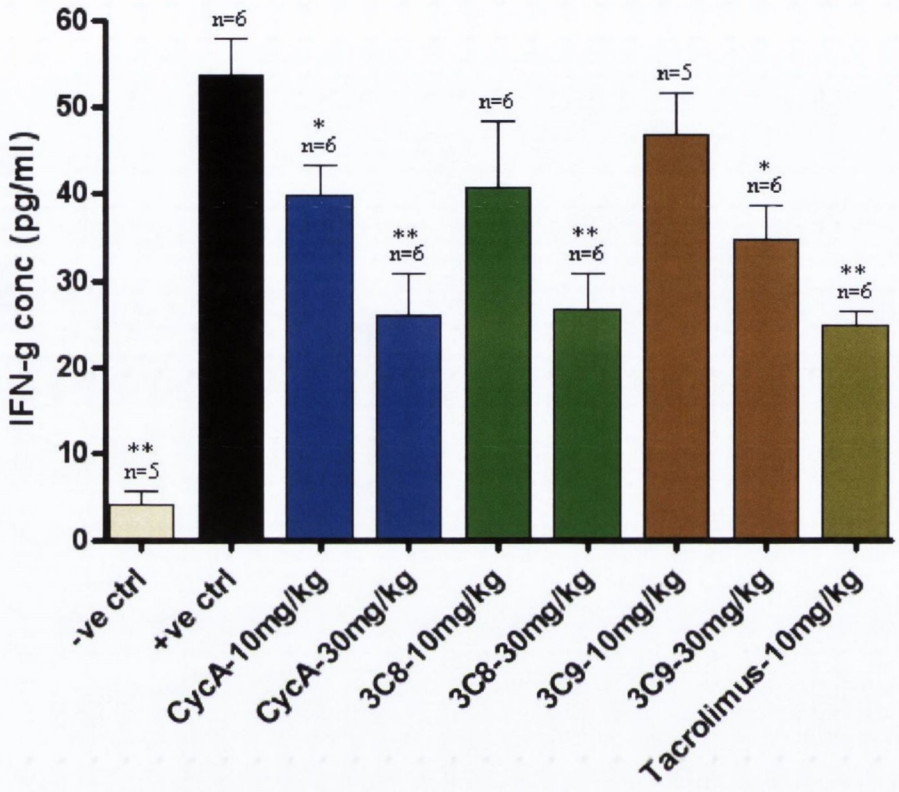
significant (P = 0.004). As found in the BAL fluid, 3C8 significantly reduced the positive control value only when dosed at 30mg/kg and tacrolimus was again significant at 10mg/kg. However, whereas CycA was only significant at 30mg/kg in the BAL fluid, it was significant at both doses in the lung extracts. Also, 3C9 was found to have a significant impact at 30mg/kg in the lung extracts whereas it was insignificant at both doses in the BAL fluid. The greatest reduction in IFNγ levels found in the lung extracts was approximately a 55% reduction found in the group dosed with tacrolimus at 10mg/kg.

Figure 3.2.2.4.4.1 (a): IFNγ levels in BAL fluid taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the IFNγ levels (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Figure 3.2.2.4.4.1 (b): IFNγ levels in lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the IFNγ levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, using AlOH an adjuvant for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

3.2.2.4.4.2 IFNγ levels measured by ELISA in BAL fluid and lung extracts of Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

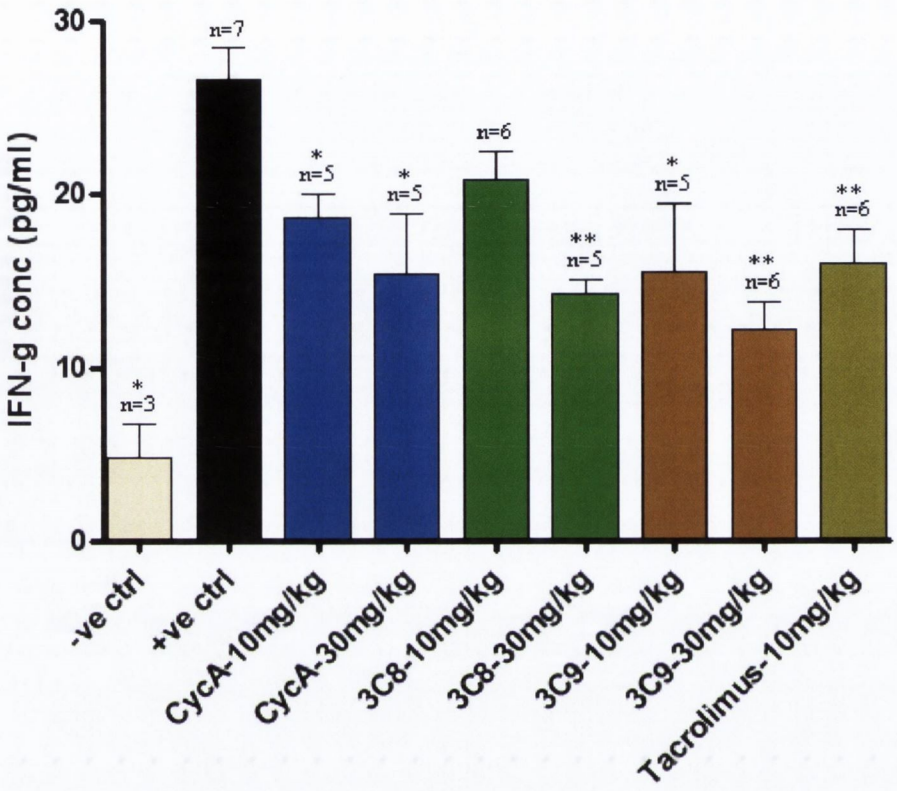
The IFNγ levels (pg/ml) measured in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants are shown in

table 3.2.2.4.4.2 (appendix 2), and illustrated in **figures 3.2.2.4.4.2 (a) and 3.2.2.4.4.2 (b)**. The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

IFNγ levels (pg/ml) in the BAL fluid are shown in **table 3.2.2.4.4.2 (appendix 2)** and illustrated in **figure 3.2.2.4.4.2 (a)**. A greater than 5-fold increase in IFNγ levels was found in the positive control group compared to the negative controls and this was significant ($P = 0.017$). CycA and 3C9 were both found to significantly reduce IFNγ levels at both tested doses. Tacrolimus also had a significant impact at 10mg/kg. 3C8 significantly reduced the positive control value only when dosing was at 30mg/kg. The greatest reduction in this value was approximately a 55% reduction seen in the group treated with 3C9 at 30mg/kg.

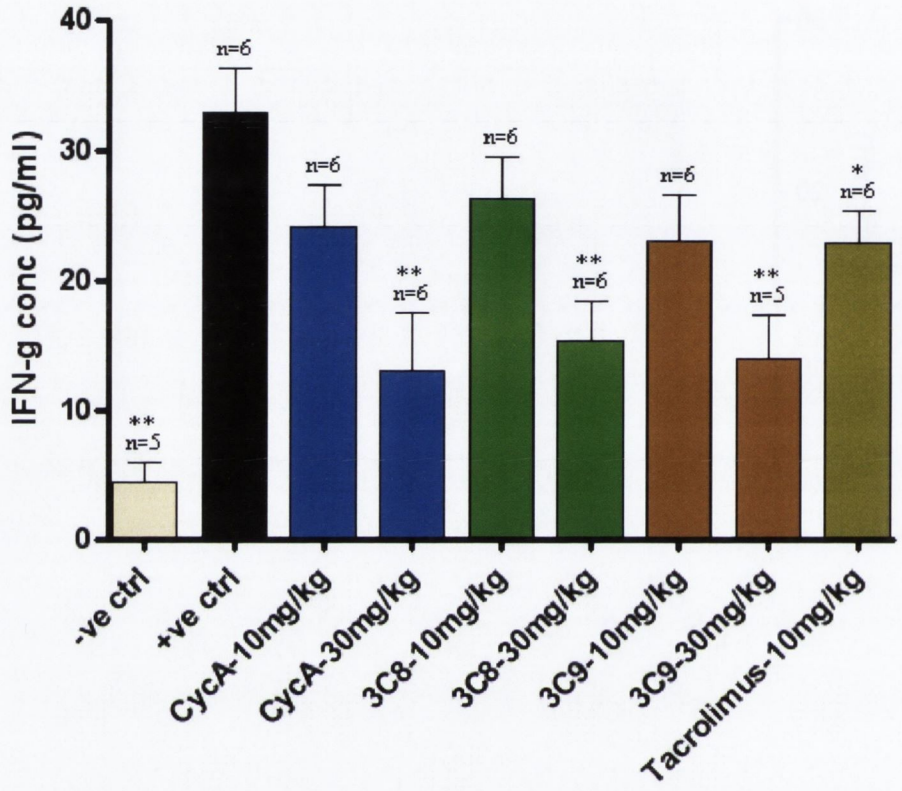
IFNγ levels (pg/ml) in the lung extracts are shown in **table 3.2.2.4.4.2 (appendix 2)** and illustrated in **figure 3.2.2.4.4.2 (b)**. Almost an 8-fold increase in IFNγ levels was found in the positive control group compared to the negative controls and this was again significant ($P = 0.004$). 3C8 only significantly reduced this positive control value when dosing was at 30mg/kg while tacrolimus had a significant impact at 10mg/kg, all of which correlates with the results found in the BAL fluid. However, while CycA and 3C9 had a significant impact at both doses in the BAL fluid, neither was significant at 10mg/kg in the lung extracts. Both did have a significant effect again at 30mg/kg. The biggest reduction in IFNγ levels was a 60% reduction seen in the group dosed with CycA at 30mg/kg.

Figure 3.2.2.4.4.2 (a): IFNγ levels in BAL fluid taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the IFNγ levels (mean ± SEM) found in the BAL fluid taken from Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Figure 3.2.2.4.4.2 (b): IFNγ levels in lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the IFNγ levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, both AlOH and FCA(T) as adjuvants for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

3.2.2.5 Discussion

Levels of IL-4, IL-5, IL-10 and IFNγ were evaluated in the BAL fluid and lung extracts taken from Balb/C mice in each of the mouse asthmatic models. These models and the preparation of BAL fluid and lung extracts were described in **section**

3.2.1.2. Analysis of these cytokines would hopefully provide further insight into the mechanisms of action of the novel compounds.

3.2.2.5.1 IL-4 ELISA

3.2.2.5.1.1 IL-4 levels in the BAL fluid and lung extracts of Balb/C mice sensitised to OVA using AlOH as an adjuvant

Levels of IL-4 were significantly increased in both the BAL fluid and lung extracts in the positive control group. This is as expected since we are examining a Th2 model of asthma and IL-4 is the main cytokine involved in stimulating the Th2 response. Levels of the cytokine were significantly raised in other similar models of allergic inflammation and asthma in Balb/C mice (Finotto, De Sanctis et al. 2001; Kudlacz, Whitney et al. 2002; Hellings, Vandekerckhove et al. 2003; Smit, Van Loveren et al. 2003; McKay, Leung et al. 2004; Itami, Oshiro et al. 2005) as well as in rat models of asthma (Koh, Choi et al. 2001; Zheng, Nong et al. 2001). IL-4 levels were higher in the lung extracts than in the BAL fluid of each group, a trend that was generally observed in all ELISA results. This would suggest that detection is more sensitive in lung extracts, which may therefore make them more suitable for accurate results. This may explain why a significant reduction in IL-4 levels was seen following CycA and tacrolimus treatment at 10mg/kg in the lung extracts only. At 30mg/kg, CycA significantly reduced levels of the leukocyte in both samples.

Calcineurin inhibition by both CycA and tacrolimus would inhibit mast cell degranulation and the release of many mast cell products. IL-4 levels may be reduced

since, as mentioned earlier (**section 3.1.5.1**), inhibition of mast cell degranulation would decrease the release of preformed cytokines (stored in granules) as well as inhibiting the transcription of cytokine genes (Abraham, Thankavel et al. 1997; Church, Levi-Schaffer 1997; Kalesnikoff, Huber et al. 2001). This would explain the potency of both compounds in reducing levels of the cytokine. IL-4 is crucial in the isotope switching of the B cells to produce IgE and also upregulates endothelial adhesion molecules needed for leukocyte recruitment (Vercelli, Geha 1992; Herz, Bunikowski et al. 1998). Therefore, it is no surprise that the reduction in IL-4 levels by CycA was accompanied by a reduction in BAL neutrophils and eosinophils in both the rat and mouse Th2 asthmatic models while tacrolimus also reduced BAL eosinophils in the mouse model. CycA and tacrolimus have been found to inhibit the release of cytokines (including IL-4 from mast cells) from many inflammatory cells such as monocytes, eosinophils and mast cells (De Paulis, Stellato et al. 1992; Hatfield and Roehm 1992; Sperr, Agis et al. 1996). Meanwhile, a slight but insignificant reduction was seen in the 3C8 treated groups at both doses while 3C9 had a significant impact at 30mg/kg only. A greater impact might have been expected for 3C8 given that it too reduced BAL neutrophils and eosinophils in both rat and mouse Th2 models. One explanation for this may be that 3C8 did not inhibit mast cell degranulation but was still able to inhibit leukocyte recruitment through inhibiting other mast cell products. For example, IL-5 contributes to eosinophil degranulation and chemotaxis while TNFα upregulates numerous endothelial adhesion molecules which are essential for leukocyte recruitment. Prostaglandins (PGs) and leukotrienes (LTs) produced from mast cells also play a role in this process, as described earlier. Another possibility is that 3C8 did inhibit mast cell degranulation but IL-4 levels were not significantly reduced because it came from other sources such as Th0 or Th2 cells.

Because of the importance of IL-4 in the Th2 response and allergic asthma, agents that are effective at reducing levels of the cytokine may be effective in treating the disease. Inhalation of IL-4 has been found to cause the development of sputum eosinophilia and increase AHR while neutralising antibodies against IL-4 have been found to be effective in asthma treatment (Finotto, De Sanctis et al. 2001). In addition, several IL-4 antagonists have already been developed (Izuhara, Arima et al. 2002). Therefore, the reduction in IL-4 levels caused by 3C9, in addition to its potent effects on BAL leukocyte counts (a combination also seen with CycA and tacrolimus) suggests that it may be useful in treating asthma.

3.2.2.5.1.2 IL-4 levels in the BAL fluid and lung extracts of Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

Levels of IL-4 were significantly increased upon OVA challenge as was found in the Th2 model although they were not as high. This would suggest that there is at least a partial Th2 response in this mixed Th1/Th2 model. CycA again reduced IL-4 levels although only significantly at the higher dose, while tacrolimus did not have a significant impact despite also reducing levels of the cytokine. Meanwhile, 3C9 significantly reduced IL-4 levels at 30mg/kg in the lung extracts only. Therefore, it would seem that CycA, tacrolimus and 3C9 have a greater impact on IL-4 levels in the Th2 model than in this supposed mixed response model.

