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Immunoregulation by *Mycobacterium vaccae*: Effects on CD11c+ antigen-presenting cells in a mouse model of pulmonary inflammation

A thesis submitted to the faculty of life sciences in part fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Throughout evolution, mammals have co-existed with many harmless organisms such as saprophytic mycobacteria. These "Old Friends" have helped the hosts' immune system to evolve immunoregulatory mechanisms that prevent inappropriate immune responses. Exposure, once a common occurrence has now become increasingly rare. A revised version of the hygiene hypothesis proposes that this reduced exposure may be a contributing factor to the recent increase in allergic diseases in developed countries.

In models of allergic pulmonary inflammation, treatment with *M. vaccae* inhibits airway hyperreactivity and induces allergen specific regulatory T cells (Tregs), which secrete IL-10 and depend upon production of IL-10 and TGF-β *in vivo*. Since Treg-induction is dependent on antigen presenting cells (APCs) such as CD11c+ cells, this thesis addresses the effects that *M. vaccae* has on CD11c+ APCs, in a mouse model of allergic pulmonary inflammation. *M. vaccae* treatment reduces pulmonary allergic inflammation by decreasing type-2 responses such as eosinophilia and IL-4 expression. Rather than an increase in type-1 cytokines, IL-10 is elevated in the lungs, both at the protein and message level. Characterization of pulmonary CD11c+ APCs by ELISA, FACS and real time RT-PCR, shows an immunoregulatory cytokine profile with increased expression of IL-10, TGF-β and IFN-α. In passive transfer experiments, CD11c+ cells appear not play a role as regulatory cells themselves, but may be involved in the induction of Treg through their cytokine release. *In vitro* studies show these CD11c+ APCs are capable of inducing naïve T cells to become Tregs, as measured by increased production of IL-10 and Foxp3 expression.

M. vaccae has been used in clinical trials of asthma and eczema, with encouraging results. This thesis goes some way to understanding one mechanism behind a potentially valuable form of immunotherapy.

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Abbreviations

A	adenine
Ab	antibody
Ag	antigen
AHR	airway hyperreactivity
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BCG	bacillus Calmette-Guérin
BM-DC	bone marrow derived dendritic cells
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA (reverse transcribed from mRNA)
CNS	central nervous system
CTLA-4	cytotoxic T lymphocyte associated-protein 4
Cy-5	cyanine 5
DC	dendritic cell
DLN	draining lymph node
DNA	deoxyribonucleic acid (bases A, C, G, T)
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediamine tetra-acetic acid (sodium salt)
ELISA	enzyme-linked immunosorbent assay
FACS	Fluorescent Activated Cell Sorter
FITC	fluorescein isothiocyanate
Foxp3	forkhead/winged helix transcription factor
G	Guanine
GAPDH	glyceraldehyde-2-phosphate dehydrogenase
GITR	glucocorticoid-induced TNF receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HPRT	hypoxanthine-guanine phosphoribosyl tranferase
IBD	inflammatory bowel disease
ICOS	inducible costimulatory molecule
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL-(10)	interleukin-(10)
i.p.	intra-peritoneal
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
i.t.	intra-tracheally
i.v.	intra-venous
Kb	kilobase
-L	ligand
LN	lymph node
LPS	lipopolysaccharide
MΦ	macrophages
MAb	monoclonal antibody
MACS	magnetic-activated cell sorter
MBP	myelin basic protein
mDC	myeloid DC
MHC	major histocompatibility complex
MLN	mesenteric lymph node
mRNA	messenger RNA
NK	natural killer
NKT	natural killer T cell

NO	nitric oxide
OVA	ovalbumin
PBS	phosphate buffered saline
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
pDC	plasmacytoid DC
PE	phycoerythrin
Pen/strep	Penicillin/ streptomycin
Per-CP	peridinin chlorophyll protein
PI	propidium iodide
PLA	phospholipase A2
PLP	proteolipid protein
PP	Peyer's patches
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RT	reverse transcriptase
s.c.	sub-cutaneous
SCID	severe combined immunodeficiency
SD	standard deviation
SEM	standard error of the mean
SLE	systemic lupus erythematosus
T	thiamine
TAE	tris-acetate-EDTA
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th	T helper (cell)
TLR	toll like receptor
Tm	annealing temperature
TNF-α	tumour necrosis factor
Tregs	regulatory T cells
XLAAD	X-linked autoimmunity-allergic dysregulation syndrome
-/-	denotes homozygous gene deficiency

Units of measurement

m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})
nm	nanometre
cm	centimetre
k	Kilo
l	litre
g	gram
U	units
M	molar
°C	degrees Celsius
A	ampere
v	volts
kDa	kilodalton

Chapter 1

Chapter 1

General Introduction

1. ALLERGY

The adaptive immune response offers specific protection against infection with bacteria, viruses, parasites, fungi and cancer. It has evolved to resist pathogens, and particularly protects against repeat infections. In certain circumstances, however, the immune system responds in an excessive or inappropriate manner to antigenic challenge. This kind of reaction is known as hypersensitivity. There are four types of hypersensitivity, each classified by their different responses (Table 1-1). Type I, or immediate, hypersensitivity is the most common type, and allergic reactions belong to this group [Roitt et al 2001]. The focus of this thesis will be on the immunoregulation of allergic responses.

Allergy is defined as an exaggerated immune response to exposure to specific Ags. When an allergic response occurs immediately after contact with the Ag an individual is sensitized to, this is known as atopic allergy. This includes atopic dermatitis, allergic rhinitis and asthma. In sensitized individuals, allergy occurs after challenge with the allergen. Specific Ags causing an exaggerated response are known as allergens. Allergens are varied and may include food (for example peanut Ag), house dust mite (Derp1 Ag) and bee venom.

1.1 Type I Hypersensitivity

Immediate hypersensitivity (Type I) is always associated with Th2-mediated inflammation and raised serum IgE levels. Details of the other 3 types of hypersensitivity have been described in Table 1-1. Th2-mediated inflammation results from secretion of type-2 cytokines by a number of cell types, including allergen activated CD4+ T cells. CD4+ T cells can be characterized by their particular immune properties, explained in more detail in section 2.6. Briefly, IFN-γ and IL-2 secreting CD4+ T cells are known as Th1 cells, whilst IL-4

producing CD4+ T cells are termed Th2 cells. These cell types also have an antagonistic effect on the development of each other. Secretion of IL-4, IL-5 and IL-13 by Th2 cells is essential for development of raised IgE levels, eosinophilic inflammation, mucus production and fibrosis, all of which contribute towards Th2-mediated inflammation.

1.1.1 IgE

IgE production is characteristic of a type-2 response, with expression of the IgE gene being dependent upon IL-4. Secretion of the type-2 cytokine IL-4 induces B cell production of IgE. The association between IgE and IL-4 has been investigated in a linkage analysis study involving twins. Linkage is the probability of two or more genes being inherited together. This study suggested that the IL-4 gene, on human chromosome 5q31.1, regulates IgE production in a non-specific manner [Marsh *et al* 1994]. In addition, IL-4$^{-/-}$ mice have less serum IgE levels, compared to wild-type mice [van der Weld *et al* 1994, Morawetz *et al* 1996]. The production of IgE is therefore dependent on IL-4 secretion. In addition, IFN-γ secreting Th1 cells have been shown to suppress IgE production [Geha *et al* 1992], and since IFN-γ is known to regulate IL-4 secreting cells, this supports the notion that IL-4 is important for IgE production [Mosmann *et al* 1989].

Allergic individuals produce raised IgE levels in response to exposure to allergens. The IgE binds specifically to high affinity receptors on mast cells and basophils. When an allergen and two IgE Abs crosslink with the mast cell or basophil Fc surface receptor, this leads to degranulation and release of histamine and other granule contents. Allergen exposure, therefore, results in rapid histamine release [Gauchat *et al* 1993, Platts-Mills *et al* 2001, Marone *et al* 1997]. Histamine leads to capillary dilatation, contraction of smooth muscle, and increased vascular permeability. The activation of mast cells and basophils also leads to the gradual release of cytokines, for example IL-4. These cytokines further activate bystander cells, resulting in further inflammation.

Serum IgE levels are generally very low in healthy individuals (non-atopic), compared to the other isotypes, such as IgM and IgG. A number of factors

account for this. Firstly, IgE has a shorter half-life, which is less than two days. It is also produced in small quantities and only in response to a select group of foreign Ags and pathogens, such as allergens and helminths. In addition, circulating levels are low, because IgE is held on high affinity receptors of mast cells or basophils [Roitt *et al* 2001]. Raised levels of type-2 cytokines at the site of inflammation and serum IgE levels are a good indicator of allergy severity in all types of atopic disease.

1.1.2 Histamine

During allergic inflammation, one of the effects of exposure to an allergen is the release of histamine. Allergens interact with and cross-link surface IgE on the surface of mast cells and basophils. Once the mast cell-Ab-Ag complex is formed, cell-degranulation and the release of histamine occurs. Once released, histamine can interact with cells in local or peripheral tissues through histamine receptors, causing tissue damage and inflammation. Histamine exerts its actions by ligating with 4 specific cellular receptors located on the surface of cells [Simons *et al* 2004].

The four histamine receptors described so far are H1, H2, H3 and H4, all belong to the superfamily of G-protein coupled receptors (GPCR) [Leurs *et al* 2001]. Different receptors are expressed on different cells in the body. H1 histamine receptors are the primary receptors involved in allergic rhinitis symptoms, and are found on smooth muscle cells, endothelial cells, and central nervous system cells, and ligation causes vasodilatation, bronchoconstriction, and smooth muscle activation [Reiner *et al* 1994]. H2 histamine receptors are located on parietal cells, which primarily regulate gastric acid secretion. The H3 histamine receptor is expressed in the central and peripheral nervous system, and is involved in decreased release of neurotransmitters, such as acetylcholine and serotonin [West *et al* 1990]. The exact role of H4 histamine receptors, which are found primarily in the thymus, small intestine, spleen, and colon, as well as on basophils and in the bone marrow remains unclear [Nguyen *et al* 2001].

1.1.3 Anti-histamine

An anti-histamine is a drug that serves to reduce or eliminate the effects mediated by histamine through its action on the histamine receptor. Only drugs where the main therapeutic effect is mediated by their negative modulation of histamine receptors are termed anti-histamines - other agents may have anti-histaminergic action but are not known as anti-histamines. The term anti-histamine only refers to H1-receptor antagonists, also known as H1-anti-histamines [Leurs *et al* 2002]. While H1-anti-histamines ameliorate the effects of histamine release, it is most effective if administered prior to the allergen-challenge, since it can occupy the H1 receptor and prevent histamine interacting with it.

Ligation of histamine with the H1 receptor has been observed to activate several signalling pathways, such as phosholipase D and phosholipase A_2 [Hill *et al* 1997], along with the transcription factor NF-κB [Aoki *et al* 1998, Bakker *et al* 2001]. The effect of H1-anti-histamines is H1-receptor dependant, meaning that binding of H1-anti-histamines to the H1-receptor inhibits the action of histamine on these cells. The actual mechanism is still relatively unclear, but it is thought that H1-anti-histamines downregulate the activation of NF-κB [Bakker *et al* 2001]. NF-κB is a ubiquitous transcription factor which binds to promoter regions of genes which regulate the production of a number of proinflammatory cytokines and adhesion proteins. Anti-histamines have been shown to downregulate NF-κB in parallel with inhibiting production of the cytokines IL-1β, IL-6, IL-8, TNF-α and GM-CSF [Arnold *et al* 1999, Rihoux *et al* 1999, Yoneda *et al* 1997].

An alternative to blocking the effect of histamine, is to block its release from mast cells. Sodium cromoglycate is able to inhibit release of histamine from mast cells *in vitro*. However its mode of action *in vivo* remains unknown. A number of related compounds that are better at inhibiting mediator release from mast cells *in vitro* have been found to be ineffective *in vivo*. Cromoglycate is available as an inhaler commonly known as "Intal", and as a nasal spray. The advantage of cromoglycate is its excellent safety record.

1.1.4 Other drugs used to treat allergy

Whereas anti-histamines are the most effective drugs against allergic reactions, others used to treat the symptoms include decongestants (such as pseudoephedrine), corticosteroids and salbutamol inhalers. Pseudoephedrine is a sympathomimetic amine, whose principal mechanism of action relies on its indirect action on the adrenergic receptor system. This is part of the sympathetic nervous system. The principal mechanism is to displace noradrenaline from storage vesicles in presynaptic neurons. The displaced noradrenaline is released into the neuronal synapse where it is free to activate the postsynapic adrenergic receptors [Abourashed *et al* 2003]. The result is vasoconstriction in the nasal mucosa, shrinking of swollen nasal mucous membranes, and reduction of tissue hyperaemia, oedema, and nasal congestion.

Salbutamol is a short-acting β_2 adrenergic receptor agonist, used for the relief of bronchospasm during asthmatic attacks. Salbutamol is usually given by the inhaled route for direct effect on bronchial smooth muscle. This is usually achieved through a dose inhaler or nebuliser. Salbutamol is available in the UK under the trade name Ventolin. Salbutamol acts by binding to β_2-adrenergic receptors in the airways. Activation of β_2-receptors results in dilation of bronchial smooth muscle, resulting in a widening of the airway (bronchodilatation). Inhaled salbutamol has a rapid onset of action, providing relief within 5-15 minutes of administration [reviewed in Martinez Ordaz *et al* 2005].

Corticosteroids are a class of steroid, which are hormones, naturally produced by the adrenal cortex. The target receptor for corticosteroids is the intracellular glucocorticoid receptor. In the steady state, inactive glucocorticoid receptors are located in the cytosol of the cell surrounded by multi-chaperone proteins. Through the steroid-binding domain, glucocorticoid receptors bind with corticosteroid molecules, forming a dimeric complex [Barnes *et al* 1997]. Upon binding, the corticosteroid molecule induces a conformational change in the receptor protein, causing the multi-chaperone proteins to dissociate. The activated steroid–glucocorticoid receptor complex then translocates from the

cytosol to the nucleus of the cell where it exerts its primary anti-inflammatory effects. In the nucleus, the steroid–glucocorticoid receptor complex binds to glucocorticoid response elements (GREs), which are specific DNA sequences found in the promoter region of target genes, causing the cells to repress gene transcription of pro-inflammatory cytokines and proteins and upregulate production of anti-inflammatory proteins. The activated steroid–glucocorticoid receptor complex may also directly interact with DNA-binding transcription factors such as NF-κB to suppress transcription factor-mediated pro-inflammatory protein production [Adcock *et al* 2000].