If a mixed Th1/Th2 response has actually occurred, cytokines produced in the Th2 response would inhibit the Th1 response and vice versa. IL-4 inhibits the Th1

response by downregulating IL-12 production and receptor expression while IFNγ inhibits the release and receptor expression for IL-4 (Breit, Steinhoff et al. 1996; Murphy 1998; Paludan 1998). Therefore, IL-4 levels may be elevated in this model but also inhibited due to the Th1 response. This may explain why levels of the cytokine were not as high as in the Th2 model. The apparently less potent effect of CycA, tacrolimus and possibly 3C9 in this model may be due to the inhibition of both Th1 and Th2 responses. As previously mentioned, IL-4 levels may be reduced due to inhibition of mast cell degranulation. However, a reduced Th1 response would mean less inhibition of the Th2 response and thus not as great an impact on IL-4 levels. 3C8 may also affect both the Th1 and Th2 responses, as was found in the DTH and asthmatic models, meaning that a result similar to that found for CycA, tacrolimus and 3C9 may have been expected according to this theory. However, 3C8 did not have a significant impact in the Th2 model and yet it did at 30mg/kg in the lung extracts in this mixed model. It should be pointed out that this effect was not seen in the BAL samples and a reduction (although not significant) was seen in the Th2 model.

3.2.2.5.2 IL-5 ELISA

3.2.2.5.2.1 IL-5 levels in the BAL fluid and lung extracts of Balb/C mice sensitised to OVA using AlOH as an adjuvant

A significant increase in IL-5 levels was found in this Th2 model, reaching 288.1 ± 19.8pg/ml and 231.9 ± 11.5pg/ml in the BAL fluid and lung extracts respectively. High levels of the cytokine have also been found in many other models of allergic

asthma in Balb/C mice and Brown Norway rats (Haczku, Macary et al. 1996; Hellings, Vandekerckhove et al. 2003; Smit, Van Loveren et al. 2003; McKay, Leung et al. 2004; Itami, Oshiro et al. 2005). This is usually accompanied by an increase in IL-4 levels in the lungs as was found in this model.

Tacrolimus reduced IL-5 levels significantly at 10mg/kg and was more potent than CycA which only had a significant impact on IL-5 levels in the BAL fluid at this dose. As stated earlier, tacrolimus has a broader range of actions than CycA and has been found to inhibit IL-2 induced IL-5 production in human $CD4^+$ T cells (Mori, Suko et al. 1997). This may explain the greater potency of tacrolimus in reducing levels of the cytokine in this assay. CycA did however have a great impact in both samples at 30mg/kg, reducing levels of the cytokine by 35-40%. This could be due to the inhibition of mast cell degranulation caused by both drugs or could be because of a more direct effect on IL-5. These results are in accordance with other IL-5 assays performed following CycA or tacrolimus treatment. IL-5 levels have been reduced by both CycA and tacrolimus in models of allergic asthma in mice and in peripheral blood mononuclear cells in atopic patients (Mori, Suko et al. 1994; Sano, Nakamura et al. 1995; Eum, Zuany-Amorim et al. 1997). 3C8 and 3C9 also potently reduced levels of this cytokine, each having a significant impact at 30mg/kg.

IL-5 is produced from a number of cells including mast cells and eosinophils. It is the most important of the eosinophil active cytokines, acting specifically on mature eosinophils and contributing not only to their differentiation and survival but also their chemotaxis and degranulation (Clutterbuck, Hirst et al. 1989; Sanderson 1992; Meng, Ying et al. 1997). Therefore, the reduction in IL-5 levels may be the reason for

the decrease in eosinophils found in the BAL fluid following all 4 treatments. IL-5 levels have been found to correlate significantly with eosinophilia in the BAL fluid of asthmatic children (Kim, Kim et al. 2003).

3.2.2.5.2.2 IL-5 levels in the BAL fluid and lung extracts of Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

BAL fluid levels of IL-5 were lower and lung extract levels higher than in the Th2 model. Overall, a large increase in IL-5 levels similar to that found in the Th2 model was found upon OVA challenge. This is in contrast to results found for IL-4, which was elevated to a greater extent in the Th2 model. However, unlike IL-4, IL-5 is not a Th2-specific cytokine and can be produced following both Th1 and Th2 responses. Therefore, any inhibition of the Th2 response by Th1 cells would not necessarily reduce overall IL-5 production.

All 4 tested drugs seemed to be even more potent than in the Th2 model with greater reductions in levels of IL-5 generally seen. For instance, tacrolimus reduced IL-5 levels by approximately 45% and 35% in the BAL fluid and lung extracts respectively compared to a 28% and 23% reduction in the corresponding samples in the Th2 model. A 10mg/kg dose of CycA only had a significant impact in the BAL fluid in the Th2 model but now significantly reduced IL-5 levels in both samples while 3C8 and 3C9, found not to have a significant impact at this lower dose in the Th2 model, did have a significant effect in one of the samples here. As mentioned, IL-5 can be produced in both Th1 and Th2 responses and yet all 4 compounds seemed to be more potent here than in the Th2 model. This would suggest that the reduction in IL-5 by

these drugs was not caused only by inhibition of the Th2 response (and mast cell degranulation) and may also involve Th1 inhibition. If this is true, these compounds could be of great benefit in treating both forms of asthma because of the significant role that IL-5 plays in both forms of the disease. Once again, a strong correlation can be seen between the reduction in IL-5 levels and BAL eosinophils for each drug.

3.2.2.5.3 IL-10 ELISA

3.2.2.5.3.1 IL-10 levels in the BAL fluid and lung extracts of Balb/C mice sensitised to OVA using AlOH as an adjuvant

Like IL-4, IL-10 is a Th2 cytokine which would explain the large increase found in both the BAL fluid and lung extracts upon OVA challenge. Levels of the cytokine have been found to greatly increase in similar models of allergic asthma in mice as well as in asthmatic patients (Robinson, Tsicopoulos et al. 1996; Van Scott, Justice et al. 2000; Smit, Van Loveren et al. 2003). It has been found to inhibit the DTH reaction induced by injection of Th1 clones into mouse footpads and also inhibited footpad swelling in SRBC-primed mice (Li, Elliott et al. 1994). These effects are most likely due to the inhibition of Th1 cytokine production since levels of IL-2, IFNγ and TNFα were reduced by IL-10 treatment in these experiments. In contrast, IL-10 has been found to support the growth and differentiation of mast cells and B cells (Thompson Snipes, Dhar et al. 1991; Rousset, Garcia et al. 1992). However, intranasal administration of IL-10 together with OVA has been found to suppress the recruitment of both neutrophils and eosinophils to the lungs of OVA sensitised mice while anti-IL-10 antibodies reversed this effect (Zuany-Amorim, Haile et al. 1995).

IL-10 also reduced TNFα release in the BAL fluid in this model while blockade of IL-10 caused an increase in TNFα production and an increase in neutrophil recruitment to the lungs in a rat lung inflammatory model (Shanley, Schmal et al. 1995). This could be explained by the upregulation of endothelial adhesion molecules by TNFα. Similarly, airway gene transfer of IL-10 inhibited antigen-induced airway hyper-responsiveness as well as reducing the number of eosinophils and neutrophils in the BAL fluid of OVA sensitized and challenged mice (Fu, Ye et al. 2006). These results would suggest that this anti-inflammatory cytokine regulates leukocyte infiltration into the airways and that it may have the potential to alleviate airway inflammation in allergic asthma.

CycA and tacrolimus both significantly reduced IL-10 levels in the BAL fluid and lung extracts, each having a similar potency at 10mg/kg. 3C9 also caused a significant reduction in levels of the cytokine in the BAL fluid only at 10mg/kg and in both samples at 30mg/kg. The greatest reduction in levels of IL-10 was an almost 50% reduction by tacrolimus at 10mg/kg and by 3C9 at 30mg/kg in the BAL fluid and lung extracts respectively. Because of the anti-inflammatory effect of IL-10, we might have expected one or more test compounds to raise levels of the cytokine to correlate with the inhibition of leukocyte recruitment. However, the decrease in IL-4 and IL-5 (as caused by all 3 drugs), both of which contribute to this process may have been sufficient to reduce BAL neutrophils and eosinophils despite the reduction in IL-10 levels. It is interesting that 3C8 did not significantly reduce IL-10 levels at either dose. This result, combined with its potent inhibition of IL-5 in this model may make it even more useful therapeutically and may explain the reduction in BAL neutrophils and eosinophils caused despite failing to significantly reduce levels of IL-4.

3.2.2.5.3.2 IL-10 levels in the BAL fluid and lung extracts of Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

Once again, IL-10 levels were significantly raised in the positive control group and were similar to levels found in the Th2 model. In this case, CycA caused the greatest reduction in levels of the cytokine when dosed at 30mg/kg and was also potent at 10mg/kg. Tacrolimus had a significant impact in the lung extracts only. Results for 3C8 and 3C9 were almost the opposite of what was found in the Th2 model. 3C8, which did not have any significant effect in the previous model did reduce IL-10 levels significantly in the lung extracts at the higher dose. Meanwhile, 3C9, which was very potent in the Th2 model did not have a significant impact here at either dose.

IL-10 is produced not only from Th2 cells and B cells but also from macrophages (Li, Elliott et al. 1994). Therefore, in this mixed Th1/Th2 model, the cytokine may be produced from all 3 cells. This may explain why CycA was even more potent at reducing IL-10 levels here than in the Th2 model given its anti-Th1 and anti-Th2 effects. The same may be true for 3C8 which may also be more effective here due to the inhibition of IL-10 production from macrophages. The most obvious difference between the results found in this model compared to the Th2 model was seen with 3C9. Perhaps IL-10 production from Th2 cells and/or B cells but not from macrophages was inhibited by 3C9. This could explain why the drug was potent at reducing levels of the cytokine in the Th2 model but not in this mixed model, in which IL-10 may also be produced from macrophages.

3.2.2.5.4 IFNγ ELISA

3.2.2.5.4.1 IFNγ levels in the BAL fluid and lung extracts of Balb/C mice sensitised to OVA using AlOH as an adjuvant

IFNγ is produced from Th1 cells and it is therefore surprising that a significant increase in levels of the cytokine was found in this Th2 model. In the BAL fluid of asthmatic children in which IL-5 levels were elevated, no difference was found in the levels of IFNγ (Kim, Kim et al. 2003). In addition, in a similar model of allergic asthma in Balb/C mice, IL-4 and IL-5 levels were increased but IFNγ was not detectable in the BAL fluid (McKay, Leung et al. 2004). Similar results were found in BN rats in which IL-4 and IL-5 mRNA levels were increased but IFNγ mRNA levels were significantly reduced in ovalbumin sensitised and challenged rats, again suggesting a Th2 specific response (Haczku, Macary et al. 1996). As mentioned, this result was unexpected and was generally not found in allergic models of asthma. However, IL-2 and IFNγ mRNA levels were elevated as were IL-4 and IL-5 mRNA levels 8 hours after OVA challenge in Sprague Dawley (SD) rats. This was not the case for BN rats which only demonstrated an increase in IL-4 and IL-5 mRNA levels (Renzi, al Assaad et al. 1996). In other similar experiments, the IL-4: IFNγ ratio was elevated in BN rats but was not as pronounced in SD rats due to the fact that IFNγ producing cells were not downregulated (Hylkema, Hoekstra et al. 2002). Thus, despite the fact that most authors have found an increase only in Th2 specific cytokines in OVA sensitised and challenged animals, there is some evidence of elevated levels of IFNγ in other Th2 models.

One possible explanation for the elevated levels of IFNγ found in this model is that it may have been produced from Th0 cells. As explained in **section 1.3**, the Th1 and Th2 responses are cross regulated with Th2 cytokines such as IL-4 inhibiting the Th1 response and Th1 cytokines such as IFNγ inhibiting the Th2 response. Both IL-4 and IFNγ are produced from Th0 cells and the development of either a Th1 or Th2 response depends on the ratio of IL-4 to IFNγ and IL-12. IFNγ is produced earlier from Th0 cells than IL-4 but since IL-4 is dominant over IFNγ, if enough IL-4 is present, a Th2 response will occur. Thus, despite the increase in levels of the Th1 cytokine in this model (presumably from Th0 cells), IL-4 levels which were also raised may have prevented the development of a Th1 response.

Both CycA and tacrolimus significantly reduced IFNγ levels, as did 3C8 at 30mg/kg in both the BAL fluid and lung extracts, and 3C9 at this higher dose in the lung extracts only. As explained earlier, both CycA and tacrolimus can indirectly inhibit IL-2 through binding to and inhibiting calcineurin. This would prevent IL-2 which is produced from Thp cells from stimulating IL-12 production from the antigen presenting cells (APCs), thus inhibiting the production of IFNγ from Th0 cells (also explained in **section 1.3**). This would support the theory that IFNγ is produced from Th0 cells in this model. Since 3C8 and 3C9 also had a significant impact at the higher dose, it suggests that they too can inhibit IFNγ production from Th0 cells. This could again be due to the inhibition of calcineurin or could be due to a more direct effect of these compounds on IFNγ and the Th0 cells.

3.2.2.5.4.2 IFNγ levels in the BAL fluid and lung extracts of Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

It is less surprising that IFNγ levels were significantly raised in this mixed Th1/Th2 response model. However, levels were not quite as high as in the Th2 model which was unexpected since in this case the cytokine may have been produced from both Th0 and Th1 cells. This could be because a Th2 specific response has occurred and IFNγ has been produced only from Th0 cells (as hypothesised in the Th2 model) or because the Th2 response was significantly stronger than the Th1 response. However, this may simply be due to the varying sensitivity and slightly erratic nature of the cytokine assays and the most important observation is that levels of the cytokine were significantly raised in this model. It should again be stated that this model is not established as a Th1/Th2 response model and further study would be of benefit in determining the exact response(s) generated.

Once again, CycA and tacrolimus significantly reduced IFNγ levels with a similar potency to the Th2 model, again presumably through calcineurin inhibition. 3C8 had a significant impact only at 30mg/kg, exactly as was found in the Th2 model while 3C9 was even more potent and was far more effective than in the previous model. IFNγ plays a role in upregulating the endothelial adhesion molecules and has been found to do so synergistically in the presence of TNF (Doukas and Pober 1990). Therefore, inhibition of this cytokine would also contribute to the reduction in lung eosinophil and neutrophil levels caused by these drugs. A 55-60% reduction in IFNγ levels was found for 3C9 at 30mg/kg, the greatest reduction caused by any compound in the BAL fluid and second only to CycA at 30mg/kg in the lung extracts. What this

suggests is that 3C9 may have a more potent anti-Th1 effect than the other compounds. Since it was more effective here than in the Th2 model, it may have specifically inhibited the Th1 response and subsequent IFNγ production with less effect, if any, on Th0 derived IFNγ. Also, since this compound significantly reduced IL-4 levels in the Th2 model whereas 3C8 did not, 3C9 may be beneficial in treating both intrinsic and extrinsic asthma due to its effects on both Th1 and Th2 responses.

3.2.3 Eosinophil Peroxidase (EPO) and Myeloperoxidase (MPO) assays performed on Mouse Lung Extracts following BAL

3.2.3.1 Introduction

Eosinophil peroxidase (EPO) is released from eosinophils upon degranulation along with other eosinophil derived proteins such as eosinophil cationic protein (ECP), major basic protein (MBP) and eosinophil-derived neurotoxin. As mentioned earlier, these cytotoxic proteins are believed to contribute to the development of AHR, possibly by causing epithelial shedding. Elevated levels of these proteins have been found in the BAL fluid of asthmatic patients (Durham, Kay 1985; Diaz, Gonzalez et al. 1989). Myeloperoxidase (MPO) is a neutrophil-derived protein which plays a role in the NADPH oxidase pathway, thus contributing to tissue injury through production of hypochlorous acid (HOCL). Neutrophils contribute to T cell and monocyte recruitment in inflammation (Taub, Anver et al. 1996). They are elevated in asthmatic patients but they might not be activated. There have been conflicting reports on MPO levels in asthma (DuBuske 1995; Schneider and Issekutz 1996). Therefore, evaluation

of EPO and MPO levels would hopefully determine if the corresponding leukocytes are active.

The aims of the EPO and MPO assays were to correlate eosinophil and neutrophil numbers measured in the BAL fluid in **section 3.2.1** with levels of EPO and MPO respectively and to compare these to cytokine levels measured in **section 3.2.2**. In this way, it would hopefully be determined if the eosinophils and neutrophils are active in the mouse asthmatic models. Further information regarding the modes of action of the novel compounds would also hopefully be obtained.

3.2.3.2 Methods

3.2.3.2.1 EPO Assay on mouse lung extracts following BAL

The EPO assay was performed as described by Schneider and Issekutz (Schneider and Issekutz 1996). Lung extracts were diluted 1/10 in 50mM N-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid (Hepes), pH 8.0 (EPO dilution buffer). Aliquots of 75µl of each sample were pipetted into four wells of a 96-well tissue culture plate. Cold stop solution (4N H_2SO_4, also containing 2mM resorcinol for the EPO assay) was added to two wells (150µl per well) to stop the reaction at t = 0 seconds (background O.D.). The EPO-substrate solution consisted of 50mM Hepes, pH 8.0, 6mM potassium bromide (KBr), 3mM *o*-Phenylenediamine, dihydrochloride (OPD) and 8.8mM hydrogen peroxide (H_2O_2). Substrate solution (75µl) was added to each well, the reaction was stopped after 30 seconds for EPO with 150µl of cold stop solution, and the O.D. was determined at 492nm. As an additional control, 75µl

dilution buffer was placed into four wells and 75µl substrate buffer was added followed by 150µl stop solution after 30 seconds. No colour reaction was observed in these controls. All reagents were used at room temperature and the reaction was carried out at 22°C. The enzyme activities of the lung samples were calculated by subtracting the mean background O.D. and are expressed as change of O.D./minute.

3.2.3.2.2 MPO Assay on mouse lung extracts following BAL

The MPO assay was also performed as described by Schneider and Issekutz (Schneider and Issekutz 1996). Lung extracts were diluted 1/10 in 10mM citrate buffer, pH 5.0 (MPO dilution buffer). Aliquots of 75µl of each sample were pipetted into four wells of a 96-well tissue culture plate. Cold stop solution (4N H_2SO_4) was added to two wells (150µl per well) to stop the reaction at t = 0 secs (background O.D.). The MPO-substrate solution was 3mM TMB, 120µm resorcinol and 2.2mM H_2O_2 in distilled water. Substrate solution (75µl) was added to each well, the reaction was stopped after 2 minutes for MPO with 150µl of cold stop solution, and the O.D. was determined at 450nm. As an additional control, 75µl dilution buffer was placed into four wells and 75µl substrate buffer was added followed by 150µl stop solution after 2 minutes. No colour reaction was observed in these controls. All reagents were used at room temperature and the reaction was carried out at 22°C. The enzyme activities of the lung samples were calculated by subtracting the mean background O.D. and are expressed as change of O.D./minute.

Optical densities for the EPO and MPO assays were written directly into lab books at the time of the experiments. Data was then analysed using Microsoft Excel and Graphpad Instat for statistical comparisons. All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests. Values were expressed as mean $\pm$ SEM with a P-value of less than 0.05 (compared to the positive control group) taken to be significant.

3.2.3.3 Materials

Sigma Aldrich, Airton Road, Tallaght, Dublin, Ireland.

H_2SO_4 (95-97%), H_2O_2 (30% w/w), KBr, sodium citrate and resorcinol were obtained from Sigma Aldrich.

Lennox Chemicals Ltd, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.

TMB (soluble), HEPES and OPD were supplied to Lennox Chemicals Ltd. by Merck, KgaA, 64271 Darnstedt, Germany.

3.2.3.4 Results

3.2.3.4.1 EPO and MPO assays performed on Balb/c mice sensitised to OVA using AlOH as an adjuvant

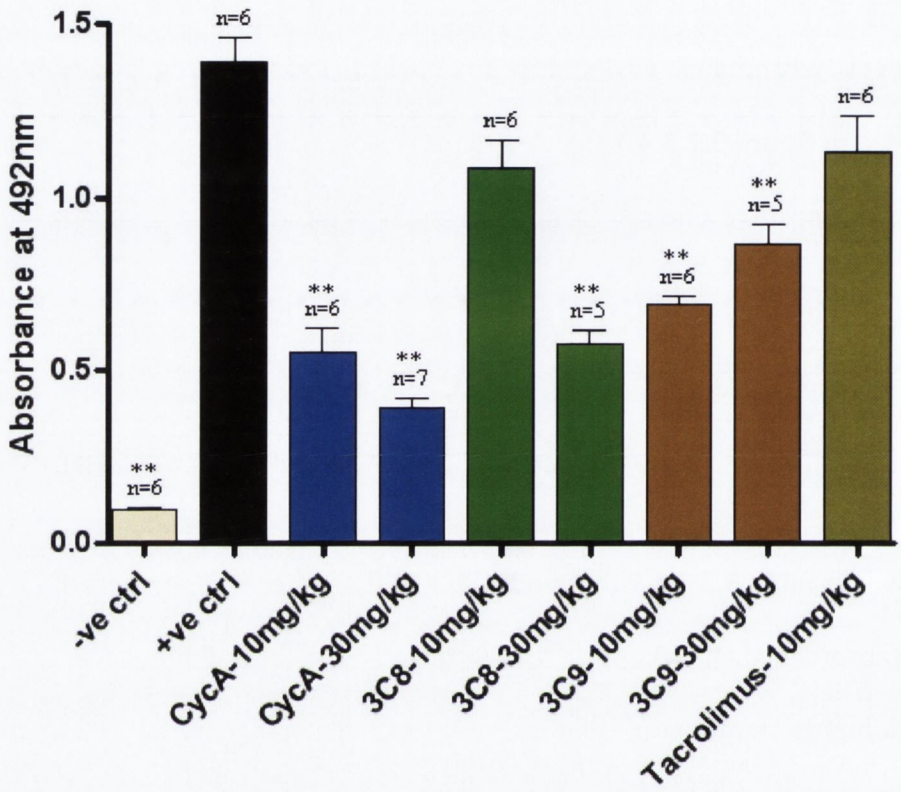
The EPO and MPO levels (mean $\pm$ SEM of O.D. at 492nm and 450nm respectively) seen in lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant are shown in **table 3.2.3.4.1 (appendix 2)**, and illustrated in **figures**

3.2.3.4.1 (a) and 3.2.3.4.1 (b). The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

EPO levels (absorbance at 492nm) are shown in **table 3.2.3.4.1 (appendix 2)** and illustrated in **figure 3.2.3.4.1 (a).** Approximately a 15-fold increase in EPO levels was seen in the positive control group compared to the negative controls and this was highly significant (P=0.004). CycA and 3C9 significantly reduced the positive control value at both 10 and 30mg/kg. 3C8 reduced this value significantly only at 30mg/kg and not at 10mg/kg while tacrolimus was not significant at 10mg/kg.

As shown in **table 3.2.3.4.1 (appendix 2)** and illustrated in **figure 3.2.3.4.1 (b),** MPO levels (absorbance at 450nm) were significantly greater in the positive control group where a highly significant 7-fold increase was seen compared to the negative control group (P=0.004). CycA, 3C8 and 3C9 significantly reduced this positive control value at both 10 and 30mg/kg, as did tacrolimus at 10mg/kg; i.e. all tested compounds were significant at all tested doses.

Figure 3.2.3.4.1 (a): EPO levels in lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



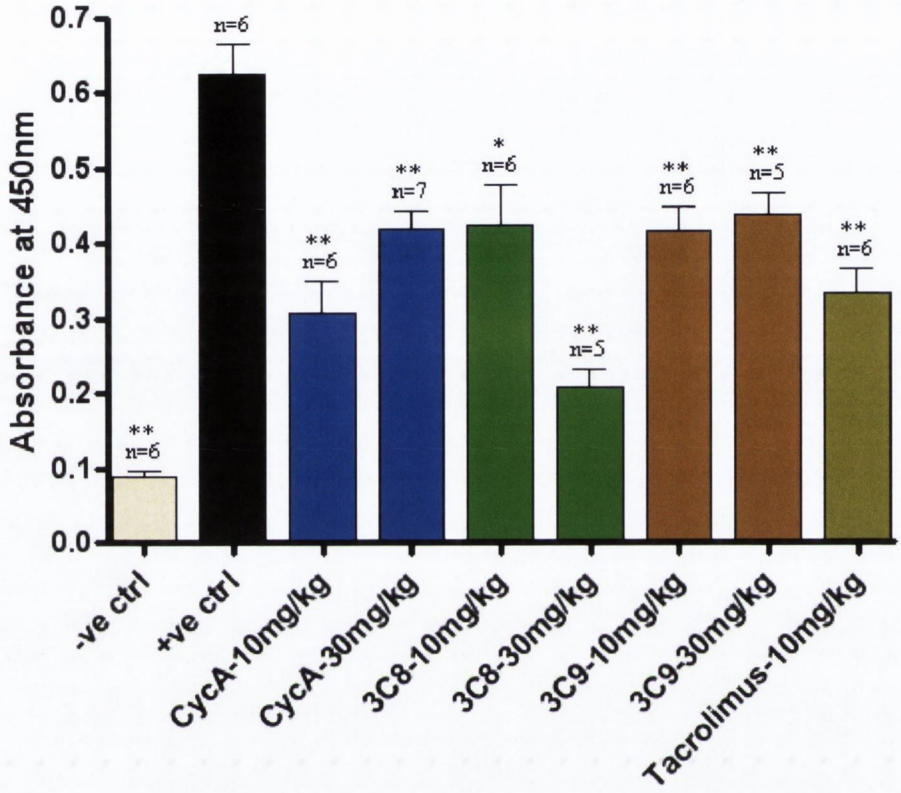
This graph represents the EPO levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Results are from 3 EPO assays.

Figure 3.2.3.4.1 (b): MPO levels in lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant



This graph represents the MPO levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, using AlOH as an adjuvant for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 MPO assays.

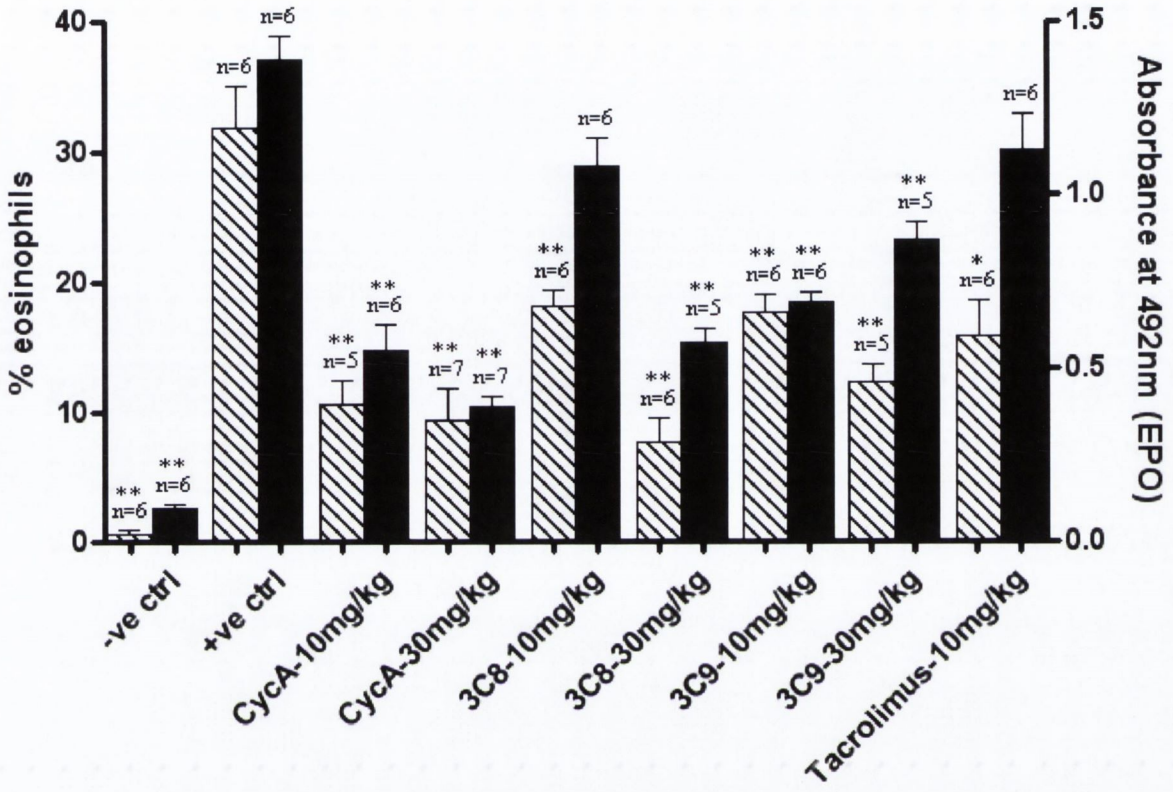
3.2.3.4.2 EPO and MPO assay results compared to eosinophil and neutrophil levels in Balb/C mice sensitised to OVA using AlOH as an adjuvant

The EPO and MPO levels shown in **section 3.2.3.4.1** are compared to the eosinophil and neutrophil levels shown in **section 3.2.1.4.1**. **Figure 3.2.3.4.2 (a)** compares the

EPO levels (mean ± SEM of O.D. at 492nm) found in the lung extracts to the eosinophil levels (mean ± SEM of % eosinophils in total cell count) found in the BAL fluid of Balb/C mice. Similarly, **figure 3.2.3.4.2 (b)** compares the MPO levels in lung extracts (mean ± SEM of O.D. at 450nm) to the BAL neutrophil levels (mean ± SEM of % neutrophils in total cell count) of the Balb/C mice.

A comparison of the statistics for EPO levels versus percentage eosinophils as well as for MPO levels versus percentage neutrophils can be seen in **table 3.2.3.4.1 (appendix 2)**. From this table, we see that a significant difference in EPO and MPO levels as well as in levels of both leukocytes can be seen in all treatment groups except for the 3C8-10mg/kg and tacrolimus-10mg/kg groups. In both these groups, there was a significant difference in eosinophil levels but not in EPO levels. Conversely, no significant difference in neutrophil levels was found while MPO levels were significantly reduced with both treatments.

Figure 3.2.3.4.2 (a): EPO levels in lung extracts compared to BAL eosinophil levels in Balb/c mice sensitised to OVA using AlOH as an adjuvant



The above graph represents the EPO levels in lung extracts and the BAL eosinophil levels (mean ± SEM) in Balb/c mice sensitised and challenged to OVA, using AlOH an adjuvant for sensitisation.