1.2 Atopic disease

Atopic diseases include atopic dermatitis, allergic rhinitis and asthma. All are defined as a Type I hypersensitivity and are all associated with high levels of IgE production. Epidemiological studies suggest that these types of allergies have more than doubled over the last 40 years, particularly in developed countries. Since this rise has happened in such a short space of evolutionary time, it is unlikely that change in genetic makeup is solely responsible. Environmental factors are likely to be involved [Arkwright *et al* 2001, Howarth *et al* 1998].

This increase in atopic disease was first reported by Strachan in a seminal manuscript published in the British Medical Journal in 1989 [Strachan *et al* 1989]. There he described the prevalence of atopic disease in a national study concerning over 17,000 British children. He observed that the prevalence of allergic rhinitis was inversely correlated to the number of other children within the same household, particularly older ones. Similar observations were made with atopic dermatitis. No data on asthma, however, were reported. Strachan suggested that 'unhygienic contact' with other siblings in large households protected against the development of atopy. In this hypothesis, termed the hygiene hypothesis, the combination of declining family size, increased standards of living and better personal hygiene is responsible for reducing the opportunity for cross infection between young family members, reducing exposure to microbes. It is this reduced exposure to microbes, which Strachan linked directly to an increase in clinical atopy [Strachan *et al* 1989].

If Strachan's hypothesis is correct, an association must exist between atopic disease and modern standards of living. Less exposure to dirt, along with an increased use of cleaning products and disinfectants must lead to lower exposure to microbes. It would therefore be of interest to determine whether the increase in atopic disease is due to a lack of microbial exposure. Geographical variation in atopic disease prevalence around the world supports Strachan's hypothesis that environmental factors, such as exposure to infections, are partly to blame for the rise in allergic disease. An international study was done in the 1990s involving over 450,000 children aged between 13 and 14 years and living in 56 countries [ISAAC *et al* 1998]. For example, atopic dermatitis varied from 0.3% of children in third world countries where exposure to infections is higher, such as China, India and Georgia, to 20.5% in countries like the UK and New Zealand. Similar trends were seen with asthma and allergic rhinitis, with developed countries having a higher prevalence for disease than developing countries [ISAAC *et al* 1998]. Of additional interest is the observation that differences in atopic disease prevalence over time can be observed within developed countries, with the incidence of asthmatic symptoms significantly increasing over a period of 20-25 years [Ninan *et al* 1992, Upton *et al* 2000].

The incidence of atopic disease differs between developed and developing countries, suggesting environmental factors play a role in disease prevalence. One such factor is increased use of antibiotics within developed countries, which might possibly have an affect on the incidence of atopic disease. For example, studies amongst the UK population between the years of 1946 and 1970 observed an increase in childhood atopic dermatitis from 5% to 12% [Taylor *et al* 1984]. A study in Australia between 1981 and 1997 observed a rise in asthma from 13% to 39% [Downs *et al* 2001]. This rapid increase coincides with a greater use of antibiotics and disinfectants by developed countries, though many other factors would show similar correlations. Evidence is compatible with the view that environmental factors limit our exposure to microbial infection, and are at least partly responsible for the increased prevalence and incidence of atopic disease.

1.2.1 Non-Atopic (intrinsic) asthma

Non-atopic asthma is often referred to as intrinsic asthma, and is characterized by inflammation that is clinically similar to allergic asthma allergy, such as similar pulmonary cellular infiltrate, eosinophilia and cytokine production [Bentley *et al* 1994, Ying *et al* 1997]. However, there is no raised IgE level in response to any external allergen, which is characterized by a negative skin prick test. It has been observed that people suffering from intrinsic asthma are more likely to be female, have a later onset of symptoms, have more severe clinical symptoms and have rhinitis associated with their asthma [Kauffmann *et al* 1995, Romanet-Manent *et al* 2002]. Similar observations have been made of people suffering from non-atopic rhinitis and dermatitis.

As mentioned above, compared to patients with atopic asthma, the cellular infiltrate into the lungs of non-atopic asthmatics is similar apart from an increase number of MΦs [Bently *et al* 1994]. In addition, despite the observed absence of IgE, patients suffering with intrinsic asthma have equivalent levels of IL-4, IL-5 and IL-13 in the bronchial mucosa at the mRNA and protein level [Humbert *et al* 1996, Ying *et al* 1997, Humbert *et al* 1997, Wuthrich *et al* 1996]. Levels of IL-10 and IL-12 secreting cells are also reported to be low in the sputum of non-atopic patients compared to atopic asthmatics [Zeibecoglou *et al* 2000], which may explain why there is continued airway inflammation despite the absence of any recognised external or endogenous allergen.

1.2.2 Contact dermatitis

Several non-atopic allergic diseases, which are not dependent on Th2 or IgE responses, are thought to be largely attributable to Type IV Hypersensitivity, or delayed type Hypersensitivity (DTH). This type of Hypersensitivity occurs when an Ag interacts with Ag-specific lymphocytes that release inflammatory and toxic substances, which attract other leukocytes and results in tissue injury. CD8+ cytotoxic T cells and CD4+ helper T cells recognize either intracellular or extracellular Ag when it is presented by class I or class II MHC molecules, respectively. Cytotoxic T cells cause direct damage to surrounding tissue by releasing cytotoxic substances, such as perforin. Th1 cells secrete cytokines,

which activate cytotoxic T cells, and recruit and activate monocytes and MΦs. MΦs function as APCs, further presenting allergen to T cells, and also release IL-1, which promotes the proliferation of Th1 cells. Th1 cells then release interferon-γ and IL-2, which together regulate delayed Hypersensitivity reactions centred on MΦ activation and T cell mediated immunity [Belsito *et al* 1989, Grabbe *et al* 1998]. There are three variants of Type IV Hypersensitivity: Contact Hypersensitivity, tuberculin-type Hypersensitivity and granulomatous Hypersensitivity

1.2.3 Type IV Hypersensitivity

Contact Hypersensitivity is characterized by a reaction at the site of contact with the allergen (such as contact dermatitis in response to nickel or poison ivy). It is an epidermal response most often elicited by molecules called haptens. Haptens interact with host molecules to create new Ag determinants. The cell involved in Ag presentation at this site is the Langerhans cell, a type of DC present in the epidermis. The pathway from initial allergen exposure involves sensitization (with Langerhans cells presenting Ag to CD4+ helper T cells), followed by recruitment of T cells and MΦs around blood vessels in the epidermis and ensuing edema. A variety of cytokines are involved in this process, including IL-2, IL-3, IFN-γ and GM-CSF, and chemokines, such as RANTES and MCP-1. The reaction resolves within 48-72 hours following exposure [Hoefakker *et al* 1995, Schwarzenberger *et al* 1996].

Tuberculin Hypersensitivity was first observed when soluble Ags from microorganisms such as mycobacteria were administered intradermally to patients. In these individuals fever and hard swelling at the site of injection was observed after approximately 48-72 hours. This skin reaction is frequently used to test for recall responses to microorganisms that a person has been exposed to, such as for example during the Mantoux test which determines exposure to Mycobaterium tuberculosis [Kindler *et al* 1989].

Granulomatous Hypersensitivity is characterized by persistence of intracellular microorganisms or other foreign Ag within MΦs. Such Ags can include particulate matter such as talc and silica, or mycobacteria. In cases of

Granulomatous Hypersensitivity, a granuloma is usually formed, self-containing the MΦs. The characteristic cells found in these lesions are epitheloid cells (derived from activated MΦs) and giant-cells (multi-nucleated MΦs). The granuloma consists of a hard core of cells sometimes with a necrotic centre. This core is surrounded by lymphocytes with a deposition of collagen fibres, caused by proliferation of fibroblasts and increased collagen synthesis [Yamamura *et al* 1991, Romagnani *et al* 1997].

1.3 Atopic Dermatitis

1.3.1 Prevalence

As with all atopic diseases there has been an increase in atopic dermatitis over the last few decades. Several studies on the incidence of atopic dermatitis have reported increased levels in northern Europe. Interestingly, the incidence of atopic dermatitis in children up to the age of 7 years varied depending on the decade they were born. If born before 1960, the incidence was less than 3%. This increased to 4-8% in children born between 1960 and 1970, to 8-12% for those born after 1970 and is over 15% in children born in recent decades [Ninan *et al* 1992, Upton *et al* 2000, Nystad *et al* 1997, Diepgen *et al* 2000]. Even when methodological errors have been taken into account, the overall agreement is that there is an authentic and significant increase in the prevalence of atopic dermatitis over the last 40 years in developed countries [Diepgen *et al* 2000].

1.3.2 Symptoms

Atopic dermatitis, sometimes referred to as eczema, is an allergy affecting the skin. Symptoms include dryness and itching, which are caused by defective lipid barriers in the epidermis, and are often intensified by scratching. There is an extensive list of further symptoms, ranging from an inflamed red rash, oozing and crusting blisters resulting from scratching, dry and leathery skin patches, discolouring and thickening of the skin (known as hyperkeratonic eczema), to discharge or bleeding from the ears resulting from eczema in the ear canal. In addition, atopic dermatitis sufferers often have increased *Staphylococcus*

aureus colonization of their skin. These bacteria release inflammatory substances which further irritate the skin. The onset of atopic dermatitis usually occurs in infancy, but in some cases can occur for the first time after puberty.

The extent and severity of atopic dermatitis are clinically assessed by the SCORAD index, which is an abbreviation of SCORing Atopic Dermatitis. This index was developed by the European Task Force on Atopic Dermatitis in 1993 to allow consistent assessment of eczema severity. SCORAD is calculated by taking into account the area the eczema has spread, its severity and patients' symptoms [Gelmetti *et al* 2004].

1.3.3 Controlling Symptoms

The simplest way of preventing eczema is by avoiding the allergen that triggers it. Unfortunately for patients, items that trigger eczema are common and can include woollen clothes, carpets and animal hair, chlorinated water, cosmetics and perfumed soaps, as well as certain foods. Skin dryness can be aggravated by excessive heating in the home, contributing to further skin irritation.

Colonization of the skin by *Staphylococcus aureus* is common in eczema sufferers, and can frequently be the cause of an eczema flare up. Therefore, treatments can include antibiotics. Systemic antibiotics, like erythromycin or cloxacillin are often used, and sometimes a prolonged course of antibiotics is required [Correale *et al* 1999, Health Encyclopaedia].

The inflammatory aspect of atopic eczema is commonly treated with topical application of corticosteroids. These reduce the swelling, itching and redness of the rash. The mildest example of a corticosteroid is Hydrocortisone, whilst one of the strongest is Betamethasone dipropionate. Betamethasone dipropionate is 600 times more potent than Hydrocortisone, and as with all strong steroids can only be obtained by prescription. The strength of the steroid needed depends on the stage and location of the eczema.

The use of steroids, however, only provides symptomatic relief, since steroids do not target the cause of disease. In addition to only providing symptomatic

relief they can have side effects, including thinning of the epidermis, tachyphylaxis (tolerance of the skin to the vascoconstrictive action of the steroid), allergic reactions and facial flushes. Alternative therapies include the use of Chinese herbs, evening primrose oil and behavioural therapy [Health Encyclopaedia]. Non-steroidal topical products, such as Tacrolimus, have also been shown to reduce skin susceptibility to irritants by providing a protective barrier to the skin and increasing the degree of hydration.

1.3.4 Immunology

Atopic dermatitis is characterized by inflammation of the different layers of the skin. This is caused by migration of eosinophils, mast cell precursors, macrophages and lymphocytes (particularly Th2 cells) across the skin epithelia. These recruited cells release cytokines, for example IL-4, which increases IgE levels leading to degranulation of mast cells and basophils within the skin epidermis. Histamine, released from activated mast cells and basophils, is one of the major causes of atopic dermatitis symptoms following contact with an allergen. Histamine stimulates sensory nerves and induces itching. It also leads to vasodilatation, which results in reddening and facilitates inflammation of the skin.

1.4 Allergic Rhinitis

1.4.1 Prevalence

Allergic rhinitis is today one of the most prevalent chronic diseases affecting developed countries. There is no universally agreed definition of rhinitis and so its prevalence is difficult to assess. A number of studies have however demonstrated an increase in disease since the 1960s, suggesting it now affects between 15% and 25% of people in developed countries, such as the USA, Britain and Australia [Downs *et al* 2001, Upton *et al* 2000, Ninan *et al* 1992]. This is a more rapid increase than is observed in studies of asthma or atopic dermatitis.

1.4.2 Symptoms

Often referred to as hay fever, rhinitis is an inflammation of the lining of the nose in response to an irritant or allergen. Symptoms include irritated eyes, nose and throat, watering eyes, nasal blockage or rhinorrhoea (running nose) and frequent sneezing. Wheezing may also accompany these symptoms in severe cases. There are two types of rhinitis, seasonal (hay fever) which occurs in response to pollens released at certain times of the year, for example tree or grass pollen, and perennial, a chronic all year round problem. This is usually the result of permanent, indoor allergens, like house dust mite protein or animal hair. The onset of allergic rhinitis usually occurs during childhood, but sensitisation in adulthood is becoming more common [Health Encyclopaedia].

1.4.3 Controlling symptoms

The simplest way of preventing rhinitis is by avoiding the allergen which triggers the symptoms. Skin prick tests can identify the allergen responsible by transferring a very small amount of Ag into the skin, which becomes inflamed if the patient is allergic. Even if the identity of the specific allergen is unknown, behavioural changes, such as wearing a pollen mask when mowing the grass or cleaning the house, may reduce exposure to the most common allergens. In addition, exposure can be reduced by installing an air purifier which remove airborne allergens or enclosing sleeping mattresses in plastic and using cotton or synthetic materials such as Dacron in pillows and beddings.

Although avoidance of the allergen in the most effective way to prevent allergy, this is often impossible. There are a number of treatments available. For example, flushing the nose with salt water can reduce nasal inflammation. In addition, it washes out thickened nasal secretions, irritants (smog, pollens, etc.), bacteria, and mucus crusts from the nose and sinuses to prevent nasal blockage. Non-prescription nasal sprays (for example Ocean spray, Ayr, Nasal) can be used frequently to reduce the local inflammation in the nasal passage, and are very convenient to obtain and use. Anti-histamines are drugs that block the action of histamine released during inflammation. These medications work best when given prior to exposure. Anti-histamines can be divided into two

groups; sedating (for example Benadryl, ChlorTrimetron, Tavist), and non-sedating (Claritin, Hismanal). The effectiveness of anti-histamines is hindered by the need of an individual to drive or use dangerous equipment if the anti-histamines are sedating ones. In the case of non-sedating, they can have serious drug interactions. Nasal steroids (for example Vancenase, Beconase, Flonase, Nasacort, Rhinocort) also reduce allergic inflammation, and being effective locally they do not have the side effects that systemic steroid treatment has. However, sometimes their use leads to nasal bleeding. If this happens in children, anti-allergic drugs such as sodium cromoglycate and nedocromil sodium are used, which are preventative drugs. They are taken regularly to prevent inflammation, so decrease the need for other drugs.