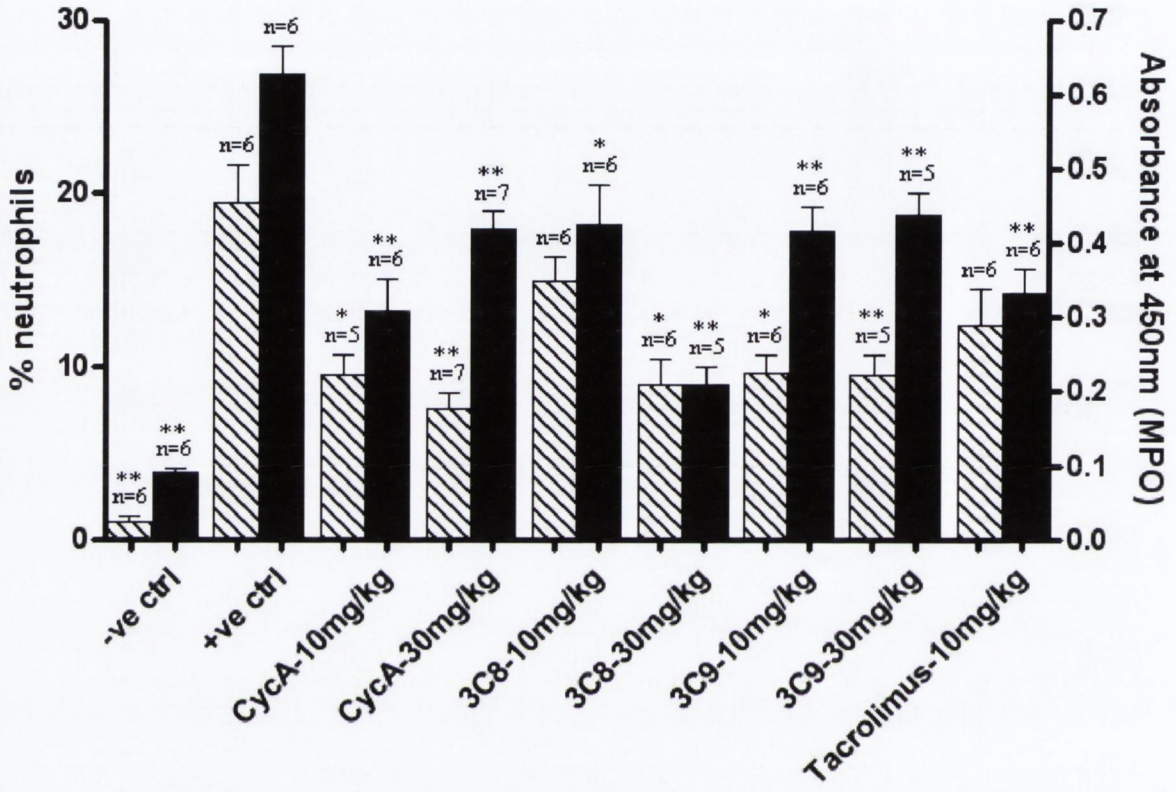
▨ = eosinophils; ■ = EPO

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Figure 3.2.3.4.2 (b): MPO levels in lung extracts compared to BAL neutrophil levels in Balb/c mice sensitised to OVA using AlOH as an adjuvant



The above graph represents the MPO levels in lung extracts and the BAL neutrophil levels (mean ± SEM) in Balb/c mice sensitised and challenged to OVA, using AlOH an adjuvant for sensitisation.

▨ = neutrophils; ■ = MPO

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

3.2.3.4.3 EPO and MPO assays performed on Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

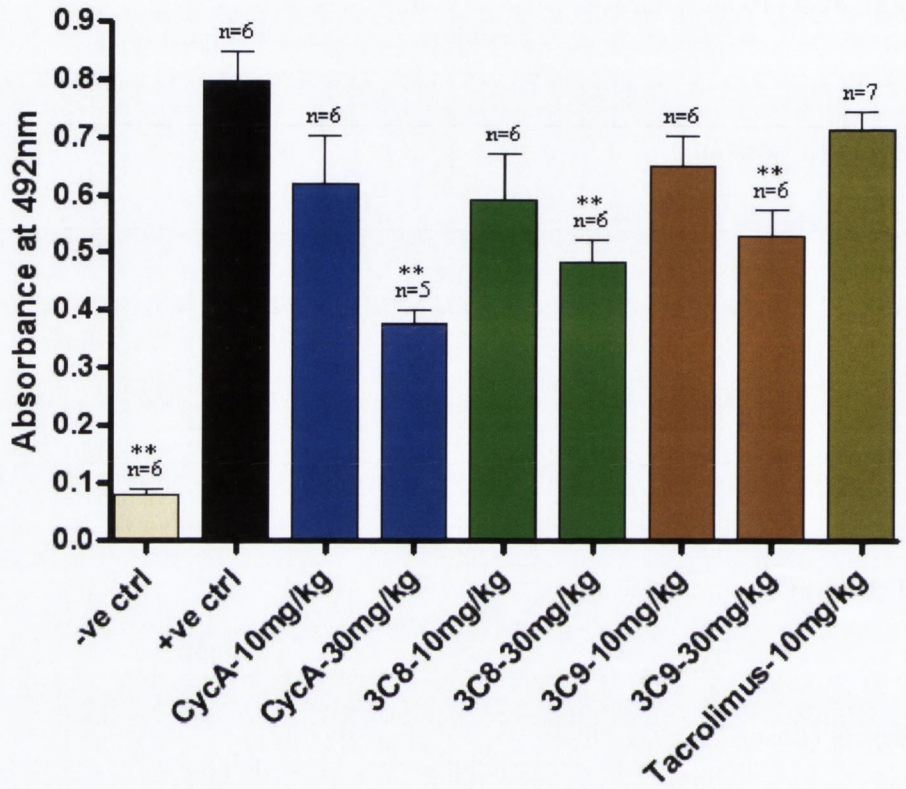
The EPO and MPO levels (mean ± SEM of O.D. at 492nm and 450nm respectively) seen in lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and

FCA(T) as adjuvants are shown in **table 3.2.3.4.2 (appendix 2)**, and illustrated in **figures 3.2.3.4.3 (a) and 3.2.3.4.3 (b).** The 9 groups are the negative control (–ve ctrl), the positive control (+ve ctrl group), CycA (10 and 30mg/kg), 3C8 (10 and 30mg/kg), 3C9 (10 and 30mg/kg) and tacrolimus (10mg/kg).

EPO levels (absorbance at 492nm) are shown in **table 3.2.3.4.2 (appendix 2)** and illustrated in **figure 3.2.3.4.3 (a).** Just over a 10-fold increase in EPO levels was seen in the positive control group compared to the negative controls and this was highly significant (P=0.004). CycA, 3C8 and 3C9 were all unsuccessful at significantly reducing this positive control value at 10mg/kg but all three were successful when dosing was at 30mg/kg. Tacrolimus also failed to significantly reduce the positive control value at 10mg/kg.

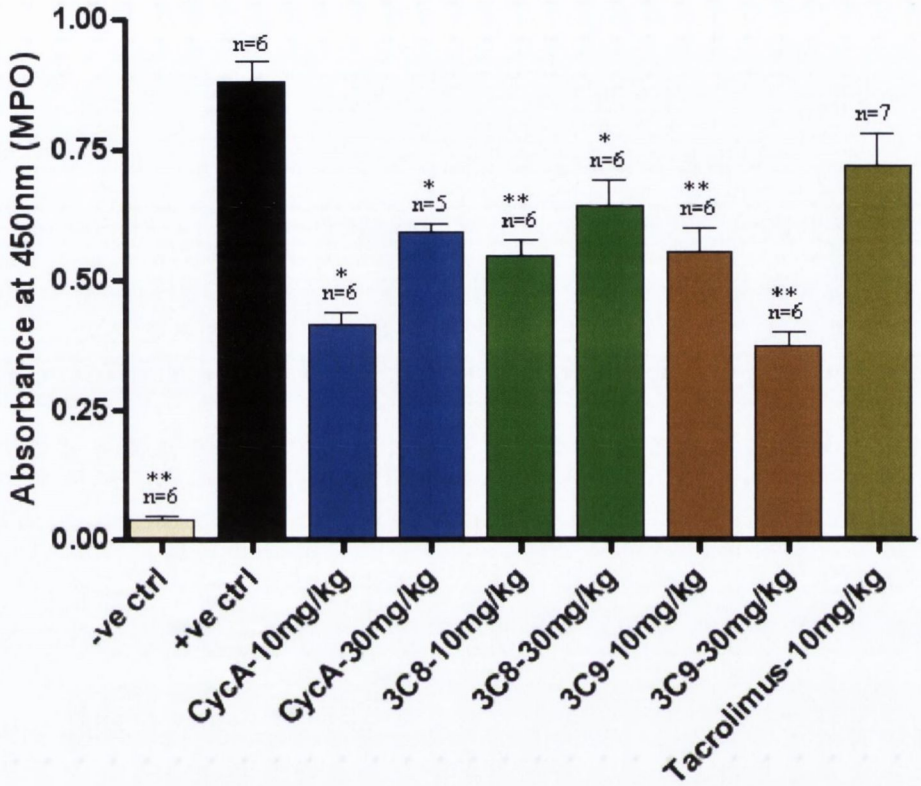
MPO levels (absorbance at 450nm) are shown in **table 3.2.3.4.2 (appendix 2)** and illustrated in **figure 3.2.3.4.3 (b)**. The positive control group showed almost a 25-fold increase in MPO levels compared to the negative controls and this was again highly significant (P=0.008). CycA, 3C8 and 3C9 all significantly reduced this value whether dosing was at 10mg/kg or 30mg/kg. Tacrolimus did not have a significant impact on the positive control value when administered at 10mg/kg.

Figure 3.2.3.4.3 (a): EPO levels in lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



This graph represents the EPO levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 EPO assays.

Figure 3.2.3.4.3 (b): MPO levels in lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



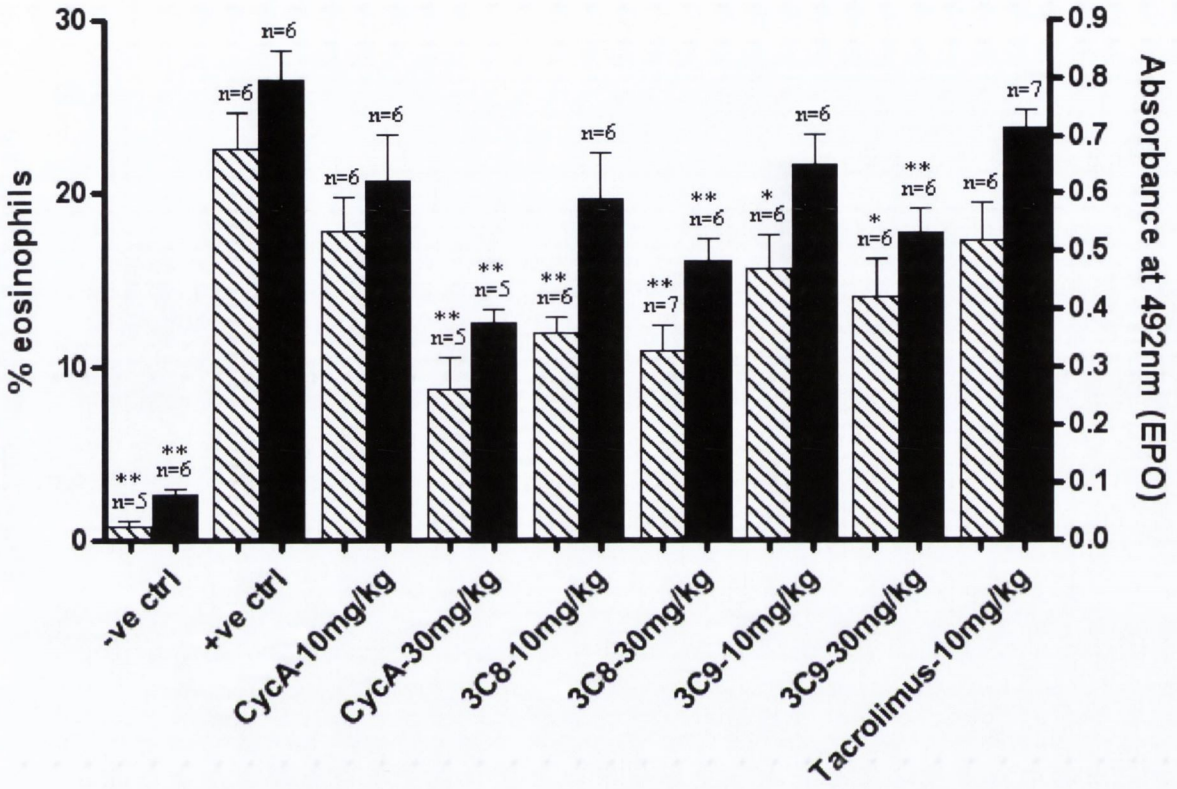
This graph represents the MPO levels (mean ± SEM) found in the lung extracts taken from Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.
* indicates statistical significance with a P-value of less than 0.05 compared to the positive control
** indicates statistical significance with a P-value of less than 0.01 compared to the positive control
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 MPO assays.

3.2.3.4.4 EPO and MPO assay results compared to eosinophil and neutrophil levels in Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

The EPO and MPO levels shown in **section 3.2.3.4.3** are compared to the eosinophil and neutrophil levels shown in **section 3.2.1.4.2**. This is done in exactly the same way as for **section 2.5.3.1**. **Figure 3.2.3.4.4 (a)** compares the EPO levels (mean $\pm$ SEM of O.D. at 492nm) found in the lung extracts to the eosinophil levels (mean $\pm$ SEM of % eosinophils in total cell count) found in the BAL fluid of Balb/C mice. Similarly, **figure 3.2.3.4.4 (b)** compares the MPO levels in lung extracts (mean $\pm$ SEM of O.D. at 450nm) to the BAL neutrophil levels (mean $\pm$ SEM of % neutrophils in total cell count) of the Balb/C mice.

The statistics for EPO levels versus percentage eosinophils as well as for MPO levels versus percentage neutrophils can be compared in **table 3.2.3.4.2 (appendix 2)**. A significant difference in EPO and MPO levels as well as in the levels of both leukocytes was seen only in the negative control, CycA-30mg/kg and 3C9-30mg/kg groups. At 10mg/kg, CycA only had a significant impact on the neutrophil and MPO levels while 3C9 only significantly reduced eosinophil and MPO levels at the same dose. 3C8 significantly reduced all four values at 10mg/kg except for EPO levels while at 30mg/kg, neutrophil levels were the only value not significantly reduced. Finally, tacrolimus was not found to have any significant impact on either the EPO or MPO levels or the percentage of either leukocyte.

Figure 3.2.3.4.4 (a): EPO levels in lung extracts compared to BAL eosinophil levels in Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



The above graph represents the EPO levels in lung extracts and the BAL eosinophil levels (mean ± SEM) in Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.

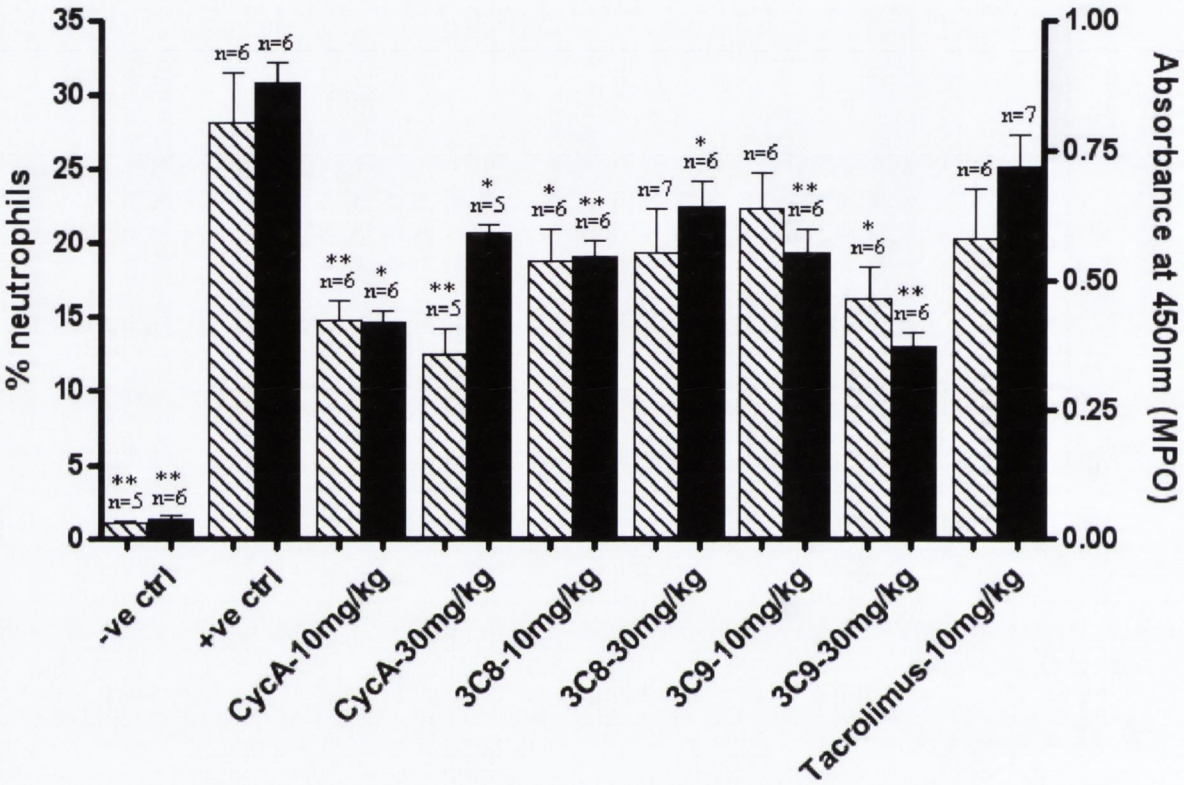
▨ = eosinophils; ■ = EPO

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Figure 3.2.3.4.4 (b): MPO levels in lung extracts compared to BAL neutrophil levels in Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants



The above graph represents the MPO levels in lung extracts and the BAL neutrophil levels (mean ± SEM) in Balb/c mice sensitised and challenged to OVA, using both AlOH and FCA(T) as adjuvants for sensitisation.

▨ = neutrophils; ■ = MPO

* indicates statistical significance with a P-value of less than 0.05 compared to the positive control

** indicates statistical significance with a P-value of less than 0.01 compared to the positive control

All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

3.2.3.5 Discussion

EPO and MPO levels were measured in lung extracts taken from each of the mouse asthmatic models. It was hoped that a correlation would be found with the

corresponding eosinophil and neutrophil levels, thus confirming the effects of the test compounds on BAL leukocyte numbers and possibly determining if these leukocytes are active.

3.2.3.5.1 EPO and MPO assays performed on Balb/c mice sensitised to OVA using AlOH as an adjuvant

EPO and MPO levels were significantly raised upon OVA challenge. A significant reduction in EPO levels was found for CycA and 3C9 at both doses as well as for 3C8 at 30mg/kg, and this correlated well with the reduction in eosinophils and neutrophils caused by these treatments. The increase in EPO levels found upon OVA challenge may mean that eosinophil degranulation has taken place, causing the release of EPO as well as ECP, eosinophil-derived neurotoxin, MBP and other mediators such as eicosanoids and various cytokines. The reduction in EPO levels caused by these treatments would then suggest that there is a reduction not only in eosinophil chemotaxis but also in numbers of active degranulating eosinophils. This would be of obvious benefit in treating asthma due to the roles that these eosinophil-derived mediators play in airway hyper-responsiveness (AHR) and the pathogenesis of asthma. However, Malm-Erjefalt and co-workers suggested that while EPO activity may reflect a higher activation state of the eosinophils, it may also reflect traumatic rupture of eosinophils caused by the processing procedure (Malm-Erjefalt, Persson et al. 2001). If this is the case, EPO levels would only be a measure of the presence of eosinophils and not of their activation state. EPO assays have been used to determine eosinophil levels in many mouse models of allergic asthma (Iwasaki, Tanaka et al. 2001; Itami, Oshiro et al. 2005). In addition, a strong correlation has been found

between EPO and eosinophil levels in other murine models of allergic asthma (Hellings, Vandekerckhove et al. 2003; Itami, Latinne et al. 2003; Konduri, Nandedkar et al. 2003).

At 10mg/kg, 3C8 and tacrolimus reduced EPO levels although not significantly, despite the potent effects of these treatments on eosinophil levels. This is surprising since a reduction in eosinophils should have a direct impact on levels of EPO, an eosinophil-specific mediator. It is possible that some eosinophils which had degranulated were not recognisable in the BAL fluid, thus lowering eosinophil counts without reducing EPO levels. The exact opposite was found for MPO and neutrophil levels, with both treatments significantly reducing the former and not the latter. This may mean that while neutrophil chemotaxis was not significantly altered, there was a significant reduction in neutrophil degranulation. However, eosinophils were distinguished from neutrophils primarily based on the presence of red granules in the cytoplasm [as illustrated in **figure 3.1.4.1 (c)**]. As mentioned earlier, incomplete staining of some eosinophils could have caused them to be mistaken for neutrophils. If this was the case and such an error was avoided, eosinophil levels would be higher and neutrophil levels lower than was found in the BAL cell counts and these results would then correlate better with the EPO and MPO levels. This would suggest that the EPO and MPO assays may be more accurate than BAL cell counts for estimating eosinophil and neutrophil levels. Use of these assays could therefore be of great benefit for screening the effects of novel compounds since they could eliminate time consuming slide preparations and cell counts (Varga, Beckman et al. 2002).

Meanwhile, MPO levels were significantly reduced (as were EPO levels) by CycA and 3C9 at both doses and by 3C8 at 30mg/kg. These also correlated with the potent effects of these treatments on BAL neutrophil levels, therefore confirming these results. This could mean that all 3 drugs had a potent effect against neutrophil recruitment and degranulation. EPO and MPO assays have been used for the specific detection and quantitation of eosinophils and neutrophils in the lungs of allergen-challenged, sensitised BN rats (Schneider and Issekutz 1996). In a similar allergic model in BN rats, EPO and MPO levels were elevated and correlated with eosinophil and neutrophil levels (Tigani, Hannon et al. 2003).

Neutrophils contribute to T cell and monocyte recruitment in inflammation (Taub, Anver et al. 1996). Activated neutrophils, in addition to releasing MPO, would also release eicosanoids and PAF (Lewis, Austin 1984). In this way, they could act in a similar way to eosinophils in contributing to the development of asthma. MPO itself is involved in the NADPH oxidase pathway, converting H_2O_2 to HOCL, which causes further oxidative tissue injury. However, while eosinophils and their products are known to contribute to the disease, the role of neutrophils and MPO in asthma is unknown and levels of MPO, in contrast to levels of eosinophil-derived proteins, have not been found in the BAL fluid of asthmatic patients (Durham, Kay 1985; Diaz, Gonzalez et al. 1989; DuBuske 1995). It should therefore be taken into account that, as with the EPO assay (and corresponding eosinophils), it is possible that neutrophils may have ruptured in the processing procedure, thus releasing MPO, meaning that elevated MPO levels may simply reflect an increase in neutrophils.

3.2.3.5.2 EPO and MPO assays performed on Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

Once again, both EPO and MPO levels were significantly raised in the positive control group. EPO levels were lower and MPO levels higher than in the Th2 model, exactly as was found for BAL levels of the corresponding leukocytes. Interestingly, this trend was also found in all 3 rat asthmatic models, with eosinophil levels found to be lower and neutrophil levels higher when FCA(T) was used for sensitisation. As previously mentioned, eosinophils play a large role in both forms of asthma but are particularly prominent in the intrinsic form. We might therefore have expected greater levels of this leukocyte in a Th1 or mixed response model than in a Th2-specific model. Once again, it should be stated however, that the responses generated in these FCA(T) models (and particularly the mixed response model) are not fully understood and further analysis of these responses would be beneficial for a clear interpretation of these results.

Tacrolimus was the only agent that failed to significantly reduce levels of either peroxidase and this was also consistent with BAL cell counts. CycA, 3C8 and 3C9, tested at both 10 and 30mg/kg, potently reduced MPO levels, while all significantly reduced EPO levels only at the higher dose. The only results that did not correlate with BAL leukocyte counts were the insignificant impacts of 3C8 and 3C9 on EPO levels at the lower dose and the potent reduction in MPO levels caused by 3C8 at 30mg/kg and 3C9 at 10mg/kg. This highlights another interesting trend found in the EPO and MPO assays in both mouse asthmatic models. As shown in **tables 3.2.3.4.1 and 3.2.3.4.2**, whenever a correlation was not found between EPO or MPO levels and

their corresponding leukcoytes, it was either due to a significant reduction in eosinophils and not in EPO or due to a significant reduction in MPO and not in neutrophils. This consistent trend would support the theory that some eosinophils may have been mistaken for neutrophils in BAL cell counts. In this case, the EPO and MPO assays, which as mentioned earlier may simply reflect levels of eosinophils and neutrophils, may be more suitable for accurate leukocyte counts while also providing quicker results.

Chapter 4

Final Discussion

Chapter 4: Final Discussion

This study set out to examine the effects of current immunosuppressants and novel compounds on animal models of DTH and asthma. Compounds were evaluated in 3 models of DTH; first in the mBSA model, and then in the SRBC and oxazolone models. CycA and tacrolimus are known immunosuppressants that act through inhibition of calcineurin and the NF-AT pathway, leading to inhibition of IL-2 gene transcription and therefore reduced IL-12 production from the antigen presenting cells (APCs). Both cytokines stimulate the production of IFNγ, an essential component of the Th1 response, while IL-2 contributes to macrophage activation. This anti-Th1 effect would explain the potent anti-inflammatory actions of both drugs in these Th1 models of DTH. Although tacrolimus did not have a significant impact in the mouse ear oxazolone model, it was more potent than CycA in the mBSA and SRBC models. This difference may be explained by the additional actions of tacrolimus in blocking cytokine receptor expression and inhibiting the effects of cytokines on target cells. For example, tacrolimus has been found to inhibit IL-2 induced IL-5 production in human $CD4^+$ T cells and has also been shown to inhibit T cell proliferation stimulated by IL-2 and IL-7 (Mori, Suko et al. 1997; Almawi and Melemedjian 2000).

3C8 and 3C9 also proved to be effective in 2 models of DTH but were not effective in the oxazolone model. This is exactly what was found for tacrolimus, which may suggest that all 3 compounds have similar modes of action. However, the exact mechanisms of action of these novel compounds are still unknown and neither compound was as potent as tacrolimus in the other 2 models of DTH. These results

confirm previous tests in our laboratory, in which some of these novel compounds were found to be effective in inflammatory models such as the carrageenin induced rat paw oedema model and the AA-induced mouse ear oedema model and showed a partial anti-Th1 effect through the inhibition of IL-2 release but not TNFα (Frankish, Sheridan unpublished data). In addition, several related compounds were found to inhibit compound 48/80-stimulated histamine release from rat peritoneal mast cells (Frankish, Farrell et al. 2004). Compounds that are effective in these DTH models may be useful in treating intrinsic asthma since it is believed to follow a Th1 response.

Following these DTH experiments, asthmatic models were set up in both rats and mice. Three rat asthmatic models were set up to try to mimic extrinsic asthma (Th2) and intrinsic asthma (Th1), while the third model would theoretically generate a mixed Th1/Th2 response. CycA and 3C8 were potent in reducing neutrophil levels, eosinophil levels or both in all three models. Of particular relevance is the fact that 3C8 significantly reduced eosinophil levels in both the Th2 and mixed Th1/Th2 response models whereas CycA only had this effect in the Th2 model. Eosinophils play a large role in the pathogenesis of both forms of asthma through the release of various cytotoxic proteins and eicosanoids while cytokines released include TNFα, which causes tissue remodelling and is important in leukocyte recruitment. The potency of 3C8 in reducing BAL levels of this leukocyte in two asthmatic models may therefore make it useful for asthma treatment. These results were later confirmed in equivalent Th2 and mixed Th1/Th2 models of asthma in mice, in which EPO and MPO levels correlated well with eosinophil and neutrophil BAL cell counts. Reduced EPO levels could mean that numbers of active degranulating eosinophils have been

reduced. However, some reports suggest that the processing procedure may rupture the leukocyte and EPO levels may therefore only be a measure of the presence of eosinophils and not of their activation state (Malm-Erjefalt, Persson et al. 2001).

Further analysis of the compounds involved examining BAL and lung tissue levels of IL-4, IL-5, IL-10 and IFNγ. IL-4 is crucial for the development of the Th2 response and also contributes to eosinophil recruitment by upregulating VCAM-1. IL-5 contributes to the differentiation, activation and survival of the eosinophils as well as inducing their degranulation and chemotaxis. IL-10 is, like IL-4, a Th2 cytokine which may inhibit the Th1 response. It has been found to inhibit production of the Th1 cytokines IL-2, IFNγ and TNFα as well as reducing footpad swelling in a mouse model of DTH (Li, Elliott et al. 1994). Finally, IFNγ is crucial in the development of the Th1 response and inhibits IL-4 secretion and receptor expression while also contributing to leukocyte recruitment through the upregulation of endothelial adhesion molecules (Paludan 1998; Doukas and Pober 1990). All 4 cytokines were significantly reduced in either the BAL fluid or lung extracts by CycA and tacrolimus, confirming the anti-Th1 and anti-Th2 effects of both compounds.

Mast cell degranulation is part of the early phase of extrinsic asthma and mediators released would include a number of cytokines, prostaglandins and leukotrienes, all of which contribute to leukocyte recruitment. Platelet activating factor (PAF) and the cysteinyl leukotrienes (cLTS) increase vascular permeability while PAF and leukotriene B_4 (LTB_4), also produced following mast cell degranulation, increase leukocyte adherence, therefore contributing further to leukocyte recruitment. The prostaglandins (PGs) cause vasodilation, which will allow for a greater concentration

of leukocytes at the site of inflammation. Inhibition of calcineurin would not only inhibit the Th1 response but also the Th2 response since it is a necessary target for the inhibition of mast cell degranulation as caused by both drugs. This would explain the potency of these immunosuppressants for reducing levels of these cytokines, including IL-4, the main Th2 cytokine and IFNγ, which is crucial for the Th1 response. Certain cytokines produced from mast cells may be released in part from preformed stores (for example TNFα), while others may be newly synthesised. Since mast cell degranulation is linked to the transcription of cytokine genes; inhibition of degranulation would not only result in the reduced release of preformed cytokines, but also in a reduced production of newly synthesised cytokines.