1.4.4 Immunology

Like atopic dermatitis, allergic rhinitis is characterized by the infiltration of cells into the nasal mucosa. Superficially, the mechanisms of inflammation are similar in both atopic dermatitis and allergic rhinitis. Cytokines, such as IL-4, are released and IgE levels increased, which leads to degranulation of mast cells and basophils. Histamine release causes inflammation in the nasal passage, and stimulates sensory nerves inducing sneezing, nasal itch, vasodilatation and mucus production. These symptoms of histamine release in the nose are called rhinorrhea. Histamine also has a pro-inflammatory role through up-regulation of adhesion molecules such as ICAM and further release of IL-4. Lipid compounds known as leukotrienes are also generated by mast cells, eosinophils, macrophages, neutrophils and epithelial cells, and are released into nasal tissue. They exacerbate inflammatory responses and are also important later in the inflammatory reaction, causing nasal obstruction, mucus secretion and leading to further inflammatory cell recruitment [Health Encyclopaedia].

2. ASTHMA

Asthma causes morbidity and mortality throughout the developed world. It is no more prevalent than dermatitis or rhinitis, but its symptoms are far more severe.

Novel treatments and therapies are constantly being sought. Asthma forms the focus of this thesis and this section is more detailed than that for the others.

2.1 Prevalence

Asthma has only been defined as a disease entity in its own right since the mid 1950s, so there is little information about it prior to that time. However, since the 1960s, there has been a reported progressive increase in the prevalence of asthma in westernised societies. In the past 20 years, the prevalence of asthma has almost doubled, so much so that today asthma affects approximately 8% to 10% of the population in the United States for example. It is now the leading cause of hospitalisation among young children in developed countries such as USA and Britain [Cohn *et al* 2003, Arkwright *et al* 2003].

2.2 Asthmatic symptoms

Asthmatic symptoms are characterized by laboured breathing accompanied by wheezing, a sense of constriction in the chest, and often attacks of coughing or gasping. During an attack, the airways are severely inflamed. Tightening of muscles surrounding the airways (also known as bronchoconstriction) causes them to narrow. Goblet cells in the airways secrete mucus, which 'plugs up' or partly blocks the airways. Asthma attacks can occur very rapidly, or develop over several hours or even days. This is dependent upon the extent to which an individual is allergic to a particular Ag, and to the length of time for which they are exposed to it. Asthmatic symptoms may also take just as long to reverse after the initial asthma attack has passed. This can be explained by the finding that Ag presentation may be prolonged due to a small population of resident airway APCs that can present Ag for up to eight weeks following exposure [Julia *et al* 2002]. There are a variety of stimuli that can trigger an asthmatic attack. Common allergens include grass pollen, house dust mite and animal hair.

2.3 Controlling asthmatic symptoms

As with other allergies, avoiding allergens is the best option to prevent symptoms. Eliminating other irritants from every day life, for example tobacco smoke and animal hair, and exercising regularly may also reduce the risk of an asthma attack. As most allergens are airborne avoiding them becomes difficult, and medical treatment is often necessary. Treatments fall into two categories, controlling and relieving medicines. Controlling medicines prevent the asthma attacks from starting, whereas relievers reverse the symptoms. Usually combinations of both are used to treat asthmatic patients.

Controlling medicines are usually anti-inflammatory drugs, which are taken regularly even without any symptoms of asthma, and prevent the likelihood of an attack occurring. The most effective and most commonly used anti-inflammatory medicines are inhaled glucocorticosteroids, such as Budesonide, Beclomethasone and Fluticasone. Glucocorticosteroids are defined as steroids that regulate carbohydrate, lipid and protein metabolism. These medicines help to prevent severe asthma attacks if taken regularly.

Relieving medicines are bronchodilators. These are medicines that help to prevent attacks progressing further by quickly opening up the narrowed airways. They do this by relaxing the muscles surrounding the airways. Most bronchodilators only have a short-term effect and should not be used regularly as controllers. Long-acting bronchodilators, including Formoterol and Salmeterol, are effective when they are used with an inhaled glucocorticosteroid. These can be used for both the immediate relief of symptoms, and alongside controlling medicine to keep asthmatic symptoms under control.

2.4 Immunology of asthma

During an asthma attack, the airway is infiltrated with excessive numbers of CD4+ Th2 cells, mast cells, MΦs, plasma cells and eosinophils. In the lumen of the lung, these cells infiltrate the airway mucosa [Cohn *et al* 2004]. The presence of so many inflammatory cells causes the airway wall to thicken 10% -

300% compared to that of non-asthmatic patients, resulting in a reduction in diameter of the lumen and small airways [Homer *et al* 2000, Roberts *et al* 1997]. Besides inflammatory cells, other elements in the lung contribute to the thickening of the lumen wall. For example, mucus glands undergo hypertrophy, and goblet cells in the airway epithelium secrete excessive mucus. There is deposition of collagen (types I, III and V), fibronectin and tenascin (a protein that interacts with collagen) under the basement membrane in the lumen, resulting in thickening and hardening of the airways [Roberts *et al* 1997, Roche *et al* 1989]. In addition myofibroblasts become hyperplastic, so small muscle mass, vascular permeability and airway oedema increase [Black *et al* 1997, Homer *et al* 2000].

Asthma is triggered when individuals come into contact with otherwise harmless allergens to which they are sensitized. Genetic predisposition to asthma is common. At least a dozen polymorphic genes associated with asthma have been identified, controlling mechanisms behind inflammatory responses, IgE and cytokine production, and AHR [Cookson *et al* 1999, van Eerdewegh *et al* 2002, Fahy *et al* 2000]. For example, the *ADAM33* gene belongs to a family of proteases, and has been identified as an asthma susceptibility gene. A genome screen using linkage analysis of 401 microsatellite markers in 460 Caucasian families was carried out. Each family had at least 2 siblings with doctor-diagnosed asthma. Strong linkage was seen between asthma and the region of chromosome 20p13. Forty genes were characterized in this region, and polymorphisms in the *ADAM33* gene had the highest significant association with asthmatic symptoms [van Eerdewegh *et al* 2002]. Although the exact function of the ADAM33 protein is still unknown, a number of possible roles have been addressed. ADAM33 may influence remodelling of the airway, as its selective expression in mesenchymal cells strongly suggests that alterations in activity may underlie abnormalities in the function of airway smooth muscle. In addition, it may alter release of cytokines and growth factors. Other ADAM proteins have this capacity, like ADAM17, which is responsible for generating soluble TNF-α [Cakebread *et al* 2004].

Another example of a gene potentially associated with asthma is CD14. CD14 is expressed and secreted by myeloid cells, usually monocytes and MΦs. In

some, but not all [Sengler *et al* 2003], studies a polymorphism in the promoter region of CD14 is associated with raised levels of IgE in peripheral blood [Baldini *et al* 1999]. In addition, during pregnancy and breast-feeding, low levels of secreted CD14 in the amniotic fluid and in breast milk have been linked with an increased risk of the child developing atopic dermatitis when born [Jones *et al* 2002].

2.5 Initiating excessive Th2 response

In healthy individuals, inhaled protein allergens induce tolerance. The respiratory mucosa is not completely impenetrable, and microbes and foreign Ags can enter the tissue. For example, some aeroallergens possess a protease activity that allows them to overcome the protective mucosal barrier. Therefore, pulmonary immune responses must be suppressed by active mechanisms. For example, inhaled Ag has been observed to induce immune unresponsiveness in naïve T cells, with both Ag specific and Ag non-specific "bystander" effects [Wolvers *et al* 1994]. In addition, APCs at mucosal surfaces have the unique ability to secrete IL-10, promoting the development of T cells with regulatory properties [Iwasaki *et al* 1999, Stumbles *et al* 1998]. This leads to tolerance at the mucosal surface in healthy people. In the lungs of asthmatic patients, tolerance is not induced in response to specific allergens. Tissue resident APCs present these allergens to T cells, resulting in a type-2 mediated airway inflammation [Hewitt *et al* 1995, Machado *et al* 1996].

APCs are defined as cells that can present Ag to a T cell via the MHC-TCR complex. The most efficient presenters of Ag are the DCs, which internalise all Ag, process it and present it on the MHC surface molecules. Immune responses during asthma attacks are initiated by APCs, as they are necessary for allergen presentation to T cells. The importance of APCs in asthma initiation is supported by the observation that DCs are found in increased numbers in the lung biopsies of asthmatic patients [Tunon-De-Lara *et al* 1996]. These DCs also express increased levels of markers, for example MHC II, compared to the DCs in the lungs of non-asthmatic patients. [Tunon-De-Lara *et al* 1996, Moller *et al* 1996]. Tissue resident APCs pick up exogenous allergen then migrate to the lymphoid organs, where they present the Ag on the MHC II to CD4+ T cells.

Once a CD4+ T cell is activated, it proliferates and secretes either type-1 or type-2 cytokines. The genetics of an individual may play a key role in biasing CD4+ T cell responses, to Th1 or Th2. In allergic individuals, CD4+ T cells are more commonly directed down a Th2 pathway. A number of genes have been closely linked with asthmatic traits. These include a cytokine cluster on chromosome 5q31, which contains genes for the type-2 cytokines IL-4, IL-5 and IL-13 [Song *et al* 1996, Rosenwasser *et al* 1997, Kawashima *et al* 1998]. Polymorphisms in these genes result in an increased incidence of asthma.

Although lymphocytes make up only a small percentage of the cells in the lung, CD4+ T cells are significantly increased in the airways of asthmatic patients, and they express activation markers such as CD25 (IL-2 receptor α chain) [Walker *et al* 1991, Corigan *et al* 1988]. Secretion of Th2 cytokines, such as IL-4, IL-5 and IL-13 by CD4+ T cells activates eosinophils, mast cells and induces B cell production of IgE. These further contribute to pulmonary inflammation. A cascade of cell activation and infiltration into the lungs then occurs, creating a positive feedback loop. For example, activated mast cells and NK T cells can further promote Th2 cell development by secretion of cytokines, such as IL-4 and IL-13 [Zuany-Amorim *et al* 1998, Akbari *et al* 2003b, Mosmann *et al* 1989]. In a murine model of OVA-induced pulmonary inflammation, development of AHR and airway inflammation by respiratory exposure to OVA required the activation of pulmonary NK T cells, producing a combination of IL-4 and IL-13 [Akbari *et al* 2003b]. Although CD4+ Th2 cells initiate allergic asthma, many activated cells contribute to airway inflammation.

The inflammatory response observed in allergic pulmonary disease is initiated and maintained by a number of redundant mechanisms. The pulmonary inflammation and increased vascular permeability results in rapid migration of more inflammatory cells into the airways. This promotes continued Th2 cell activation in the lungs. Damaged epithelial cells secrete GM-CFS, which also induces further DC maturation [Huh *et al* 2003, Cella *et al* 1996]. Memory CD4+ Th2 cells that already reside in the airways can also be activated locally by DCs, thereby leading to rapid induction of cytokine release and AHR [Huh *et al* 2003, Julia *et al* 2002, Salek-Ardakani *et al* 2003, Gonzalo *et al* 2002]. Ag

presentation may be prolonged due to a small population of airway APC that can present sequestered Ag for up to eight weeks following exposure [Julia *et al* 2002].

2.6 AHR

Airway hyper-responsiveness (AHR) has been identified as an important and defining feature of asthma. In terms of allergic asthma, AHR describes the increased ability of the airways to narrow after exposure to an allergen, and is an indication of the increased sensitivity of the airways to that allergen. The extent of AHR is indicated by the amount of allergen necessary to induce a bronchoconstrictor response, the steepness of the slope when the dose of allergen or agonist is plotted against the maximal response [Woolcock *et al* 1984].

2.6.1 Measuring AHR in humans

In humans, the severity of AHR is generally measured by patients inhaling increasing concentrations of a bronchoconstrictor stimulus, such as histamine or methacholine, until a given level of bronchoconstriction is achieved. This level is usually measured as a 20% fall in the Forced Expiratory Volume in one second (FEV_1) (PC_{20}) [Cockcroft *et al* 1977]. Asthmatic patients generally have a PC_{20} of <8mg/ml of histamine or methacholine, whereas non-asthmatics have a PC_{20} >16mg/ml. This method of measuring AHR has been used not only to measure AHR in asthmatics, but also in patients suffering from airway obstruction due to chronic obstructive pulmonary disease (COPD) [Ramsdale *et al* 1984].

This method, however, does have a limitation in determining AHR correctly. There is sometimes an overlap in PC_{20} between asthmatic and non-asthmatic patients, so defining an exact level of AHR is not always possible. This is because there appears to be a continuous distribution of non-specific AHR in the general population, with asthmatic patients in one tail of this distribution. This makes distinguishing asthmatics from non-asthmatics difficult [Cockcroft *et al* 1983].

2.6.2 Measuring AHR in animals

There are several procedures used to determine AHR in animals. Methods include *in vitro* measurement of intracheal smooth muscle contractility after electrical field stimulation [Larsen *et al* 1992], *in vivo* measurement of lung resistance after i.v. injection of bronchoconstrictive agents (as described above in humans) [Martin *et al* 1988, Renz *et al* 1992], and *in vivo* measurement of peak airway opening pressure [Levitt *et al* 1988, Hamelmann *et al* 1997]. Most experiments for measuring AHR have been carried out using murine models of airway inflammation, including mice, rats and guinea pigs. The use of murine models does present some limitations, which are discussed later.

Measurement of AHR by intracheal smooth muscle contractibility involves placing tracheal smooth muscle segments from allergen sensitized and challenged mice in an organ bath, on wire supports and attached via a gold chain to a FT.03 isometric force transducer. Tissues are then submerged in a Krebs-Henseleit solution, and each tracheal smooth muscle segment is equilibrated in the bath for 90 minutes at a tension of 1.5 g. A stimulus is applied through the tissue by means of parallel platinum electrodes. The optimal resting length for each tissue is established by assessing its maximal contractile response to the electrical field stimulation (in Hz). Recordings of resting tensions and all contractile responses are made on a dynagraph recorder. At the end of each experiment, the tracheal smooth muscle segment is weighed. All tensions were expressed as grams isometric tension per gram tracheal smooth muscle weight (g/g). The frequency that caused 25%, 50%, and 75% of the maximal contraction is calculated from linear plots of the contractile response versus the frequency of electrical field stimulation, and expressed as the ES_{25}, ES_{50}, and ES_{75} [Larsen *et al* 1992]. Animals sensitized and challenged with allergens have a reduced ES_{25}, ES_{50} and ES_{75} compared to animals that are challenged but not sensitized.

Measuring lung function *in vivo*, using increasing concentrations of a bronchoconstrictor stimulus, often requires animals to be anaesthetized, tracheotomized and ventilated. The tracheotomy tube is passed into a plethysmograph chamber and connected to a ventilator. A bronchoconstrictor

stimulus, such as histamine or methacholine, is dissolved in saline, diluted to different concentrations and injected individually i.p. Changes in lung pressure are recorded by detecting chamber pressure via a pressure transducer and flow is measured by electronic differentiation of the volume signal [Martin *et al* 1988]. As described above, the lower the concentration of the bronchoconstrictor needed to induce a 20% drop in lung function, the greater the severity of AHR.

The two methods described above for measuring AHR in animal do have limitations. The *in vitro* technique of measuring intracheal smooth muscle contractibility after electrical field stimulation has been shown to correlate with allergic airway sensitisation [Larsen *et al* 1992]. However, using this technique does not take into account the influence of other changes within the airway, such as mucus production. The *in vivo* method of measuring lung function in response to histamine or methacholine is limited by the fact that animals need to be anaesthetized and ventilated. The influence of the anaesthetic and of the operating procedures on lung function is unknown. In addition, this is a time consuming protocol [Martin *et al* 1988, Renz *et al* 1992].

These problems can be overcome by using a method, where unrestrained and conscious animals can be monitored. This technique is similar to the *in vivo* measurement of lung function in response to a bronchoconstrictor, except the pressure measurements are obtained using barometric whole-body plethysmography (WBP). Conscious mice are placed in the chamber of the whole-body plethysmograph. The pressure differences between the main chamber of the WBP and an empty reference box are measured. This is known as the known as the box pressure signal, and is caused by volume and resultant pressure changes in the main chamber during the respiratory cycle of the animal. Aerosolized methacholine is then pumped into the chamber in increasing concentrations and readings taken [Hamelmann *et al* 1997].

The changes in pressure are used to determine several aspects of the animals' lung function. Inspiration and expiration are recorded, by establishing start and end of inspiration as where the box pressure/time curve crosses zero (see Figure 1-1). The time of inspiration or expiration is the time from the start of inspiration or expiration to the end (T_i or T_E); the maximal box pressure signal in

one breath is defined as peak inspiratory or expiratory pressure (PIP or PEP); relaxation time is the time of pressure decay to 36% (T_r). During bronchoconstriction, the main alteration in the signal occurs during early expiration and leads to changes in the waveform of the box pressure signal (see Figure 1-1) [Hamelmann *et al* 1997].

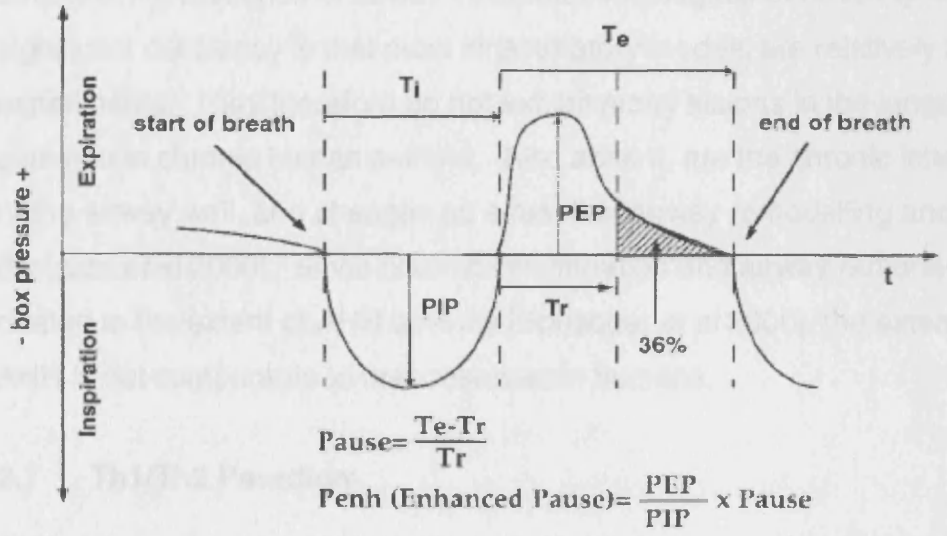


Figure 1-1: Parameters measured by barometric plethysmography. Schematic diagram of box pressure waves in inspiration (down) and expiration (up). T_I = inspiratory time; T_E = expiratory time; PIP = peak inspiratory pressure; PEP = peak expiratory pressure; T_r = relaxation time.

Subtracting the Tr from TE and dividing the whole number by Tr can determine the measurement Pause. This can then be multiplied by (PEP/PIP) to give the Enhanced Pause (Penh). Penh reflects the changes in the waveform of the signal pressure from both inspiration and expiration (PIP and PEP) and combines it with the timing of early and late expiration (pause). AHR is often visualised or plotted as a fold increase for each concentration of methacholine compared to Penh values after challenge [Hamelmann *et al* 1997, Kearley *et al* 2005]. Although the calculations of this method are complicated, a lot of data can be obtained without having to terminate the animal. The protocol is also less time consuming and does not put unnecessary stress on the animals by requiring anaesthesia or tracheotomy.

2.6.3 Limitations of measuring AHR in animals

All the above methods of measuring AHR have been described using rodents. However, there are some severe limitations in using murine models to measure AHR. For example, murine studies may not clearly distinguish between AHR originating from the airways, and that due to contractile elements in the lung parenchyma, which is responsible for a significant component of resistance to airflow in the presence of airway inflammation [Nagase *et al* 1994]. Another significant deficiency is that most inflammatory models are relatively short-term experiments. They therefore do not exhibit many lesions in the lungs that are common in chronic human asthma. Also absent, are the chronic inflammation of the airway wall, and changes as a result of airway remodelling and fibrosis [Holgate *et al* 2000]. Since chronic inflammation and airway remodelling are be related to the extent of AHR severity [Bousquet *et al* 2000], the extent of murine AHR is not comparable to that observed in humans.

2.7 Th1/Th2 Paradigm

CD4+ T cells can be characterized by the cytokines they secrete. Cells producing IL-2 and IFN-γ are known as Th1 cells, and those producing IL-4 as Th2 [Mosmann *et al* 1989]. These CD4+ Th cells produce cytokines, which differentiate naïve T cells into the same T cell subtype, enhancing their own cell type proliferation. Each cell type also down-regulates the other. For example, Th1 IFN-γ secretion is shown to suppress Th2 proliferation and IL-4 secretion *in vitro* [Mosmann *et al* 1989, Fitch *et al* 1993, Abbas *et al* 1996] and, in a separate study, supernatants removed from Th2 clone cultures and added to Th1 clone cultures inhibited IFN-γ production by Th1 cells [Fiorentino *et al* 1989].

Reciprocal regulation between Th1 and Th2 cells *in vivo* remains, however, a matter of debate. Whereas supporting data have so far been obtained from *in vitro* studies. A number of observations *in vivo* appear to contradict the Th1/Th2 paradigm. For example, chronic atopic dermatitis, rather than being a Th2-mediated disease, is associated with an increase in both Th1 and Th2 cytokines, IFN-γ and IL-4 respectively [Hamid *et al* 1994]. Furthermore, OVA-

induced Th2 mediated AHR in a murine model of pulmonary inflammation failed to be reversed by the addition of Th1 cells. Rather, Th1 cells contributed to the severity of the disease [Hansen *et al* 1999]. In a similar study the passive transfer of OVA-specific Th1 cells i.v. resulted in enhancement of an originally Th2 mediated airway inflammation. This effect was observed regardless of whether the T cells were transferred before sensitisation or after airway inflammation was already established. The transfer of Th1 cells also resulted in increased recruitment of both Th1 and Th2 cells to the airways [Randolph *et al* 1999]. Finally, in contrast to what was predicted based on the classification of asthma as a type-2 disease, IFN-γ has been detected in the lungs of asthmatic patients [Krug *et al* 1996].

The lack of agreement between *in vitro* and *in vivo,* data suggests a more complicated balance between Th1 and Th2 responses. When limited to an *in vitro* system, with controlled cell types and environment, Th1 and Th2 cells may easily antagonise each other's development and expansion. However, in an *in vivo* model, responses would not be physically contained and other "bystander" cells would be affected by and react to changes in immune responses. Therefore, decreases in type-2 responses may not be due to changes in Th1 and Th2 cell activation alone. If *in vivo* Th1 and Th2 cells are not regulating each other then some other mechanism must be involved. Allergy has been described as a disease of immunodysregulation, where the self-regulation mechanisms of the immune system are dysfunctional [Rook *et al* 2002]. One theory is that Tregs are involved in the reversal of allergic inflammation, as well as autoimmune disease, where their role has been firmly established [Robinson *et al* 2004, Curotto *et al* 2002, Wraith *et al* 2004]. This theory is further described in section 4.

2.8 Immunosuppressive cytokines

Immunosuppressive cytokines, so-called for their ability to suppress immune responses, play a major part in the self-regulation of the immune system. The best described cytokines with such abilities are IL-10 and TGF-β. Both have been observed to play essential roles in regulation of Th2-mediated responses [Ludviksson *et al* 2000, John *et al* 1998]. More recently another cytokine, IFN-

α, has also been put forward as an immunoregulatory cytokine [Lombardi *et al* 2000].

Originally IL-10 had been classified as a Th2 cytokine. It is secreted by IL-4 producing Th2 cells and has an inhibitory effect on Th1 responses [Elliott *et al* 1994]. More recently IL-10 secretion by IFN-γ producing Th1 cells was also observed [Gerosa *et al* 1999]. IL-10 is secreted by a variety of cells, including T cells, APCs and NK cells. The regulatory functions of IL-10 are discussed in more detail later in sections 3 and 4 [Read *et al* 2001, Roncarolo *et al* 2000]. IL-$10^{-/-}$ mice develop severe allergen-induced airway inflammation, with excessive IL-4, IL-5 and IFN-γ production [Borish *et al* 1998]. Stampfli and colleagues have suggested that IL-10 is essential to downregulate inflammation in the lungs of OVA sensitized and challenged mice. Addition of IL-10 via an adenoviral vector alleviated airway inflammation, by inhibiting cytokine and chemokine production, and by downregulating CD80 and CD86, co-stimulatory ligands involved in T cell activation, hence preventing efficient Ag presentation to the T cell [Stamfali *et al* 1999]. A protective role for IL-10 has also been observed in asthmatic patients, where the use of the inhaled corticosteroid Budesonide, an anti-inflammatory controlling medicine for asthma, elevates IL-10 protein levels in the lungs [John *et al* 1998]. Together these observations suggest an important role for IL-10 amelioration of symptoms of asthma.

TGF-β is a cytokine secreted by a number of different cells types, such as T cells, APCs and eosinophils. TGF-β, like IL-10, has also been observed to regulate immune responses. TGF-$\beta^{-/-}$ mice develop a multifocal wasting disease, with severe multiorgan inflammation and tissue necrosis twenty days after birth. The inability to suppress the immune response leads to organ failure and rapid death [Shull 92, Prud'homme *et al* 2000]. TGF-β also has an inhibitory effect on T cells undergoing differentiation. When CD4+ T cells isolated from naïve transgenic DO11.10 mice were treated with OVA and TGF-β, a significant reduction was observed in T cell proliferation, compared to CD4+ T cells treated with just OVA. This result occurred even when the cells were also treated with IL-4 and IFN-γ [Ludviksson *et al* 2000]. Treatment with IFN-γ

or IL-4 would have favoured Th1 or Th2 development, but the presence of TGF-β prevented this, suggesting an immunoregulatory role for TGF-β.

IFN-α has also been described as having immunoregulatory properties. This cytokine is secreted by APCs, usually in response to viral infections. Together with IL-10, exogenous IFN-α enables the differentiation of human T cells with regulatory abilities. In experiments where naïve CD4+ T cells were cultured in the presence of exogenous IL-10 and IFN-α, the resulting T cells secreted elevated amounts of IL-10 and TGF-β, suggesting that the presence of IL-10 and IFN-α induces Tregs. When these Tregs were added to co-cultured APCs and naïve CD4+ T cells, they significantly inhibited CD4+ T cell proliferation [Levings *et al* 2001]. IFN-α also upregulates the secretion of IL-10 by DCs, further promoting induction of T cells with regulatory properties [Bilsborough *et al* 2003]. IFN-α has also been observed to promote survival of anergic T cells. Indeed, apoptosis of anergic T cells is blocked by addition of IFN-α to culture medium [Lombardi *et al* 2000].

3. Antigen Presenting Cells

APCs are a large and heterogeneous group of cells. A wide spectrum of cells can present Ag, but APCs consist of three main groups; DCs, MΦs and B cells. They present Ag to T cells initiating T cell development. MHC is the molecule necessary for presentation of Ag to the T cell via the TCR [Bryant *et al* 2002]. The type of immune response triggered depends on the molecular context in which the Ag is presented. For example if an intracellular pathogen is detected, signalling through the MHC I-TCR activates CD8+ cytotoxic T cells, which leads to destruction of the cell. In contrast, extracellular foreign Ag is presented by the APC to the T cell by the MHC II molecule, which initiates a CD4+ Th response, either Th1 or Th2 depending on the cytokine environment.

APCs pick up exogenous Ag in the peripheral tissue, and degrade the Ag into peptides, before they migrate to the T cell rich zones of the LNs. Once in the LNs, APCs upregulate activation and co-stimulatory cell surface markers like MHC II, andCD80 and CD86. DCs alone are actually capable taking

exogenous Ag up from outside the cell and presenting it to T cells through interaction with MHC I, and triggering CD8 T cell activation, normally activated by the MHC I pathway. This is known a cross-priming, and results in activation of CD8+ cytotoxic T cells that are capable of killing cells by degranulation of substances like granzyme B and perforin [Zinkernagel *et al* 2002]. DCs are also the most efficient APC at presenting Ag to T cells. In addition they play vital roles in other aspects of the immune response, for example in cell regulation and peripheral tolerance.

3.1 Role of APCs in tolerance

The immune system comes into constant contact with food Ags and aeroallergens at mucosal surfaces. APCs at mucosal surfaces have unique features that stimulate the development of tolerance and may provide homeostatic regulation [Bilsborough *et al* 2003]. The generation of tolerance in the airways appears to be predominantly under the control of mucosal DCs, and may be achieved by a number of mechanisms.