3C8 and 3C9 also reduced levels of all 4 cytokines in one or both mouse asthmatic models. 3C8 failed to significantly reduce IL-4 and IL-10 levels in the Th2 model but reduced levels of all 4 cytokines in the mixed response model. This could mean that 3C8 is more potent at inhibiting the Th1 response than the Th2 response. 3C9 seemed to be more effective at inhibiting both responses since all 4 cytokines were significantly reduced in both models except for IL-10 in the mixed response model. Therefore, 3C9 may have a broader range of actions than 3C8 and might act in a similar way to tacrolimus by inhibiting cytokine effects on target cells and/or blocking cytokine receptors. One or both of these compounds might inhibit calcineurin (like CycA and tacrolimus), therefore inhibiting the Th1 response (through inhibition of the NF-AT pathway and IL-2) and the Th2 response (through inhibition of mast cell degranulation). Alternatively, they might inhibit the formation of the calcium/calmodulin complex by reducing the availability of i.c. calcium (possibly by decreasing the release of calcium from i.c. stores or by reducing the influx of calcium

from e.c. sources) or by inhibiting calcium/calmodulin binding. This would also inhibit both the Th1 and Th2 responses since calcineurin is a calcium/calmodulin dependant phosphatase and the complex is also essential for mast cell degranulation. However, the exact mode(s) of action of these compounds are unknown and unlike the Th2 model, the Th1 and mixed Th1/Th2 response models have not been established as such. Further testing of BAL and lung cytokine levels may help in determining the exact response(s) generated.

4.1 Key Findings and Possible Modes of Action of Novel Compounds

A summary of all the results found for CycA, tacrolimus, 3C8, 3C9 and 6C6 is illustrated in **table 4.1 (appendix 2)**. As already mentioned, some of the novel compounds may have a similar mode of action to CycA and tacrolimus by inhibiting calcineurin and therefore inhibiting IL-2 gene transcription and the Th1 response as well as mast cell degranulation and the Th2 response. However, as can be seen in **table 4.1 (appendix 2)**, no two compounds produced identical results in all experiments and each may therefore have a different mode(s) of action. It should be pointed out that the novel compounds have completely differing structures to those of CycA and tacrolimus and are much smaller molecules. Also, CycA and tacrolimus do not possess the indane moiety.

3C8 and tacrolimus seemed to have similar effects on the DTH models, significantly reducing oedema in all but the oxazolone model. The oxazolone CHS model is a slightly different model of DTH and appears to involve IL-4 and Fas/Fas ligand binding. However, what may also be significant is the fact that Balb/C mice were used

in this model whereas CD-1 mice were used in the other 2 models of DTH. Using outbred CD-1 mice, which would show diversity in gene functions, may more closely mimic what would happen in humans. However, inbred Balb/C mice should be more reliable for reproducing results and may be more suitable for justifying further drug development work. The fact that the –ve ctrl group in the SRBC model showed some signs of oedema would also suggest that using inbred Balb/C mice would be more suitable for all three DTH models.

In comparing 3C8 and tacrolimus further, it is clear that despite the similar results found for both drugs in the DTH models, tacrolimus was more effective at reducing cytokine levels in the mouse asthma models. At 10mg/kg, tacrolimus significantly reduced IL-4 levels in the Th2 model as well as levels of IL-5, IL-10 and IFNγ in both Th2 and Th1/Th2 models. At the same dose, apart from the significant impact on IL-5 levels in the Th1/Th2 model, 3C8 failed to significantly reduce levels of any of the four cytokines. By inhibiting IL-2 induced IL-5 production in T cells, tacrolimus would inhibit eosinophil chemotaxis and degranulation. This could inhibit the release of more IL-4 and IL-5 from eosinophils. Meanwhile, inhibition of IL-2 stimulated T cell proliferation may explain the greater potency of tacrolimus in reducing IFNγ levels. Perhaps 3C8 successfully inhibited mast cell degranulation without reducing cytokine production from eosinophils or Th2 cells. This could have occurred due to inhibition of calcium/calmodulin formation or due to inhibition of another factor(s) involved in the degranulation pathway such as lyn, syk, PLC or IP_3. 3C8 could have inhibited IFNγ production through inhibition of calcineurin without having an impact on calcineurin-independent pathways such as the JNK and p38 pathways, which seem

to be essential for cytokine production in T cell activation. This could explain why IFNγ levels were reduced by 3C8 only at 30mg/kg.

As a pair of diastereoisomers, 3C8 and 3C9 should have similar physiochemical properties. Both compounds showed similar results in the DTH models as well as on their impact on BAL eosinophil levels in both mouse asthma models. Cytokine levels were also similar following treatment with both compounds but with two interesting differences. Compound 3C9 was more potent than 3C8 at reducing IFNγ levels in the Th1/Th2 model and also significantly reduced IL-4 levels in the Th2 model, which 3C8 did not, suggesting differing pharmacology between the two diastereoisomers. Therefore, 3C9 may be more potent at inhibiting both Th1 and Th2 responses. Perhaps 3C9, in contrast to 3C8, was able to inhibit IL-4 release from mast cells, Th2 cells and eosinophils. It may also have inhibited the JNK and p38 pathways (calcineurin-independent), therefore having a greater impact on IFNγ levels than 3C8.

Another interesting observation from these experiments is that 6C6 was the only novel compound to have a significant impact in the oxazolone model. This may relate to its structure since it is different to 3C8 and 3C9 and is the only one of these compounds to contain nitrogen. Perhaps 6C6 was able to regulate COX-2, TNFα or IL-1β produced by macrophages and/or IFNγ or IL-4 produced by Th cells since mRNA levels for COX-2 and all four cytokines were induced in similar oxazolone models (Wee, Shin et al. 2005). It is also possible that 6C6 may have inhibited the Fas/Fas ligand pathway since this has been shown to partially contribute to the development of CHS in other murine oxazolone models (Xu, Bulfone-Paus et al. 2003).

4.2 Future Studies

As mentioned earlier, the Th1 and mixed Th1/Th2 asthmatic models have not been established as such. One problem is that the Th1 model should theoretically represent intrinsic asthma, which is not universally accepted as following a Th1 cytokine profile. Another problem is trying to establish the response(s) evoked in the Th1 and Th1/Th2 asthma models. Further testing could involve examining levels of TNFα and perhaps levels of other cytokines involved in the Th1 response, such as IFNα, TNFβ and IL-12. By carrying out protein estimation for each sample, cytokine levels could be expressed per unit protein (pg/unit protein) instead of per unit volume (pg/ml). This would make comparisons between different drug treatment groups more meaningful, thus improving future ELISA experiments.

One way to improve the asthmatic models in the future would be to purify OVA. OVA has been found to contain high levels of endotoxin contamination. Watanabe and co-workers found that levels of LPS in commercial OVA (grade 5, as was used for the rat and mouse asthmatic models in this study) were sufficient to activate endothelial cells and suppress the inflammatory effect of OVA (Watanabe, Miyazaki et al. 2003). Purified OVA was found to be more effective than commercial OVA in stimulating IgE production and lung inflammation in a murine model of AHR. Therefore, endotoxin contamination of OVA would seem to induce tolerance in this model. Consequently, elimination of endotoxin in future animal models of allergic asthma could ensure that the response generated is as pure a Th2 response as possible. While it may be impossible to completely eliminate endotoxin from OVA (Watanabe, Miyazaki et al. 2003), measures can be taken to minimise such contamination.

Mangan and co-workers described how endotoxin-free reagents were used in the preparation of OVA while all glassware and equipment were decontaminated in 0.5mM NaOH. In addition, endotoxin was partially removed using Detoxi-gel endotoxin removing gel columns and OVA preparations were dialysed against sterile PBS. This procedure reduced OVA endotoxin levels from ~1.5–3 endotoxin units/mg to less than 0.5 endotoxin units/mg (Mangan, van Rooijen et al. 2006). Murine models of AHR using commercial OVA may reflect human allergic asthma since simultaneous exposure to endotoxin and antigen would occur through organic dusts (Watanabe, Miyazaki et al. 2003). However, such models would not be an accurate reflection of the effect of antigen alone.

Another way in which the animal models of asthma could be improved in future studies would be to use flow cytometry to differentiate BAL cells. Counting and identifying cells in BAL cytospin preparations is not only time consuming but also highly subjective. Distinguishing between neutrophils and eosinophils can be particularly difficult if the red granules of the eosinophils are not clearly visible. Flow cytometry can be used to identify BAL fluid cells based on forward scatter (FSC) and side scatter (SSC) characteristics, autofluorescence of macrophages and simultaneous staining with various antibodies, including CCR3-PE for eosinophils. This method has been found to correlate very well with differential cell counts performed on BAL cytospin preparations in an animal model of asthma (van Rijt, Kuipers et al. 2004). It has also been used to identify cells in lung digests in a similar allergic model in mice (Mangan, van Rooijen et al. 2006). Eosinophils were distinguished as SSC^{high}, $CD19^{-}$, $CD4^{-}$, $CD8^{-}$ nonautofluorescent granulocytes that stain positive for CCR3. Flow cytometric determination of BAL fluid cells is a rapid method which could eliminate

the need for time consuming and subjective BAL cell counts. In addition, airways resistance could be measured in the asthma models by whole body plethysmography (Featherstone, Parry et al. 1996). This would act as an indicator of airways obstruction.

The DTH models could be improved in future studies by using the same mouse strain in each model. Inbred Balb/C mice, as used in the oxazolone model, would be suitable and should be more reliable than outbred CD-1 mice for reproducing results and justifying further drug development work. In order to further improve the accuracy and consistency of results in the mBSA and SRBC paw swelling models, the challenged paw could be measured before and after challenge. This would eliminate the possibility of inaccurate results due to size differences between the left and right paws of the animal.

All novel compounds were administered i.p in every model since they are non-polar and it was not known if they would be orally active. However, subsequent unpublished results from our laboratory have shown 3C9 to have efficacy in an *in vivo* inflammatory model when administered orally. In addition, PH44, a closely related compound to 3C8 and 3C9, showed good permeability through caco-2 cell layers, again suggesting oral availability (Frankish, Sheridan unpublished data). However, there is no current estimation of bioavailability. Future testing of novel compounds could therefore involve oral administration in animal models of DTH and asthma.

Side effects of the novel compounds could include nephrotoxicity, hepatotoxicity and hypertension, as seen with CycA. However, in acute toxicity tests performed in our

laboratory in Balb/C mice, no observable morbidity was found following a single 300mg/kg i.p. dose of 3C8. There was also no mortality and no observable morbidity following daily treatment with 3C9 for 5 days, again at 300mg/kg i.p. and only slight sedation was observed following injection (Frankish, Sheridan unpublished data).

4.3 Final Conclusion

The current study has shown extremely promising results for novel compounds, in particular 3C8 and 3C9, in animal models of DTH and asthma. Both diastereoisomers significantly reduced oedema in 2 DTH models and also significantly reduced BAL eosinophil levels in the Th2 and Th1/Th2 asthma models. The reduction in eosinophil levels is particularly relevant due to the significant role that the leukocyte plays in the pathogenesis of asthma. In addition, these results correlated with the significant impact of both drugs on BAL fluid and/or lung extract levels of IL-4, IL-5, IL-10 and IFNγ, cytokines which play crucial roles in the development of Th1 and Th2 responses. These findings suggest that the novel compounds may be of great benefit in treating asthma. Due to their significant impact on both Th1 and Th2 models, these compounds may also be suitable for treating organ transplantation rejection as well as autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.

Chapter 5

Bibliography

Chapter 5: Bibliography

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Appendices

Appendix 1

Table 3.1.2.1 (a): Sensitisation of rats to OVA using AlOH as an adjuvant

OVA/AlOH sensitisation mixture – administered 1ml i.p.	
OVA	**AlOH**
1mg/ml	100mg/ml

Table 3.1.2.1 (b): Vehicle/Drug treatment and OVA challenging procedure of rats sensitised to OVA using AlOH as an adjuvant

Group	Vehicle/drug treatment i.p. 1 hour prior to challenge	Challenging (30 mins by nebuliser)
Negative control	Vehicle (1% CMC)	dH_20
Positive control	Vehicle (1% CMC)	5% OVA
CycA	10mg/kg (in 1% CMC)	5% OVA
3C8	10mg/kg (in 1% CMC)	5% OVA

Table 3.1.2.2 (a): Sensitisation of rats to OVA using FCA(T) as an adjuvant

OVA/FCA(T) sensitisation mixture – administered 1ml i.p.	
OVA	**FCA(T)**
1mg/ml	66% (v/v); 660µl/ml

Table 3.1.2.2 (b): Vehicle/Drug treatment and OVA challenging procedure of rats sensitised to OVA using FCA(T) as an adjuvant

Group	Vehicle/drug treatment i.p. 1 hour prior to challenge	Challenging (30 mins by nebuliser)
Negative control	Vehicle (1% CMC)	dH_20
Positive control	Vehicle (1% CMC)	5% OVA
CycA	10mg/kg (in 1% CMC)	5% OVA
3C8	10mg/kg (in 1% CMC)	5% OVA

Table 3.1.2.3 (a): Sensitisation of rats to OVA using AlOH and FCA(T) as adjuvants

OVA/AlOH/FCA(T) sensitisation mixture – administered 1ml i.p.		
OVA	**AlOH**	**FCA(T)**
1mg/ml	100mg/ml	66% (v/v); 660µl/ml

Table 3.1.2.3 (b): Vehicle/Drug treatment and OVA challenging procedure of rats sensitised to OVA using AlOH and FCA(T) as adjuvants

Group	Vehicle/drug treatment i.p. 1 hour prior to challenge	Challenging (30 mins by nebuliser)
Negative control	Vehicle (1% CMC)	dH_20
Positive control	Vehicle (1% CMC)	5% OVA
CycA	10mg/kg (in 1% CMC)	5% OVA
3C8	10mg/kg (in 1% CMC)	5% OVA

Table 3.2.1.2 (a): Sensitisation of mice to OVA using AlOH as an adjuvant

OVA/AlOH sensitisation mixture – administered 200µl i.p.	
OVA	**AlOH**
100µg/200µl	4.5mgs/200µl

Table 3.2.1.2 (b): Sensitisation of mice to OVA using both AlOH and FCA(T) as adjuvants

OVA/AlOH/FCA(T) sensitisation mixture – administered 200µl i.p.		
OVA	**AlOH**	**FCA(T)**
100µg/200µl	4.5mgs/200µl	66% (v/v)

Table 3.2.2.2: Preparation of mouse antibodies used in ELISA experiments

Specificity	Type	Reconstituted Concentration	Working Concentration
Mouse IL-4	Monoclonal (capture)	720μg/ml in 1ml PBS	4μg/ml in 10mls PBS
Mouse IL-4	Biotinylated (detection)	72μg/ml in 1ml diluent	400ng/ml in 10mls diluent
Mouse IL-4	Recombinant (standard)	100ng/ml in 0.5mls diluent	0-1000pg/ml in diluent
Mouse IL-5	Monoclonal (capture)	180μg/ml in 1ml PBS	1000ng/ml in 10mls PBS
Mouse IL-5	Biotinylated (detection)	18μg/ml in 1ml diluent	100ng/ml in 10mls diluent
Mouse IL-5	Recombinant (standard)	60ng/ml in 0.5mls diluent	0-2ng/ml in diluent
Mouse IL-10	Monoclonal (capture)	360μg/ml in 1ml PBS	2μg/ml in 10mls PBS
Mouse IL-10	Biotinylated (detection)	72μg/ml in 1ml diluent	400ng/ml in 10mls diluent
Mouse IL-10	Recombinant (standard)	70ng/ml in 0.5mls diluent	0-2ng/ml in diluent
Mouse IFNγ	Monoclonal (capture)	720μg/ml in 1ml PBS	4μg/ml in 10mls PBS
Mouse IFNγ	Biotinylated (detection)	72μg/ml in 1ml diluent	400ng/ml in 10mls diluent
Mouse IFNγ	Recombinant (standard)	45ng/ml in 0.5mls diluent	0-2ng/ml in diluent

Appendix 2

Table 2.1.4: Percentage increase in paw oedema in CD-1 mice sensitised and challenged with mBSA using FCA(B) as an adjuvant for sensitisation

Treatment	n	Unchallenged Left Paw (mls)	Challenged Right Paw (mls)	% Paw Swelling	P-value
+ve ctrl	23	0.099	0.204	107.3 ± 6.4	---
CyclosporinA-50mg/kg	14	0.090	0.116	29.4 ± 4	<0.0001
Tacrolimus-1mg/kg	12	0.088	0.144	63.2 ± 7	0.0002
Tacrolimus-3mg/kg	12	0.101	0.145	44.8 ± 6.3	<0.0001
Tacrolimus-10mg/kg	11	0.098	0.136	38.4 ± 5.8	<0.0001
3C8-3mg/kg	6	0.119	0.219	85.4 ± 12	0.119
3C8-10mg/kg	21	0.117	0.198	71.5 ± 7.3	0.0002
3C8-30mg/kg	6	0.122	0.191	56.9 ± 8.1	<0.0001
3C8-100mg/kg	5	0.115	0.205	76.3 ± 15.2	0.045
3C9-10mg/kg	12	0.106	0.181	70.8 ± 6.3	0.001
3C4-30mg/kg	4	0.114	0.218	90.3 ± 13.9	0.531
7C9-10mg/kg	10	0.111	0.183	66.4 ± 9.3	0.006
7C9-30mg/kg	6	0.111	0.228	106.5 ± 18.8	0.694
6C6-10mg/kg	10	0.109	0.166	50.4 ± 7.3	<0.0001
6C6-30mg/kg	6	0.110	0.183	68.8 ± 14.5	0.036
7C17-30mg/kg	6	0.113	0.222	98.6 ± 17.5	0.628
6C7-30mg/kg	6	0.118	0.221	88.7 ± 17.1	0.142
5C4-30mg/kg	5	0.108	0.219	102.4 ± 17.9	0.954

The +ve ctrl group was untreated (received only 1% CMC). All groups were sensitised and challenged with mBSA.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 4-6 groups at a time with n=6 per group.

Table 2.2.4: Percentage increase in paw oedema in CD-1 mice sensitised and challenged with Sheep Red Blood Cells

Treatment	n	Unchallenged Left Paw (mls)	Challenged Right Paw (mls)	% Paw Swelling	P-value
-ve ctrl	13	0.127	0.157	24.4 ± 3.8	<0.0001
+ve ctrl	11	0.119	0.221	86.4 ± 7.2	---
CyclosporinA-50mg/kg	11	0.118	0.184	56.8 ± 6.2	0.008
CyclosporinA-100mg/kg	6	0.098	0.150	51.6 ± 6.0	0.010
Tacrolimus–10mg/kg	11	0.121	0.193	58.9 ± 7.8	0.014
Tacrolimus–30mg/kg	6	0.119	0.171	44 ± 2.7	0.001
3C8-10mg/kg	11	0.111	0.192	73.3 ± 5.0	0.217
3C8-30mg/kg	6	0.114	0.183	59.8 ± 7.2	0.038
3C9-10mg/kg	6	0.105	0.181	72.8 ± 9.5	0.180
3C9-30mg/kg	6	0.114	0.174	52.5 ± 7.4	0.028

The –ve ctrl group was not sensitised (received only PBS) and was challenged with sheep red blood cells. All other groups were sensitised and challenged with sheep red blood cells. The –ve ctrl and +ve ctrl groups were untreated (received only 1% CMC).

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 4-6 groups at a time with n=6 per group.

Table 2.3.4: Percentage increase in ear swelling in Balb/C mice sensitised and challenged with oxazolone

Treatment	n	Unchallenged Left Ear		Challenged Right Ear		% Ear Swelling	
		Thickness (μm)	Weight (mgs)	Thickness (μm)	Weight (mgs)	Thickness	Weight
+ ve ctrl	6	30.7	8.0	58	17.1	90.2 ± 6.6	108.7 ± 14.1
CyclosporinA-15mg/ml	6	30	4.7	47.3	7.7	58.4 ± 4.7	64.3 ± 5.3
P-value						0.009	0.026
Tacrolimus-15mg/ml	6	28.5	4.7	50.8	8.8	78.5 ± 4.8	88.8 ± 5.9
P-value						0.132	0.240
Dexamethasone-15mg/ml	6	22.2	10.1	33.3	16.2	51.1 ± 8.1	59.9 ± 6.7
P-value						0.009	0.026
3C8-15mg/ml	6	25.7	11.3	45.2	22.9	76.7 ± 8.4	104.4 ± 15.3
P-value						0.240	0.937
3C9-15mg/ml	6	24.8	11.3	46	24.9	86.0 ± 7.9	121.3 ± 7.2
P-value						0.575	0.485
6C6-15mg/ml	6	25.7	11.7	41.5	19.3	62.3 ± 6.0	65.4 ± 3.4
P-value						0.015	0.041

The +ve ctrl (positive control) group was untreated (received only acetone). All groups were sensitised and challenged with oxazolone.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 3-4 groups at a time with n=6 per group.

Table 3.1.4.1: Percentage leukocytes in BAL fluid taken from BN rats sensitised to OVA using AlOH as an adjuvant

		% Leukocytes in BAL fluid				
Treatment	**n**	**Neutrophils**	**Eosinophils**	**Small lymphocytes**	**Large Lymphocytes**	**Monocytes**
- ve ctrl	8	3.6 ± 0.78	1.2 ± 0.5	26.8 ± 1.9	5.2 ± 1.5	63.1 ± 3.1
P-value		<0.0001	<0.0001	<0.0001	0.414	<0.0001
+ ve ctrl	13	39 ± 2.7	24.7 ± 1.8	13.4 ± 1.3	6.1 ± 0.9	14.8 ± 2.9
CycA-10mg/kg	8	10.5 ± 1.4	5.5 ± 0.5	25.4 ± 3.9	2.6 ± 0.7	54.5 ± 2.6
P-value		<0.0001	<0.0001	0.003	0.005	<0.0001
3C8-10mg/kg	12	14.6 ± 4.3	10.1 ± 3.5	26.8 ± 4.2	2.5 ± 0.8	42.8 ± 7.8
P-value		0.0001	0.002	0.001	0.001	0.001

The –ve ctrl group was sensitised to OVA but was not challenged (received only dH_2O). All other groups were sensitised and challenged with OVA. The –ve ctrl and +ve ctrl groups were untreated (received only 1% CMC).

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 2-4 groups at a time with n=6 per group.

Table 3.1.4.2: Percentage leukocytes in BAL fluid taken from BN rats sensitised to OVA using FCA(T) as an adjuvant

		% Leukocytes in BAL fluid				
Treatment	**n**	**Neutrophils**	**Eosinophils**	**Small lymphocytes**	**Large Lymphocytes**	**Monocytes**
- ve ctrl	6	0.5 ± 0.3	0.06 ± 0.07	2.7 ± 0.9	0.2 ± 0.1	96.6 ± 1.1
P-value		<0.0001	<0.0001	0.045	0.007	<0.0001
+ ve ctrl	15	73.1 ± 2.6	6.8 ± 1.1	5.6 ± 0.8	1.5 ± 0.3	12.8 ± 2
CycA-10mg/kg	9	33.9 ± 8.7	6.6 ± 1.5	17 ± 4.2	0.6 ± 0.2	41.5 ± 9.4
P-value		0.001	>0.9999	0.007	0.043	0.003
3C8-10mg/kg	12	40.5 ± 7.2	10.4 ± 3.2	11.7 ± 1.6	0.9 ± 0.3	36.3 ± 5.4
P-value		0.0003	0.905	0.007	0.113	<0.0001

The –ve ctrl group was sensitised to OVA but was not challenged (received only dH_2O). All other groups were sensitised and challenged with OVA. The –ve ctrl and +ve ctrl groups were untreated (received only 1% CMC).

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 2-4 groups at a time with n=6 per group.

Table 3.1.4.3: Percentage leukocytes in BAL fluid taken from BN rats sensitised to OVA using both AlOH and FCA(T)as adjuvants

		% Leukocytes in BAL fluid				
Treatment	**n**	**Neutrophils**	**Eosinophils**	**Small lymphocytes**	**Large Lymphocytes**	**Monocytes**
- ve ctrl	6	1.3 ± 0.3	0.73 ± 0.18	15 ± 0.9	1.5 ± 0.3	81.5 ± 1.5
P-value		0.001	0.001	0.001	0.181	0.001
+ ve ctrl	7	51.7 ± 2.2	11.2 ± 1.3	8.3 ± 1.1	1.0 ± 0.3	27.8 ± 2.5
CycA-10mg/kg	8	19.6 ± 3.5	15.4 ± 2.2	27.3 ± 3.1	1.9 ± 0.3	35.7 ± 3.8
P-value		0.0003	0.072	0.0003	0.094	0.094
3C8-10mg/kg	8	35.1 ± 5.9	3.8 ± 1.3	23.9 ± 6.3	0.7 ± 0.3	36.4 ± 7.1
P-value		0.054	0.001	0.054	0.336	0.463

The –ve ctrl group was sensitised to OVA but was not challenged (received only dH_2O). All other groups were sensitised and challenged with OVA. The –ve ctrl and +ve ctrl groups were untreated (received only 1% CMC).

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 2-4 groups at a time with n=6 per group.

Table 3.2.1.4.1: Percentage leukocytes in BAL fluid taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant

		% Leukocytes in BAL fluid				
Treatment	**n**	**Neutrophils**	**Eosinophils**	**Small lymphocytes**	**Large Lymphocytes**	**Monocytes**
- ve ctrl	6	1.0 ± 0.3	0.5 ± 0.3	6.3 ± 0.6	0.2 ± 0.1	88.8 ± 3.2
P-value		0.002	0.002	0.699	0.370	0.002
+ ve ctrl	6	19.4 ± 2.4	31.8 ± 3.5	7.0 ± 1.2	0.7 ± 0.4	41.1 ± 2.9
CycA-10mg/kg	5	9.5 ± 1.3	10.5 ± 2.1	3.8 ± 0.4	0.3 ± 0.3	75.9 ± 3.4
P-value		0.017	0.004	0.030	0.710	0.004
CycA-30mg/kg	7	7.6 ± 0.9	9.3 ± 2.6	10.1 ± 3.4	1.0 ± 0.7	72.1 ± 3.0
P-value		0.002	0.001	0.945	0.717	0.001
3C8-10mg/kg	6	15 ± 1.4	18 ± 1.3	10.8 ± 1.2	0.3 ± 0.2	55.9 ± 1.9
P-value		0.093	0.004	0.394	0.516	0.004
3C8-30mg/kg	6	9.0 ± 1.7	7.7 ± 2.1	15.1 ± 5.0	1.9 ± 1.2	66.3 ± 4.2
P-value		0.038	0.010	0.171	0.392	0.010
3C9-10mg/kg	6	9.7 ± 1	17.7 ± 1.5	17.3 ± 5.1	0.3 ± 0.2	55.1 ± 4.2
P-value		0.015	0.004	0.026	0.567	0.026
3C9-30mg/kg	5	9.5 ± 1.3	12.2 ± 1.6	13.4 ± 5.4	0.8 ± 0.6	64.1 ± 5.7
P-value		0.009	0.004	0.247	>0.9999	0.017
Tacrolimus-10mg/kg	6	12.4 ± 2.4	15.7 ± 3.3	9.0 ± 1.5	0.9 ± 0.7	62.0 ± 2.8
P-value		0.114	0.019	0.352	0.914	0.010

The –ve ctrl group was sensitised to OVA but was not challenged (received only dH_2O). All other groups were sensitised and challenged with OVA. The –ve ctrl and +ve ctrl groups were untreated (received only 0.5% CMC).

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 2-5 groups at a time with n=6 per group.

Table 3.2.1.4.2: Percentage leukocytes in BAL fluid taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

		% Leukocytes in BAL fluid				
Treatment	**n**	**Neutrophils**	**Eosinophils**	**Small lymphocytes**	**Large Lymphocytes**	**Monocytes**
- ve ctrl	5	1.0 ± 0.2	0.7 ± 0.4	5.9 ± 0.8	0.5 ± 0.3	91.9 ± 0.8
P-value		0.004	0.004	0.428	0.783	0.004
+ ve ctrl	6	29 ± 4.4	22.6 ± 2.8	10.4 ± 3.4	0.6 ± 0.3	37.3 ± 8.1
CycA-10mg/kg	6	14.8 ± 1.5	17.9 ± 2.1	3.3 ± 0.9	0.6 ± 0.4	63.4 ± 2.6
P-value		0.004	0.178	0.009	0.7829	0.004
CycA-30mg/kg	5	12.4 ± 2.1	8.7 ± 2.1	9.6 ± 2.0	3.6 ± 1.8	65.6 ± 5.1
P-value		0.01	0.01	0.914	0.165	0.01
3C8-10mg/kg	6	21.7 ± 10.2	12.5 ± 4.3	12 ± 2.0	0.0 ± 0.0	53.8 ± 12.5
P-value		0.015	0.004	0.394	0.123	0.015
3C8-30mg/kg	7	19.3 ± 3.2	10.9 ± 1.7	12.1 ± 2.5	0.8 ± 0.5	57 ± 2.7
P-value		0.073	0.002	0.445	0.611	0.008
3C9-10mg/kg	6	22.3 ± 2.6	15.7 ± 2.2	11.5 ± 2.2	2.1 ± 0.8	48.4 ± 3.9
P-value		0.180	0.041	0.394	0.128	0.310
3C9-30mg/kg	6	16.2 ± 2.3	14 ± 2.4	12.2 ± 3.4	2.2 ± 1.0	55.3 ± 6.8
P-value		0.030	0.015	0.818	0.469	0.093
Tacrolimus-10mg/kg	6	20.3 ± 3.7	17.3 ± 2.4	4.6 ± 1.4	0.3 ± 0.3	57.4 ± 4.6
P-value		0.178	0.178	0.178	0.518	0.052

The –ve ctrl group was sensitised to OVA but was not challenged (received only dH_2O). All other groups were sensitised and challenged with OVA. The –ve ctrl and +ve ctrl groups were untreated (received only 0.5% CMC).

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Experiments were performed on 2-5 groups at a time with n=6 per group.