One possible mechanism of tolerance may be through APC expression of co-stimulatory molecules. DCs at mucosal surfaces have been observed to express low levels of MHC II and co-stimulatory molecules, such as CD80 and CD86 [Stober *et al* 1998, Janeway *et al* 1994]. These molecules are essential for naïve T cell activation. The low levels of these markers expressed by mucosal DCs leads to reduced activation in response to harmless Ags. This suggests that DC expression of co-stimulatory molecules may influence activation of Th2 mediated inflammation.

Another possible mechanism involves APC production of immunosuppressive cytokines. For example, oligosaccharides derived from the helminth *Schistosoma mansoni* can induce APCs in the peritoneal cavity, which express low levels of proinflammatory cytokines, such as IL-12 and IFN-γ, and increased levels of the regulatory cytokines IL-10 and TGF-β. These APC have been shown to mediate regulatory processes. For example, when cultured *in vitro* together with anti-CD3 and anti-CD28 stimulated naïve CD4+ T cells, they also suppressed T cell proliferative responses [Terrazas *et al* 2001]. This suggests

that secretion of immunoregulatory cytokines by APCs may have a suppressive effect on T cell activation.

In addition to regulating immune responses themselves, APCs have been observed to induce T cells with regulatory properties. Mucosal DCs that produce IL-10 have been observed to promote the generation of Tregs [Iwasaki *et al* 1999, Stumbles *et al* 1998]. The regulatory properties of these Tregs arise from their secretion of immunosuppressive cytokines, such as IL-10 and TGF-β. These cytokines would have further suppressive effects on "bystander" cells. IL-10 producing DCs have been reported following respiratory exposure to OVA. These in turn stimulated the development of IL-10 secreting Tregs. Adoptive transfer of these DCs to recipient animals with allergic pulmonary inflammation induced Ag specific unresponsiveness [Akbari *et al* 2001, Akbari *et al* 2002]. This suggests that IL-10 secretion by Tregs could lead to immune tolerance, when occurring at the mucosal surface.

4. Regulatory T cells

Suppression of immune responses to self or foreign harmless Ag is known as tolerance. Two main kinds of tolerance exist, central and peripheral tolerance. Central tolerance involves negative selection of autoreactive thymocytes, deleting these cells by apoptosis [Kappler *et al* 1987]. In contrast, peripheral tolerance occurs once the cells have migrated away from the thymus into the periphery. Several mechanisms can result in peripheral tolerance, including suppression of cell activation [Earle *et al* 2005]. In this thesis, peripheral tolerance is concentrated on.

Tolerance to both self and environmental Ags in the periphery is important, particularly at mucosal surfaces. T cells exist that have the ability to suppress immune responses and these are known as Tregs. Recently, the term "Treg" has been reserved, by some authors, for constitutive CD25+, Foxp3+ regulatory T cells. In this thesis Treg is used for all types of regulatory T cell. Several types of Tregs exist, each characterized by their different cytokine secretions and cell surface marker expression. All Tregs do, however, have similar functions, and are capable of suppressing immune responses. These cells are

probably identical to the suppressor cells studied in the late 1970's and early 1980s by Gershon and colleagues. However "suppressor cells" went out of fashion for a decade, until workers such as Weiner and colleagues at Harvard University rediscovered them in a model of autoimmunity. They were by Weiner and colleagues using MBP in an SJL mouse model to induce EAE. MBP is one of the components of myelin, a substance that forms part of the layer around the nerve axons. EAE is an animal model for multiple sclerosis, a cell mediated autoimmune disease that results in demyelination of axons. This group observed that feeding mice with EAE low doses of MBP Ag suppressed symptoms, by inducing a peripheral tolerance that was mediated by the differentiation of an Ag specific Tregs. These Tregs secreted both IL-4 and TGF-β [Chen *et al* 1994].

Tregs have since been described to secrete IL-10. Naïve T cells isolated from the spleens of OVA-TCR transgenic DO11.10 mice were repetitively stimulated with OVA and IL-10. The cytokine profile of these Tregs was characterized by IL-10, with little IL-2 and no IL-4. This cytokine profile distinguishes this Treg type from Th1 or Th2 cells. When these cells were injected i.p. into a murine model of Th1 mediated colitis, the development of the disease was inhibited, demonstrating immunosuppressive properties [Groux *et al* 1997, Cottrez *et al* 2000]. Tregs have also been described in humans. When human peripheral T cells were stimulated with exogenous IL-10, they also secreted the IL-10 themselves [Groux *et al* 1997]. The secretion of TGF-β and IL-10 by different types of Tregs demonstrates that immunosuppressive cytokines are important in the maintenance of peripheral tolerance.

Experiments have been performed that directly demonstrate the importance of IL-10 and TGF-β Treg-induced peripheral tolerance. T cells have been engineered to secrete IL-10 and TGF-β using a retrovirus. These T cells have the ability to inhibit AHR and airway inflammation, when adoptively transferred into animals with allergic pulmonary inflammation. This suppression is reversed by the addition of anti-IL-10 or anti-TGF-β Ab. This suggests that these cytokines may have a suppressive effect of inflammatory responses [Hansen *et al* 2000, Nakao *et al* 2000, Oh *et al* 2002, Stampfli *et al* 1999]. Tregs have been hard to study, mainly due to the difficulty in culturing them *in vitro*. Despite

these problems, several subtypes of T cell with regulatory properties have been described.

4.1 Natural CD4+CD25+ Tregs

In 1995 Sakaguchi and colleagues originally described natural Tregs as a small fraction, approximately 10%, of CD4+ T cells present in the periphery [Sakaguchi *et al* 1995]. They constitutively express the IL-2 receptor α chain, CD25, and are selected in the thymus. In addition, they have been observed to express a variety of cell surface markers and transcription factors, some more commonly associated with activated and memory T cells. These include $CD45RB^{Low}$, CD62L, CD103, CTLA-4, GITR and *Foxp3* (Table 1-3) [Powrie *et al* 1993, Sakaguchi *et al* 1995, Takahashi *et al* 2000, McHugh *et al* 2002, Khattri *et al* 2003]. *In vitro,* naturally occurring CD4+CD25+ Tregs operate in a contact dependent manner [Shevach *et al* 2002, Thornton *et al* 1998], although secretion of IL-10 has been observed [Ling *et al* 2004]. The mechanism of action adopted by naturally occurring Tregs *in vivo* is still unclear.

Natural CD4+CD25+ Tregs have the ability to prevent organ-specific autoimmunity. This was demonstrated when CD4+ T cell populations were depleted of CD25+ cells and transferred to recipient mice. These mice were shown to develop a variety of autoimmune diseases, including thyroiditis, gastritis and polyarthritis. Rapid reconstitution of CD4+CD25+ T cells prevented development of these autoimmune diseases [Sakaguchi *et al* 1995]. The importance of the thymus as the centre of origin for naturally occurring Tregs has been highlighted by experiments in thymectomized mice and rats [Asano *et al* 1996, Seddon *et al* 1999]. Mice thymectomized 3 days after birth spontaneously develop autoimmune diseases, like gastritis. They also have no detectable levels of CD25+ cells in the peripheral blood. If splenic CD25+ T cells isolated from healthy animals are immediately transferred into thymectomized mice, autoimmunity is prevented. Interestingly, mice thymectomized at day 7, have a decreased likelihood of developing autoimmunity, and no difference is seen in the number of CD25+ cells in the periphery. This may be due to a sufficient number of Tregs circulating in the periphery by day 7. This suggests that natural CD25+ T cells are important in

the maintenance of peripheral tolerance, and that development is completed by day 7 [Asano *et al* 1996]. These data emphasise the role of natural CD4+CD25+ cells in peripheral tolerance.

Naturally occurring CD4+CD25+ Tregs operate by cell contact dependent mechanisms *in vitro*. Shevach and colleagues observed that CD4+CD25+ cells purified from the LNs of naïve mice failed to proliferate after stimulation with either IL-2 or anti-CD3. When co-cultured together with CD4+CD25- cells, CD4+CD25+ cells significantly suppressed CD4+CD25- cell proliferation and IL-2 production, as measured by ELISA. Cytokines were believed to have no effect on the suppressive ability of the Tregs [Thornton *et al* 1998]. Indeed, CD4+CD25+ cells isolated from IL-$4^{-/-}$ or IL-$10^{-/-}$ mice and co-incubated with anti-CD3 and CD4+CD25- cells from control animals retained the ability to suppress proliferation of CD4+CD25- cells [Thornton *et al* 1998]. Cytokine mediated mechanisms may therefore be an unlikely mode of action of natural Tregs.

4.2 Inducible Tr1 cells

Tr1 cells are one of two inducible types of CD4+CD25+ Tregs described. Unlike natural CD4+CD25+ Tregs, Tr1 cells are induced in response to specific, non-self Ag [O'Garra *et al* 2004a, O'Garra *et al* 2004b]. Development of Tr1 cells is still observed in thymectomized TCR transgenic after treatment with influenza HA peptide, suggesting that Tr1 cells are induced locally, rather than in the thymus [Apostolou *et al* 2004]. This suggests that the thymus is not essential for the development of Tr1 cells, and that Tregs can still be induced by foreign Ag in animals that lack naturally occurring Tregs. This distinguishes these Tr1 cells from natural CD4+CD25+ Tregs.

Tr1 cells were first described by Groux and colleagues in 1997. They showed that chronic activation of both human and mouse CD4+ T cells in the presence of IL-10 gave rise to a T cell with a low proliferative capability, and which secreted large amounts of IL-10, no IL-4 and low levels of IL-2. Proliferation of naïve murine CD4+ T cells, in response to OVA presented by splenic APCs, was significantly reduced when Tr1 cells were present in the culture. This effect on proliferative responses was neutralized by the addition of anti-IL-10 and anti-

TGF-β Abs, suggesting that both IL-10 and TGF-β are essential for the development and function of Tregs [Groux *et al* 1997]. Tr1 cells have also been shown to be Ag specific. For example, transfer of OVA specific Tr1 cells inhibited the onset of IBD in SCID mice, but only when mice had been pre-feed with OVA [Groux *et al* 1997].

Powrie and colleagues have observed similar T cells in a murine model of IBD. In their model, colitis was induced by transferring CD4+CD45RB^{High} T cells i.v. from BALB/c mice, into transgenic SCID mice. The SCID background was used, since it limits the number of lymphocytes naturally produced by the host immune system. Transfer of CD4+CD45RB^{High} cells induced inflammation in the bowel, simulating human IBD. When CD4+CD45RB^{Low} T cells were transferred, the inflammation was prevented. These cells were also observed to secrete IL-10 [Powrie *et al* 1994]. This suggests that these CD45RB^{Low} Tregs are the same as Tr1 cells.

4.3 Th3 cells

Chen and colleagues originally described Th3 cells in 1994. These cells were induced in the MLNs by oral administration of MBP Ag, and produced high levels of TGF-β with varying amounts of IL-10 and IL-4. In addition they suppressed the development of EAE in SJL mice, orally sensitized to MBP, a protein component of myelin [Chen *et al* 1994]. MBP specific T cells, which secreted TGF-β, were isolated from the MLNs of these mice. These MBP specific T cells were also effective in suppressing the symptoms of PLP-induced EAE. PLP is a protein making up the myelin layer of the neurone. When injected i.p., PLP induces EAE in SJL mice [Chen *et al* 1994]. The suppression of non-Ag specific EAE suggests that Th3 cells operate by cytokine mediated mechanisms.

4.4 Treg markers

The molecular basis for the induction of Tregs is still not clear. Several molecules have been implicated in the development of Tregs, including CTLA-4, GITR, TGF-β and IL-10. However, none of these are specific to Tregs, and are

also expressed by other cell types. The possible mechanisms triggered by the cytokines IL-10 and TGF- β will be discussed in Chapter 3 and Chapter 4.

CTLA-4 and GITR are both implicated in Treg activity [Sakaguchi *et al* 2001, Shevach *et al* 2002, Maloy *et al* 2001]. GITR was originally identified as a marker constitutively expressed by naturally occurring CD4+CD25+ Tregs [McHugh *et al* 2002]. Blockade of GITR with a mAb (known as clone DTA-1) results in the blockade of CD4+CD25+ Treg function *in vitro* [McHugh *et al* 2002]. *In vivo* injection of DTA-1 leads to induction of autoimmune diseases, such as autoimmune gastritis [Shimizu *et al* 2002]. However, GITR has also been observed to be upregulated on non-regulatory CD4+ T cells. Recent contradictory evidence suggests that ligation of GITR actually occurs on activated T cells, and not on Tregs. This work suggests that ligation of GITR and GITR-L renders effector T cells resistant to suppression by Tregs [Stephens *et al* 2004]. This may suggest that GITR is not a reliable marker for Treg activity.

CTLA-4 is a negative regulator, which causes suppression of activated T cell signals. CTLA-4 has a high affinity for the co-stimulatory B7 ligands, CD80 and CD86. The activation receptor CD28, which also binds the B7 ligands, promotes T cell activation. Since CTLA-4 binds these ligands with 500 times more avidity than CD28, CTLA-4 can out compete CD28 and trigger a negative regulatory signal [Greenwald *et al* 2002]. In addition to this, CTLA-4 has been shown to trigger the induction of the enzyme IDO when interacting with CD80 and CD86 on the APC. IDO depletes tryptophan and catalyses tryptophan to kynurenine and other metabolites, which have potent immunosuppressive effects on T cells. This may be another mechanism by which Tregs exert suppression via CTLA-4 [Grohmann *et al* 2002, Munn *et al* 2004]. However, both CTLA-4 and GITR are also expressed by other effector and memory CD4+ T cells, and are not unique to Tregs.

4.4.1 CD45

CD45 is a protein tyrosine phosphatase (PTP), expressed by most hematopoietic cells, except erythrocytes. The protein tyrosine phosphatases

constitute a family of receptor-like and cytoplasmic inducing enzymes that catalyze the dephosphorylation of phosphostyrosine residues and are characterized by homologous catalytic domains [Barford *et al* 1994]. CD45 is made of a single chain type-1 transmembrane protein, which is approximately 1100-1300 amino acids long. The extracellular domain of this protein contains amino acids that range from 300-500 amino acids and a long cytoplasmic tail with approximately 700 amino acids that contains repeats for the two phosphatase domains [Okumura *et al* 1996].

The role and function of CD45 is complex and still poorly understood. It is understood that CD45 is required for both B and T cell signalling. CD45 functions to regulate Src kinases required for T and B cell receptor signal transduction [Kung *et al* 2000]. In T cells, the members of the Src family kinases regulated by CD45 are Lck, and Fyn and in B cells the Src family kinases are as Lyn and Blk. In T cells and B cells, the phosphorylation of the immunoreceptor tyrosine bases motifs (ITAMS) is an intracellular signal that alerts the cell that the lymphocyte has detected a specific Ag. When ITAMS's tyrosines are phosphorylated by the receptor associated tyrosine kinases, the ITAMS become able to bind second family protein tyrosine phosphatase such as CD45 [Janeway *et al* 2001]. In T cells, CD45 in particular dephosphorylates Csk, an inhibitory protein tyrosine kinase. This activation is particularly important because it controls levels of tyrosine kinase activity in lymphocytes. In B cells, Ca2+ is transduced by the B cell receptor and this signalling is believed to induce the expression of CD45 [Justement *et al* 1991].

4.4.2 CD45 isoforms

The CD45 protein has several isoforms. Expression of the different CD45 isoforms can be seen at different stages of cell differentiation, and hematopoietic cells can express more than one isoform [Virts *et al* 1997]. CD45 is abundantly distributed within the cell membrane, and constitutes approximately 10% of all the molecules on the surface of expressing cells. The mammalian *CD45* gene consists of 35 well characterized exons. The exons which determine which CD45 isoform is produced is found on the plasma membrane are located in the N terminus of the CD45 protein. These are exons

4-6, although there is some evidence that exon 7 is involved in alternative splicing, since an isoform lacking exons 4, 5, 6 and 7 has been observed at the mRNA level [Chang *et al* 1991, Virts *et al* 1998]. Although theoretically a large number of isoforms should exist, only a few are usually observed. These include CD45RO, CD45RA, CD45RB CD45RC, and CD45RABC. Each isoform differs in their extracellular domains because of alternative splicing of exons 4 (CD45RA), 5 (CD45RB) and 6 (CD45RC). CD45RO results when exons 4, 5 and 6 are all spliced out and CD45RABC when all exons are transcribed [Reviewed in Hermiston *et al* 2003].

4.4.3 CD45 isoform function

Expression of the CD45 isoforms is tightly regulated in peripheral T cells [Justement *et al* 1991]. The different CD45 extracellular domains are expressed in a cell type and activation state dependent manner. This is conserved across species, suggesting an important function *in vivo* [Trowbridge *et al* 1994, Fukuhara *et al* 2002]. Double negative T cells and single positive (CD4+ or CD8+) T cells predominantly express the larger CD45 isoforms, such as CD45RA. In contrast, activated and memory T cells express CD45 isoforms encoded by mRNAs lacking most or all of the variable exons, such as CD45RO [Fukuhara *et al* 2002, reviewed in Hermiston *et al* 2003]. This makes CD45 a useful marker to distinguish between different T cell activation states, such as naïve (CD45RA+ T cells) and memory T cells (CD45RO+ T cells).

The function of the different isoforms of CD45 is still relatively unclear. Smaller isoforms tend to be more prone to homodimerization, which has been shown to inhibit intracellular phosophotase activity [Pingel *et al* 1989, Stamenkovic *et al* 1991]. Thus, alternation between expression of larger and smaller isoforms appears to affect the amount of intracellular phosphotase activity, and hence the efficiency of TCR signalling. T cells expressing the larger isoforms are therefore more likely to have high phosphatase activity and maintain the TCR in a state primed for Ag recognition [Pingel *et al* 1989]. This possible role of CD45 in immune homeostasis or tolerance is supported by studies that correlate defects in CD45 splicing to susceptibility to various autoimmune diseases, such as multiple sclerosis [Chang *et al* 1991].

4.5 Foxp3

Foxp3 is a gene for the transcription factor protein forkhead/winged-helix, or Scurfin, which is highly expressed in naturally occurring CD4+CD25+ Tregs [Hori *et al* 2003, Ling *et al* 2004]. In contrast to other markers used to identify Tregs, *Foxp3* is not simply a marker of activation. For example, CD4+CD25- cells do not usually express *Foxp3* message when they are stimulated. *Foxp3* is necessary for Treg development and activity [Khattri *et al* 2003, Fontenot *et al* 2003], for example mutations in the *Foxp3* gene in the mutant mouse strain *scurfy* lead to rapid development of autoimmune diseases [Khattri *et al* 2003, Brunkow *et al* 2001]. In humans, the genetic disease IPEX (sometimes referred to as XLAAD) is due to a mutation in the human homologue of *Foxp3*, and results in onset of autoimmune diseases such as type-1 diabetes, autoimmune endocrinopathy and, in some cases, severe atopy [Chatila *et al* 2000, Bennett *et al* 2001, Wildin *et al* 2001, Gambineri *et al* 2003]. These observations have led to the hypothesis that this transcription factor is important for Treg development.

The lymphoproliferative disease seen in *scurfy* mice and *Foxp3*$^{-/-}$ mice is similar to that seen in humans with IPEX, and also shares characteristics seen in CTLA-4 and TGF-β deficient mice [Fontenot *et al* 2003, Shevach *et al* 2002]. CD4+CD25+ cells isolated from *Foxp3*$^{-/-}$ mice have the ability to proliferate themselves, and do not have the ability to suppress proliferation of other T cells, suggesting that they are not Tregs [Fontenot *et al* 2003, Khattri *et al* 2003]. The lymphoproliferative disorder observed in *Foxp3*$^{-/-}$ mice is inhibited by the transfer of Tregs from control wild-type mice, suggesting that *Foxp3* is essential for functional Tregs [Fontenot *et al* 2003].

4.6 CD8+ Tregs

In addition to CD4+ Tregs, there is also evidence that CD8+ T cells with regulatory abilities exist [Zhang-Hoover *et al* 2004, Bisikirska *et al* 2005, Bienvenu *et al* 2005]. For example, in a murine model of MPB-mediated EAE, APCs were observed to induce Ag-specific CD8+ Tregs. Transfer of these CD8+ Tregs to MBP immunised mice inhibited the development of EAE. This

effect was not observed when CD4+ T cells were transferred to recipient mice[Faunce *et al* 2004]. Transfer of APC-induced CD8+ Tregs has also been shown to suppress pulmonary fibrosis [Zhang-Hoover *et al* 2004]. In MHC $II^{-/-}$ mice, CD8+CD25+ Tregs have been observed in the periphery, which express CTLA-4, GITR and Foxp3, and secrete IL-10. When cultured together with irradiated APCs, CD25- cells and anti-CD3, these CD8+CD25+ Tregs inhibited T cell proliferation. This was observed with both CD4+ and CD8+ naïve T cells [Bienvenu *et al* 2005]. The term Treg generally refers to CD4+CD25+Foxp3+ T cells, but CD8+ T cells with regulatory properties have now been observed. In this thesis, the term Treg refers to CD4+CD25+ T cells.

Table 1-2 Cell surface markers and cytokines expressed by CD4+ Tregs

	Natural Tregs	**Tr1**	**Th3**
Cell surface markers			
CD4	+++	+++	+++
CD25	+++	+++	+
$CD45RB^{Low}$	+	+	?
CD62L	+	?	?
CD103	+	?	?
CTLA-4	+	-	++
GITR	+	+	?
Foxp3	++	+/-	+
Cytokines			
IL-10	+/-	+++	+
TGF-β	+/-	+	+++
IL-4	-	-	+
IL-5	-	+	-
References	Sakaguchi *et al* 1995, Powrie *et al* 1993, McHugh *et al* 2002, Khattri *et al* 2003	Groux *et al* 1997, Cottrez *et al* 2004 Zuany-Amorim *et al* 2002b, Apostolou *et al* 2004	Chen *et al* 1994, Fossat *et al* 2003, Perez-Machado *et al* 2003

4.7 NK T cells

NK T cells are a unique subset of cells that express both typical NK receptors, including NK1.1, and T cell markers such as the TCR. Unlike NK cells, NK T cells develop in the thymus. In contrast to typical T cells, NK T cells respond to Ag presented by the MHC I-like molecule, CD1d, and express only intermediate levels of TCR. In addition, NK T cells are either CD4+ or CD4-CD8-. Most notably, NK T cells express an extremely limited T cell repertoire, since they bind mainly lipids, glycolipids, or highly hydrophobic peptides presented by CD1d molecules [Park *et al* 2000, Porcelli *et al* 1999].

Structurally, there are several subsets of NK T cell, depending on their specific expression of an invariant TCR chain. The most studied NK T cell in mice has a rearrangement of the variable region Vα14 to the joining region Jα18 of the TCR, to form an invariant complementarity-determining region 3α (CDR3α), which is usually referred to as a Vα14*i* TCR [Lantz *et al* 1994, Brossay *et al* 1998]. These cells preferentially co-express either Vβ8.2, Vβ7 or Vβ2 with Vα14*i*, and are autoreactive for the MHC I-like molecule CD1d [Bendelac *et al* 1995]. In humans, this region is referred to as Vα24-Jα15 [Spada *et al* 1998, Brossay *et al* 1998]. Vα14*i* NK T cells have unique properties compared to other NK and NK T cell subpopulations, in that they are capable of producing large quantities of both IFN-γ and IL-4 [Yoshimoto *et al* 1995, Chen *et al* 1997].

4.7.1 Regulation of responses by NK T cells

Increasing evidence suggests that NK T cells are essential not only for defence against pathogens, but also for the regulation of adaptive immune responses. NK T cells do this by producing specific cytokines after exposure to Ags [Medzhitov *et al* 1998]. Upon stimulation, NK T cells rapidly produce substantial amounts of IL-4. For example, injection of mice with both anti-CD3 mAb and Staphylococcal enterotoxin B induces IL-4 production from CD4+ NK1.1+ cells in the spleen [Yoshimoto *et al* 1994]. An increased production of IL-4 can be beneficial for certain diseases. For example, the high susceptibility of SJL/J mice to EAE, which is characterized by inflammation and demyelination in the

central nervous system following immunization with myelin Ag MPB, correlates with a fewer number of IL-4 secreting NK T cells [Yoshimoto *et al* 1995].

The regulation of type-1 response by NK T cells, possibly through the secretion of IL-4, is highlighted by the alteration in the numbers or function of NK T cells in human systemic sclerosis [Sumida *et al* 1995], insulin-dependent diabetes mellitus [Wilson *et al* 1998], and spontaneous autoimmune diseases in mice [Mieza *et al* 1996, Baxter *et al* 1997, Hammond *et al* 1998]. In addition, mouse models with alterations in the level or function of NK T cells are important in defining the role of these cells in susceptibility or resistance to various infectious diseases and autoimmune disorders, such as EAE or type I diabetes [Baxter *et al* 1997, Hammond *et al* 1998]. Indeed, adoptive transfer of NK T cell rich populations to NOD mice protects against the development of disease conditions mediated by type-1 responses such as in diabetes. Activation of Vα14*i*+ NK T cells has also bee observed to protect against type-1 mediated diseases [Baxter *et al* 1997, Hammond *et al* 1998, Lehuen *et al* 1998].

4.7.2 NK T cells in asthma

Based on the evidence presented above, suggesting that NK T cells are capable of inhibiting Th1 responses by IL-4 production, the role of NK T cells in allergy has been investigated. NK T cell-deficient mice (Vα14$i^{-/-}$) do not develop allergen-induced AHR, a major characteristic of asthma. This is not due to an inability of these mice to produce effective type-2 responses, since NK T cell-deficient mice that are immunized s.c. at non-mucosal sites, such as footpads, produced normal Th2-biased responses in the draining lymph nodes. AHR can be induced in NK T cell-deficient mice by adoptive transfer of tetramer-purified NK T cells that secrete IL-4 and IL-13, from the spleens of wild type mice. In addition, administration of recombinant IL-13, which directly affects airway smooth muscle cells, to NK T-cell-deficient mice also induces AHR. Thus, it appears that Vα14*i* NK T cells are crucial in the regulation and development of asthma [Akbari *et al* 2003]. Human studies support this hypothesis. Sen *et al* were the first reported a significant increase in Vα14*i* NK T cells in asthmatic patients, measured by both mRNA and Northern and Western blotting. Compared to non-asthmatic volunteers, there was a significant increase in

Vα14*i*+ NK T cells in the submucosa of patients suffering with asthma [Sen *et al* 2005]. Other groups have confirmed this observation. Akbari *et al* used CD1d-tetramers, Abs specific for NK T cells, and RT-PCR analysis for invariant TCRs, such as Vα24, to assess the frequency and distribution of NK T cells in the lungs and in the circulating blood of 14 patients with asthma. Approximately 60% of the pulmonary CD4+ cells observed in patients with asthma were not class II MHC–restricted CD4+ T cells but, rather, NK T cells. The NK T cells expressed an invariant T cell receptor and produced the type-2 cytokines IL-4 and IL-13, but not IFN-γ [Akbari *et al* 2006]. These data suggest that NK T cells play a predominant role in the immunopathology of asthma.

5. THE HYGIENE HYPOTHSIS

Asthma has been on the increase over the last few decades, nearly doubling since the 1980s. In evolutionary terms, a few decades are too short an amount of time for rises in allergic disease to be attributed solely to mutations in human genetic make up. Environmental factors must therefore play a pivotal role. The increased incidence of allergic disease in developed countries, when compared to developing countries [ISAAC *et al* 1998], suggests that changes in living conditions plays a role in this increase in allergy. The observation by Strachan that larger families had fewer allergies [Strachan *et al* 1989, Strachan *et al* 2000] became the basis for the hygiene hypothesis (See section 1.2).

There is a positive correlation between the increase in allergies and the excessive use of antibiotics, and obsessive "hygiene" [Farooqi *et al* 1998, Strachan *et al* 1996]. Whilst disinfectant usage is an important part of healthy living and improved medical care, overly stringent use of cleaning products and disinfectants reduces all microbe contact, even the harmless ones. The increased use of antibiotics and vaccinations also reduces exposure to microorganisms.

In modern society, interactions with animals have become classified as "unhygienic" and a potential source of microbes. Farming communities, where people are in close contact with animals much of the time from an early age, are therefore ideal testing grounds for studying the effect of microbe exposure on

allergy incidence. In such communities in Germany, the cowshed is part of the family home, usually with a connecting door in the kitchen. Studies within these communities showed that children who spent a lot of time in the cowshed when young had a lower incidence of allergy compared to other children in the community who were not exposed to the environment in cowsheds [Riedler *et al* 2001]. Similarly a study looking at children in the same farming communities examined the amount of endotoxin present in the children's mattresses, since endotoxin indicates bacterial presence. Again, a negative correlation was observed between allergy incidence and the amount of endotoxin found [Braun-Fahrlander *et al* 2002]. These studies both point to environmental factors, like changes in microbial contact, as a factor in the increase in allergy seen in recent decades.