Table 3.2.2.4.1.1: IL-4 levels in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant

Treatment	n	BAL IL-4 levels (pg/ml)	P-value	n	Lung IL-4 levels (pg/ml)	P-value
- ve ctrl	4	3.6 ± 1.4	0.010	6	5.7 ± 1.1	0.004
+ ve ctrl	6	46.6 ± 8.7	---	5	93.1 ± 3.6	---
CycA-10mg/kg	5	33.2 ± 4.4	0.429	6	74.9 ± 4.2	0.015
CycA-30mg/kg	6	24.7 ± 1.8	0.041	6	45.0 ± 2.1	0.002
3C8-10mg/kg	6	36.7 ± 2.8	0.589	6	81.8 ± 4.0	0.065
3C8-30mg/kg	7	36.0 ± 5.7	0.628	5	84.8 ± 3.3	0.178
3C9-10mg/kg	6	38.6 ± 6.9	0.749	6	81.4 ± 3.3	0.065
3C9-30mg/kg	5	27.4 ± 6.6	0.030	6	61.7 ± 4.0	0.002
Tacrolimus-10mg/kg	6	32.9 ± 4.1	0.200	6	56.7 ± 3.6	0.002

IL-4 assays were performed on BAL and lung samples taken from Balb/C mice sensitised to OVA using AlOH as an adjuvant.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Table 3.2.2.4.1.2: IL-4 levels in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

Treatment	n	BAL IL-4 levels (pg/ml)	P-value	n	Lung IL-4 levels (pg/ml)	P-value
- ve ctrl	5	3.4 ± 2.7	0.003	6	3.0 ± 1.8	0.002
+ ve ctrl	7	34.9 ± 3.4	---	6	48.5 ± 2.3	---
CycA-10mg/kg	6	27.6 ± 2.0	0.153	6	43.6 ± 1.3	0.065
CycA-30mg/kg	6	24.6 ± 2.5	0.045	6	29.7 ± 1.5	0.002
3C8-10mg/kg	6	36.4 ± 4.8	0.835	5	43.3 ± 2.0	0.082
3C8-30mg/kg	8	31.6 ± 6.1	>0.9999	6	31.6 ± 4.0	0.015
3C9-10mg/kg	5	29.5 ± 2.8	0.343	6	45.2 ± 2.8	0.485
3C9-30mg/kg	6	25.4 ± 2.2	0.051	6	30.5 ± 2.4	0.002
Tacrolimus-10mg/kg	5	26.2 ± 4.5	0.149	6	38.9 ± 4.3	0.132

IL-4 assays were performed on BAL and lung samples taken from Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Table 3.2.2.4.2.1: IL-5 levels in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant

Treatment	n	BAL IL-5 levels (pg/ml)	P-value	n	Lung IL-5 levels (pg/ml)	P-value
- ve ctrl	4	1.2 ± 1.5	0.024	4	2.9 ± 4.0	0.010
+ ve ctrl	6	288.1 ± 19.8	---	6	231.9 ± 11.5	---
CycA-10mg/kg	5	216 ± 13.8	0.017	6	223.1 ± 16.1	0.699
CycA-30mg/kg	7	169.5 ± 10.5	0.001	7	148.9 ± 7.5	0.001
3C8-10mg/kg	6	239.1 ± 7.1	0.093	6	208 ± 6.2	0.065
3C8-30mg/kg	5	222.2 ± 7.0	0.030	5	189.3 ± 10.4	0.030
3C9-10mg/kg	5	230.6 ± 5.7	0.082	5	242.7 ± 10.9	>0.9999
3C9-30mg/kg	5	199.1 ± 4.3	0.004	6	134.2 ± 20.4	0.004
Tacrolimus-10mg/kg	6	206.4 ± 6.0	0.009	6	179.3 ± 12.7	0.015

IL-5 assays were performed on BAL and lung samples taken from Balb/C mice sensitised to OVA using AlOH as an adjuvant.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Table 3.2.2.4.2.2: IL-5 levels in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

Treatment	n	BAL IL-5 levels (pg/ml)	P-value	n	Lung IL-5 levels (pg/ml)	P-value
- ve ctrl	4	1.9 ± 2.4	0.004	4	4.2 ± 3.2	0.006
+ ve ctrl	8	215.5 ± 9.4	---	7	270.9 ± 12.7	---
CycA-10mg/kg	5	176.7 ± 4.5	0.011	6	192.5 ± 10.7	0.005
CycA-30mg/kg	6	188.3 ± 8.7	0.043	5	159.4 ± 7.3	0.003
3C8-10mg/kg	6	189.4 ± 7.7	0.108	6	145.3 ± 5.1	0.001
3C8-30mg/kg	7	158.0 ± 14.1	0.004	6	196.2 ± 22.5	0.014
3C9-10mg/kg	6	145.9 ± 6.7	0.001	6	233.4 ± 7.8	0.073
3C9-30mg/kg	5	121.6 ± 17.1	0.002	5	125.2 ± 7.4	0.003
Tacrolimus-10mg/kg	5	116.8 ± 6.1	0.002	5	174.5 ± 14.7	0.003

IL-5 assays were performed on BAL and lung samples taken from Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Table 3.2.2.4.3.1: IL-10 levels in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant

Treatment	n	BAL IL-10 levels (pg/ml)	P-value	n	Lung IL-10 levels (pg/ml)	P-value
- ve ctrl	5	16.2 ± 4.7	0.003	5	2.4 ± 6.2	0.004
+ ve ctrl	7	217.9 ± 19.2	---	6	176.5 ± 20.9	---
CycA-10mg/kg	6	132.7 ± 20.3	0.014	6	117.4 ± 21.9	0.093
CycA-30mg/kg	7	118.4 ± 12.7	0.001	6	102.7 ± 13.3	0.026
3C8-10mg/kg	5	174.3 ± 18.0	0.268	6	163.9 ± 28.6	0.485
3C8-30mg/kg	6	183.3 ± 8.1	0.116	6	142.6 ± 31.1	0.485
3C9-10mg/kg	5	165.7 ± 13.3	0.042	6	153.3 ± 15.7	0.485
3C9-30mg/kg	6	144.6 ± 14.0	0.022	6	93.4 ± 21.2	0.026
Tacrolimus-10mg/kg	5	110.0 ± 13.1	0.005	6	120.2 ± 12.9	0.041

IL-10 assays were performed on BAL and lung samples taken from Balb/C mice sensitised to OVA using AlOH as an adjuvant.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Table 3.2.2.4.3.2: IL-10 levels in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

Treatment	n	BAL IL-10 levels (pg/ml)	P-value	n	Lung IL-10 levels (pg/ml)	P-value
- ve ctrl	4	26.8 ± 7.4	0.006	5	18.4 ± 12.0	0.004
+ ve ctrl	7	171.4 ± 25.5	---	6	238.4 ± 22.7	---
CycA-10mg/kg	6	82.7 ± 10.8	0.035	6	164.4 ± 21.1	0.041
CycA-30mg/kg	6	53.4 ± 5.3	0.001	6	132.7 ± 31.0	0.017
3C8-10mg/kg	5	152.1 ± 20.3	0.639	6	195.5 ± 13.5	0.132
3C8-30mg/kg	6	141.7 ± 19.0	0.445	6	158.9 ± 17.4	0.041
3C9-10mg/kg	6	125.6 ± 8.4	0.391	6	194.4 ± 27.4	0.336
3C9-30mg/kg	5	147.1 ± 22.5	0.570	5	233.3 ± 26.2	>0.9999
Tacrolimus-10mg/kg	6	107.0 ± 18.7	0.116	6	166.9 ± 14.0	0.026

IL-10 assays were performed on BAL and lung samples taken from Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Table 3.2.2.4.4.1: IFNγ levels in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant

Treatment	n	BAL IFNγ levels (pg/ml)	P-value	n	Lung IFNγ levels (pg/ml)	P-value
- ve ctrl	3	3.8 ± 1.1	0.024	5	4.0 ± 1.8	0.004
+ ve ctrl	6	30.8 ± 4.4	---	6	53.7 ± 4.1	---
CycA-10mg/kg	7	22.0 ± 2.0	0.073	6	39.8 ± 3.6	0.037
CycA-30mg/kg	6	12.6 ± 2.6	0.009	6	25.9 ± 4.9	0.002
3C8-10mg/kg	6	22.4 ± 1.6	0.109	6	40.7 ± 7.6	0.180
3C8-30mg/kg	5	14.3 ± 2.2	0.017	6	26.7 ± 4.2	0.002
3C9-10mg/kg	6	24.5 ± 3.2	0.394	5	46.8 ± 4.7	0.247
3C9-30mg/kg	6	19.1 ± 5.9	0.240	6	34.7 ± 3.9	0.016
Tacrolimus-10mg/kg	5	17.8 ± 2.0	0.017	6	24.8 ± 1.7	0.002

IFNγ assays were performed on BAL and lung samples taken from Balb/C mice sensitised to OVA using AlOH as an adjuvant.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Table 3.2.2.4.4.2: IFNγ levels in BAL fluid and lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

Treatment	n	BAL IFNγ levels (pg/ml)	P-value	n	Lung IFNγ levels (pg/ml)	P-value
- ve ctrl	3	4.8 ± 2.0	0.017	5	4.3 ± 1.6	0.004
+ ve ctrl	7	26.6 ± 1.9	---	6	32.9 ± 3.3	---
CycA-10mg/kg	5	18.7 ± 1.4	0.030	6	24.1 ± 3.2	0.093
CycA-30mg/kg	5	15.4 ± 3.5	0.018	6	13.0 ± 4.5	0.009
3C8-10mg/kg	6	20.8 ± 1.7	0.063	6	26.2 ± 3.3	0.310
3C8-30mg/kg	5	14.2 ± 0.8	0.003	6	15.4 ± 3.1	0.009
3C9-10mg/kg	5	15.5 ± 3.9	0.030	6	23.0 ± 3.6	0.093
3C9-30mg/kg	6	12.2 ± 1.7	0.001	5	13.9 ± 3.5	0.004
Tacrolimus-10mg/kg	6	16.0 ± 2.0	0.005	6	22.9 ± 2.5	0.026

IFNγ assays were performed on BAL and lung samples taken from Balb/C mice sensitised to OVA using both AlOH and FCA(T) as adjuvants.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 ELISA experiments.

Table 3.2.3.4.1: EPO and MPO levels in lung extracts taken from Balb/c mice sensitised to OVA using AlOH as an adjuvant

Treatment	n	EPO (O.D. at 492nm)	P-value	MPO (O.D. at 450nm)	P-value
- ve ctrl	6	0.09 ± 0.01*	0.004	0.09 ± 0.01^	0.004
+ ve ctrl	6	1.39 ± 0.08	---	0.63 ± 0.04	---
CycA-10mg/kg	6	0.55 ± 0.08*	0.008	0.31 ± 0.05^	0.004
CycA-30mg/kg	7	0.39 ± 0.03*	0.003	0.42 ± 0.03^	0.004
3C8-10mg/kg	6	1.08 ± 0.09*	0.056	0.43 ± 0.06	0.026
3C8-30mg/kg	5	0.58 ± 0.05*	0.008	0.21 ± 0.03^	0.004
3C9-10mg/kg	6	0.69 ± 0.03*	0.004	0.42 ± 0.03^	0.007
3C9-30mg/kg	5	0.87 ± 0.06*	0.004	0.44 ± 0.03^	0.009
Tacrolimus-10mg/kg	6	1.13 ± 0.11*	0.082	0.33 ± 0.04	0.002

* indicates that BAL eosinophils were also significantly reduced, as in table 3.2.1.4.1.
^ indicates that BAL neutrophils were also significantly reduced, as in table 3.2.1.4.1.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 EPO and 3 MPO assays.

Table 3.2.3.4.2: EPO and MPO levels in lung extracts taken from Balb/c mice sensitised to OVA using both AlOH and FCA(T) as adjuvants

Treatment	n	EPO (O.D. at 492nm)	P-value	MPO (O.D. at 450nm)	P-value
- ve ctrl	6	0.08 ± 0.01*	0.004	0.04 ± 0.01^	0.008
+ ve ctrl	6	0.79 ± 0.06	---	0.88 ± 0.04	---
CycA-10mg/kg	6	0.62 ± 0.09	0.178	0.42 ± 0.03^	0.011
CycA-30mg/kg	5	0.38 ± 0.03*	0.002	0.59 ± 0.02^	0.014
3C8-10mg/kg	6	0.59 ± 0.09*	0.065	0.55 ± 0.03^	0.008
3C8-30mg/kg	6	0.48 ± 0.04*	0.009	0.64 ± 0.06	0.016
3C9-10mg/kg	6	0.65 ± 0.06*	0.093	0.55 ± 0.05	0.008
3C9-30mg/kg	6	0.53 ± 0.05*	0.009	0.37 ± 0.03^	0.008
Tacrolimus-10mg/kg	7	0.71 ± 0.04	0.178	0.72 ± 0.07	0.151

* indicates that BAL eosinophils were also significantly reduced, as in table 3.2.1.4.2.
^ indicates that BAL neutrophils were also significantly reduced, as in table 3.2.1.4.2.

All values are expressed as mean ± SEM.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.
Results are from 3 EPO and 3 MPO assays.

Table 4.1: A summary of the results found for CycA, tacrolimus, 3C8, 3C9 and 6C6 in all experiments

		CycA		Tacrolimus			3C8		3C9		6C6	
mBSA model		50		1	3	10	10	30	10		10	30
SRBC model		50	100	10		30	10	30	10	30	---	
Oxazolone model		15		15			15		15		15	
Rat BAL Eosinophils	Th2 model	10		---			10		---		---	
	Th1 model	10		---			10		---		---	
	Th1/Th2 model	10		---			10		---		---	
Mouse BAL Eosinophils	Th2 model	10	30	10			10	30	10	30	---	
	Th1/Th2 model	10	30	10			10	30	10	30	---	
IL-4-Th2 model		10	30	10			10	30	10	30	---	
IL-4-Th1/Th2 model		10	30	10			10	30	10	30	---	
IL-5-Th2 model		10	30	10			10	30	10	30	---	
IL-5-Th1/Th2 model		10	30	10			10	30	10	30	---	
IL-10-Th2 model		10	30	10			10	30	10	30	---	
IL-10-Th1/Th2 model		10	30	10			10	30	10	30	---	
IFNγ-Th2 model		10	30	10			10	30	10	30	---	
IFNγ-Th1/Th2 model		10	30	10			10	30	10	30	---	
EPO-Th2 model		10	30	10			10	30	10	30	---	
EPO-Th1/Th2 model		10	30	10			10	30	10	30	---	
MPO-Th2 model		10	30	10			10	30	10	30	---	
MPO-Th1/Th2 model		10	30	10			10	30	10	30	---	

This table represents a summary of the most significant results found in all animal models of DTH and asthma.

Values represent the dose(s) of each drug tested in each model.
P-values were calculated for each drug treatment group versus the positive control group.
Shaded boxes indicate a P-value of less than 0.05, which is taken to be significant.
All tests carried out were unpaired, nonparametric, two-tail Mann-Whitney tests.

Appendix 3

Statistics

Statistical comparisons were carried out using Mann-Whitney tests. These tests are unpaired (compare two independent samples) and nonparametric (i.e. do not focus on any particular parameter such as the mean or median). In addition, two-tail P values were calculated since these should be more accurate than one-tail P values for comparing 3 or more groups. The following formulas were used:

Sample mean: $\bar{x} = \frac{1}{N}\sum x_i$

Sample standard deviation: $s_x = \sqrt{\frac{\sum (x_i - \bar{x})^2}{N-1}} = \sqrt{\frac{\sum x_i^2 - N\bar{x}^2}{N-1}}$

Standard error of the mean: $S.E.M. = s / \sqrt{N}$

(N = number of samples; x_i = sample value)

The mean is the average of all values in a group. The standard deviation quantifies the scatter of the data or how much the values vary from one another. The standard error of the mean (SEM) quantifies how close the sample mean is to the true population mean. As the sample size gets bigger, the SEM will get smaller whereas the standard deviation will, on average, stay the same. Expressing results as mean $\pm$ SEM should therefore give a sense of how accurately the mean has been determined.

ASLIB THESIS DATA FORM

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