The persuasive evidence from these epidemiological studies has given new impetus to the hygiene hypothesis. Over the last few years, different versions of the hygiene hypothesis have been formulated. The earliest versions suggested that a higher incidence of infection and cross infection with pathogens between individuals leads to a lower incidence of allergy [Yazdanbakhsh *et al* 2002, Strachan *et al* 1989]. A more recent interpretation suggests that our limited contact with harmless microorganisms, which we once regularly encountered, is responsible for the increase in allergies [Rook *et al* 1998]. This version has developed into the "Old Friends" Hypothesis [Rook *et al* 2003, Rook *et al* 2004].

6. "OLD FRIENDS"

The Old Friends hypothesis states that harmless microorganisms we have encountered throughout evolution have enabled our immune systems to develop mechanisms that prevent inappropriate responses to non-pathogens. Observations have been made of harmless commensal or environmental species being responsible for alleviating the symptoms of allergic or autoimmune disease. For example, germ free rats raised in sterile conditions are more prone to adjuvant arthritis than rats housed in standard conditions, where they are more likely to come into contact with non-pathogenic environmental microorganisms on a daily basis. Transfer of spleen cells from

these rats to rats in sterile conditions reduces the severity of Th1 mediated adjuvant arthritis [Moudgil *et al* 2001]. This suggests that background exposure to harmless microorganisms may affect the ability of the immune system to regulate Th1-mediated responses. Specifically studied microorganisms include some species of lactobacilli, helminths and mycobacteria [Ozedmir *et al* 2003, Schultz *et al* 2002, Wilson *et al* 2003].

Studies comparing Estonian and Swedish children in the Baltic have been carried out, investigating the effect the presence of lactobacilli in the gut has on the population. Although the populations of these countries are genetically similar, Estonia was behind the iron curtain hence less developed than neighbouring Sweden. These studies showed a higher incidence of allergy in Sweden than in Estonia. Interestingly, children from Estonia had significantly higher numbers of lactobacilli in their intestinal flora. [Sepp *et al* 1997, Bjorksten *et al* 1999]. This negative correlation between allergy and lactobacilli suggests this may be an "Old Friend", whose presence may induce tolerance against allergy. "Old Friends" have also been observed to have an affect on autoimmune disease. It has been observed that whilst colonising germ free IL-$10^{-/-}$ rats with normal pathogen-free bowel flora, leads to development of colitis, the subsequent introduction of *Lactobacillus plantarum* alleviated symptoms [Schultz *et al* 2002].

Infection with parasitic worms is rare in developed countries today, whereas it is widespread in developing countries. Most infestations are low grade and without severe consequences for the majority of infected individuals. A lower prevalence of allergies is also observed in helminth-infected populations. For example, in South America, children infected with helminths, such as *Ascaris lumbricoides* and *Trichuris trichiura,* have significantly lower skin reactivity to house dust mite Ag, compared to children who are helminth free, [Hagel *et al* 1993]. Similar findings have been recorded in children infected with *Schistosoma haematobium* and *Schistosoma mansoni* [Yazdanbakhsh *et al* 2001]. Chronic infection with helminths has been shown to be protective against allergic disease, with the protection being associated with the release of IL-10 and induction of immunoregulatory mechanisms [van der Biggelaar *et al* 2000, Yazdanbakhsh *et al* 2002, Kleij *et al* 2002, Terrazas *et al* 2001, Maizels *et*

al 2003]. Th2 mediated immunity can also be regulated by environmental microorganisms. In experimental mouse models of airway inflammation using Derp1 (house dust mite allergen) and OVA, chronic infection with *Heligmosomoides polygyrus* resulted in decreased eosinophilic inflammation. This protection was partially blocked by anti-CD25, suggesting Treg involvement [Wilson *et al* 2004, Wilson *et al* 2005].

6.1 Saprophytic Environmental Mycobacteria

Saprophytic mycobacteria are non-pathogenic and, although not part of the natural bacterial flora, are found abundantly in the environment. They consist of at least 80 species and are commonly found in soil, mud and untreated water. The lifestyle in developed countries has reduced exposure to such mycobacteria through the excessive use of concrete, and through treatment of water. Humans evolved as hunter-gatherers, and eventually became farmers. Contact with soil, and therefore saprophytic mycobacteria, was commonplace. Their presence in our evolutionary history is seen by the existence of CD1-restricted T cell subsets, which appear to only recognise mycobacterial lipids and glycolipids [Dutronc *et al* 2002]. Moreover, these lipids have been detected in human lymphoid tissue, suggesting their constant and widespread presence in human evolution [Hanngren *et al* 1987]. This thesis will focus on one species, *Mycobacterium vaccae (M. vaccae)*, which has been reported to have beneficial effects on allergic disease, supporting the "Old Friends" hypothesis.

6.2 Mycobacterium vaccae

M. vaccae is a non-pathogenic mycobacterium, first described in 1964 by Bonicke and Juhasz [Bonicke *et al* 1964]. The strain of *M. vaccae* described in this thesis is *M. vaccae* R887R, which was first isolated from samples of mud from Lake Kyoga in Uganda in 1971. Stanford and Paul first described this strain in 1973 [Stanford *et al* 1973] and deposited it, under the Budapest convention, at the National Collection of Type Culture (NCTC) in London in 1984. This particular strain of *M. vaccae* is acid fast (stains purple/red when stained by the Ziehl-Neelsen method), is a fast grower (with an optimal

temperature of 28-33°C in aerobic conditions), and produces a bright yellow-orange pigment when grown in the dark.

There are only 4 known reports of *M. vaccae* causing disease, all in the Southern United States of America and all in immunosuppressed individuals. One patient had a cutaneous infection, and three patients had cavitary lung disease. All four patients responded to treatment with minocycline or ciprofloxacin [Hachem *et al* 1996], broad-spectrum antibiotics.

The potential of *M. vaccae* as an immunotherapy for allergic asthma has been suggested, both from human clinical trials [Arkwright *et al* 2001, Camporota *et al* 2003] and from experimental animal models [Smit *et al* 2003a, Smit *et al* 2003b, Zuany-Amorim *et al* 2002a, Walker *et al* 2003]. Clinical trials in adults with grass pollen-induced asthma and rhinitis have shown that treatment with heat killed *M. vaccae* leads to fewer symptoms and less need for relief medication [Hopkins *et al* 2001]. Experiments using mouse models that develop AHR and pulmonary inflammation have been undertaken by several groups [Zuany-Amorim *et al* 2002a, Zuany-Amorim *et al* 2002b, Smit *et al* 2003a]. The overwhelming evidence suggests that *M. vaccae* is effective at ameliorating type-2 mediated airway inflammation and AHR [Zuany-Amorim *et al* 2002a].

7. HYPOTHESIS

7.1 Introduction

M. vaccae, a harmless "Old Friend" which has been present throughout human evolution, has been shown to have an immunotherapeutic effect against allergic diseases in animal models [Hopfenspirger *et al* 2002, Hopkins *et al* 2002, Zuany-Amorim *et al* 2002a, Smit *et al* 2003a, Smit *et al* 2003b, Ozdemir *et al* 2003]. In the case of allergic pulmonary inflammation, treatment with heat killed *M. vaccae* leads to decreased cellular infiltration into the lungs, reduced allergen-induced eosinophilia and an increase in IL-10 protein in the BAL fluid [Zuany-Amorim *et al* 2002a, Adams *et al* 2004]. IL-10 is associated with immunoregulation, and more recently with the development and function of

Tregs [Umetsu *et al* 1999, Mason *et al* 1998, Bach *et al* 2001]. *M. vaccae* has been shown to induce Tregs with a CD4+CD45RB^{Low} phenotype, similar to Tregs described as Tr1 cells [Powrie *et al* 1994, Groux *et al* 1997]. When adoptively transferred to allergic mice, these CD4+CD45RB^{Low} Tregs suppress pulmonary inflammation [Zuany-Amorim *et al* 2002b].

Regulatory APCs have been described which play a pivotal role in regulation of immune responses. Some secrete IL-10 [Tormey *et al* 1998] and some TGF-β [Terrazas *et al* 2001], both cytokines known to induce Tregs. IL-10 secreting DCs have even been shown to suppress T cell proliferation in certain conditions [Hawiger *et al* 2001, Liu *et al* 2002], an effect that has been proved to be transferable when such DCs are introduced into recipient animals sensitized and challenged with OVA [Akbari *et al* 2002, Akbari *et al* 2001].

7.2 Aim of thesis

This thesis investigates several aspects of the effect of *M. vaccae* on pulmonary inflammation. First, it aims to ascertain whether a decrease in the type-2 cytokines can be detected at the site of pathology, the BAL fluid and lung tissue. To determine whether a Th2 to Th1 switch occurs, type-1 responses in the lungs will be studied. This thesis also investigates whether immunoregulatory cytokines are secreted. Since Treg development is dependent on IL-10 and TGF-β [Zuany-Amorim *et al* 2002b], the source of any immunoregulatory cytokines is investigated. It is determined whether treatment with *M. vaccae* induces pulmonary CD11c+ APCs, which secrete immunoregulatory cytokines. These cells are also investigated for their cell surface marker phenotypes before and after *M. vaccae* treatment. The possible regulatory role of the pulmonary APCs is then investigated both *in vivo* and *in vitro*.

7.3 Summary

In summary, the aims of this thesis are to determine:

- Whether *M. vaccae* treatment of allergic mice reduces type-2 cytokine production at the site of inflammation. If so, whether an increase in type-1 cytokine expression is observed (Chapter 3)
- Whether *M. vaccae* treatment of mice with allergic pulmonary inflammation induces increased expression of IL-10 and TGF-β at the site of inflammation (Chapter 3)
- What the possible source of any immunoregulatory cytokines may be (Chapter 4)
- Whether *M. vaccae*-induced CD11c+ cells have the ability to regulate immune responses, and what role they play in alleviating allergic symptoms (Chapter 5)

Table 1-1 Types of hypersensitivity

TYPE	DETAILS	EXAMPLE
I	Immediate hypersensitivity, involves production of IgE against foreign Ag	Allergy, like asthma
II	Caused by production of IgG or IgM antibodies against cell surface antigens. Damage is therefore restricted to specific cells types	Haemolytic disease in newborn babies, where the mothers IgG antibodies have reacted against the babies erythrocytes in the uterus
III	Occurs when there is an excessive build up of immune complexes in circulation. Immune complexes are formed every time antibody meets antigen and are cleared up by macrophages or similar cells. If they cannot be cleared by the phagocytic system, they deposit in tissue or organs, and cause damage	SLE (Systemic Lupus Erythematosus)
IV	Takes more than 12 hours to develop, and involves cell mediated immune reactions, rather than antibody reactions to antigens	Contact hypersensitivity, characterized by an eczematous reaction at the point of contact to an allergen, like nickel

TABLE 1-3 HISTORY OF ALLERGY

Although a 21st Century epidemic, allergy is not a new disease. One of the earliest reports of an allergic reaction was recorded between 3640 and 3300BC, when King Menses of Egypt died from a wasp sting. The documented history of allergy is summarised in Table 1-3.

YEAR	EVENT	DETAILS
1819	Hay fever first described	Dr John Bostock described "seasonal Catarrh", that affected the upper respiratory tract. Today this would be called hay fever.
1873	First skin prick test	Charles Blakley performed the first skin prick test, by applying pollen through a break in the skin. This introduced the concept that pollen caused hay fever.
1902	Anaphylaxis first described	Charles Richet and Paul Portier first used the word "anaphylaxis" to describe the life threatening response observed to certain protein substances.
1906	The word "allergy" first used	Austrian Pediatrician Clemens von Pirquet first used the word 'allergy' to describe the strange, non-disease related symptoms that some diphtheria patients developed when treated with a horse serum antitoxin. The word comes from the Greek word 'alol', meaning, 'change in the original state.'
1911-1914	Immunotherapy established	Leonard Noon and John Freeman helped established the basis for immunotherapy or allergy shots, which involves injecting the allergy sufferer with small amounts of allergen. Over time, the body is supposed to become less sensitive to the allergen.

1919	Transfer of allergy first described	A physician observed, following a blood transfusion, a case of transient asthma caused by allergy to horse dander. This was the first indication of a factor in blood capable of mediating an allergic reaction.
1921	Passive transfer of allergy	Prausnitz & Küstner performed the passive transfer of allergy. Both were allergic to different allergens, and by injecting themselves with small amounts of each other's blood showed sensitivity to particular substances could be passed from one person to another through blood serum.
1923	The term "Atopy" first used	Coca and Cooke first used the term "atopy" to describe genetically inherited forms of allergy.
1937	First antihistamine drug synthesized	Daniel Bovet synthesized the first antihistamine drug. He and his colleagues discovered antihistamines, in blocking the effects of the chemical histamine also protected against some of the symptoms of anaphylaxis.
1953	Mast cell discovered	James F. Riley and Geoffrey B. West discovered the mast cell to be the major source of histamine in the body.
1967	IgE discovered	Kimishige and Teruko Ishizaka further explained the allergy process by discovering the role of IgE class Abs as the principal mediator in the allergic reaction.

Chapter 2

In this Chapter, all chemicals, equipment and supplies are ordered from the companies listed in Table 2-1.

Table 2-1 List of companies and addresses

COMPANY	ADDRESS
Amersham Pharmacia Biotec.	Uppsala, Sweden
Applied Biosystems.	Warrington, GB
Becton Dickinson.	Mountainview, USA
Bio-Tek Instruments Inc.	East Sussex, GB
Boehringer.	Bracknall, GB
Eurogentec.	Romsey, GB
Gibco-BRL.	Division of Invitrogen, Paisley, GB
Harlan.	Oxfordshire, GB
Helena Biosciences.	Sunderland, GB
Invitrogen.	Paisley, GB
Millipore.	Watford, GB
Miltenyi Biotec.	Bergisch-Gladbach, Germany

Molecular probes.	Leiden, The Netherlands
Pharmingen.	Div. of Becton Dickinson, Mountainview, USA
Promega.	Southampton, GB
Qiagen.	Crawley, GB
R&D Systems.	Oxfordshire, GB
Serotec.	Oxfordshire, GB
Serva.	Heidelberg, Germany
Shandon Scientific.	Cheshire, GB
Sigma.	Dorset, GB
SR Pharma.	London, GB
Verity Software House Inc.	Topsham, USA

Chapter 2

Materials & Methods

1.1 Animals

All experiments used female BALB/c mice, 6-8 weeks old, which were purchased from Harlan. These were housed under standard conditions. All experimental protocols complied with the Home Office 1986 Animals Scientific Act.

1.2 Antigens and *M. vaccae* suspensions

OVA (chicken egg white) was obtained from Sigma (Grade V). Sterile vials of heat-killed suspension of *M. vaccae* (10mg/ml in saline) were provided by SR Pharma.

1.3 Injection of naïve mice with *M. vaccae*

Naïve mice were treated on day 0 with *M. vaccae* s.c. (0.1mg in 100µl saline) or saline alone. Mice were sacrificed at days 1, 8 and 21 by CO_2 and the spleens removed and snap frozen in liquid nitrogen. Spleens were stored at -20°C until analysed by RT-PCR.

1.4 Murine model of allergic pulmonary inflammation

1.4.1 Preventive Protocol

Mice were treated s.c. with either *M. vaccae* (0.1mg in 100µl saline) or with saline alone on day –21. All mice were sensitized i.p. on days 0 and 12 with OVA (10µg in 100µl Alum; Serva) and challenged i.t. under light halothane anaesthesia on days 19 and 21 with either saline (negative control) or OVA

(0.5µg in 50µl). Animals were sacrificed 24 or 48 hours later by i.p. injection of sodium pentobarbitone (240mg/kg) (Figure 2-1 a).

1.4.2 Therapeutic Protocol

Mice were sensitized on days 0 and 12 with OVA (10µg in 100µl Alum). On day 21 mice were injected s.c. with either *M. vaccae* (0.1mg in 100µl saline) or saline alone. Mice were then sensitized again on days 42 and 54. Mice were challenged under light halothane anaesthesia on days 61 and 63 i.t. with either saline (negative control) or OVA (0.5µg in 50 µl) and sacrificed 24 or 48 hours later by i.p. injection of sodium pentobarbitone (240mg/kg) (Figure 2-1 b). This protocol goes some way to mimic clinical asthma, so it is essential that the treatment in the therapeutic protocol is as effective as in the preventive protocol.

1.4.3 BAL fluid collection

The trachea was cannulated and the BAL fluid obtained by washing the lungs thrice with 0.3ml of Wash Medium (Table 2-2). Recovered BAL fluids were kept on ice and processed immediately when all samples were collected. The number of cells in the BAL fluid was determined using a Neubauer Chamber. Cytospins of each BAL fluid were performed. 50µl of BAL fluid was spun at 700rpm for 5 minutes (Shandon Scientific). Slides were fixed in acetone for 2 minutes, then stained with filter sterilised Wright Giemsa stain (Sigma) for 4 minutes. Slides were washed in 1 x PBS once and distilled water twice for 30 seconds before the coverslip was applied. A differential cell count was performed using standard morphological criteria. The remaining BAL fluid was frozen and kept to determine cytokine levels by ELISA.

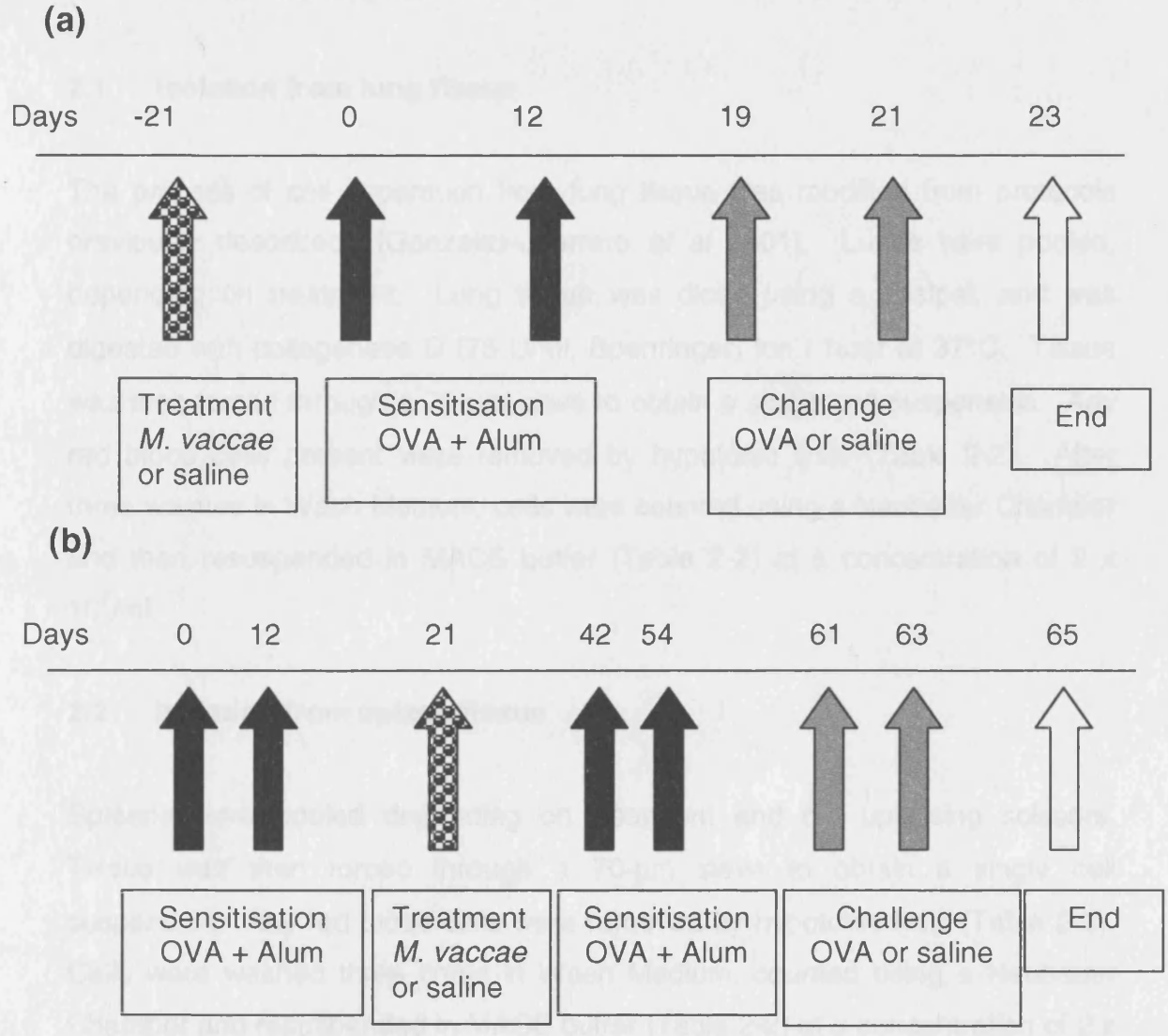


Figure 2-1: **Allergy protocols**. To determine whether treatment with *M. vaccae* is able to prevent the development of symptoms associated with allergic pulmonary inflammation, naïve mice were treated with *M. vaccae* and subsequently sensitized and challenged with OVA. This preventive protocol is schematically represented in (a). To determine whether treatment with *M. vaccae* is able to alleviate symptoms associated with allergic pulmonary inflammation, OVA sensitized mice were treated with *M. vaccae* and, following a second sensitisation, challenged with OVA. This therapeutic protocol is schematically represented in (b).

2 CD11c+ cell separation

2.1 Isolation from lung tissue

The process of cell separation from lung tissue was modified from protocols previously described [Gonzalez-Juarrero *et al* 2001]. Lungs were pooled, depending on treatment. Lung tissue was diced using a scalpel, and was digested with collagenase D (75 U/ml, Boehringer) for 1 hour at 37°C. Tissue was then forced through a 70-µm sieve to obtain a single cell suspension. Any red blood cells present were removed by hypotonic lysis (Table 2-2). After three washes in Wash Medium, cells were counted using a Neubauer Chamber and then resuspended in MACS buffer (Table 2-2) at a concentration of 2 x 10^7/ml.

2.2 Isolation from spleen tissue

Spleens were pooled depending on treatment and cut up using scissors. Tissue was then forced through a 70-µm sieve to obtain a single cell suspension. Any red blood cells were removed by hypotonic lysis (Table 2-2). Cells were washed three times in Wash Medium, counted using a Neubauer Chamber and resuspended in MACS buffer (Table 2-2) at a concentration of 2 x 10^7/ml.

2.3 Magnetic cell separation using MACS beads

Cells were resuspended in MACS buffer (Table 2-2) and incubated for 15 minutes at 4°C with anti-mouse CD11c coated microbeads, clone N418 (Miltenyi Biotec). The N418 mAb is a clone for CD11c, which has been widely used as a marker for DCs [Kelsall *et al* 1996, Marriott *et al* 1999, Williamson *et al* 2002]. After careful washing, 1 x 10^7 cells were resuspended in 500µl of MACS buffer, and applied onto the MACS MS+ separation column (Miltenyi Biotec). The column was washed three times with 500µl of MACS buffer (Table 2-2) and the flow through collected. This fraction contained CD11c- cells. The column was removed from the magnet and cells

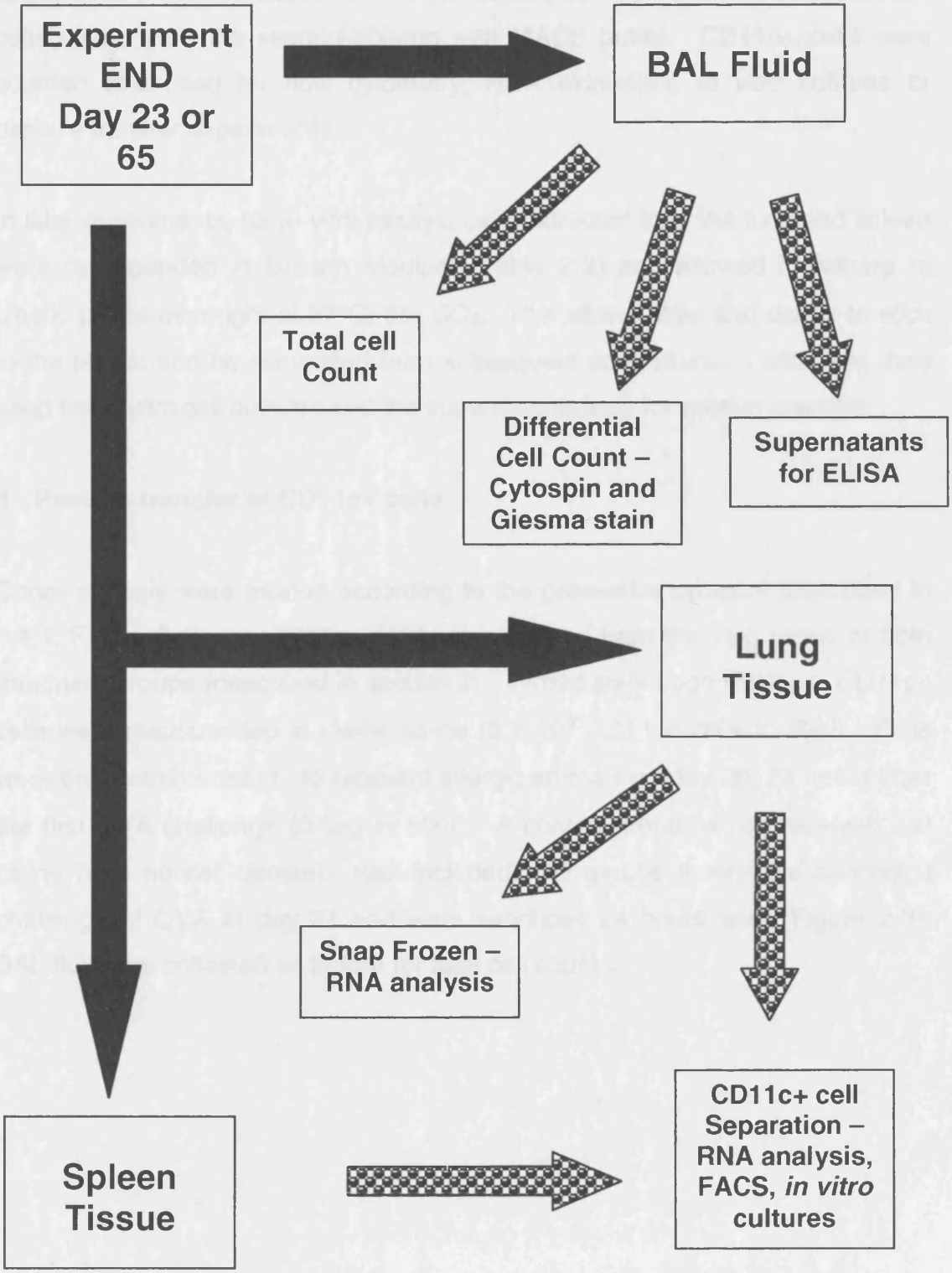


Figure 2-2: Sample Collection. Upon termination of murine allergic pulmonary inflammation protocol, mice were sacrificed and BAL fluid, lung tissue and spleen tissue were taken for further analysis. From the BAL fluid, total and differential cell counts were determined and supernatants kept for cytokine analysis. Lung tissue was frozen for RNA analysis, and CD11c+ cells were isolated from harvested lung and spleen tissue. CD11c+ cells were then used for RNA analysis, FACS staining and for setting up *in vitro* cultures.

eluted onto a second column. The cell separation was repeated and CD11c+ cells eluted from the second column with MACS buffer. CD11c+ cells were counted and used for flow cytometry, RNA extraction, *in vitro* cultures or passive transfer experiments.

In later experiments, for *in vitro* assays, cells extracted from the lung and spleen were resuspended in Growth Medium (Table 2-2) and allowed to adhere to plastic plates overnight at 37°C, 5% CO_2. This allows MΦs and debris to stick to the plastic and be eliminated from subsequent cell culture. Cells were then used for *in vitro* cell cultures and the supernatants kept for protein analysis.

3 Passive transfer of CD11c+ cells

Donor animals were treated according to the preventive protocol (described in 1.4.1, Figure 2-1) and CD11c+ cells were isolated from the lung tissue of both treatment groups (described in section 2). Immediately upon isolation, CD11c+ cells were resuspended in sterile saline (2×10^5 CD11c+ cells in 50µl). Cells were then transferred i.t., to recipient allergic animals on day 20, 24 hours after the first OVA challenge (0.5µg in 50µl). A control group, which received just saline (with no cell transfer), was included. All groups received a second i.t challenge of OVA at day 21 and were sacrificed 24 hours later (Figure 2-3). BAL fluid was collected as before for total cell counts.

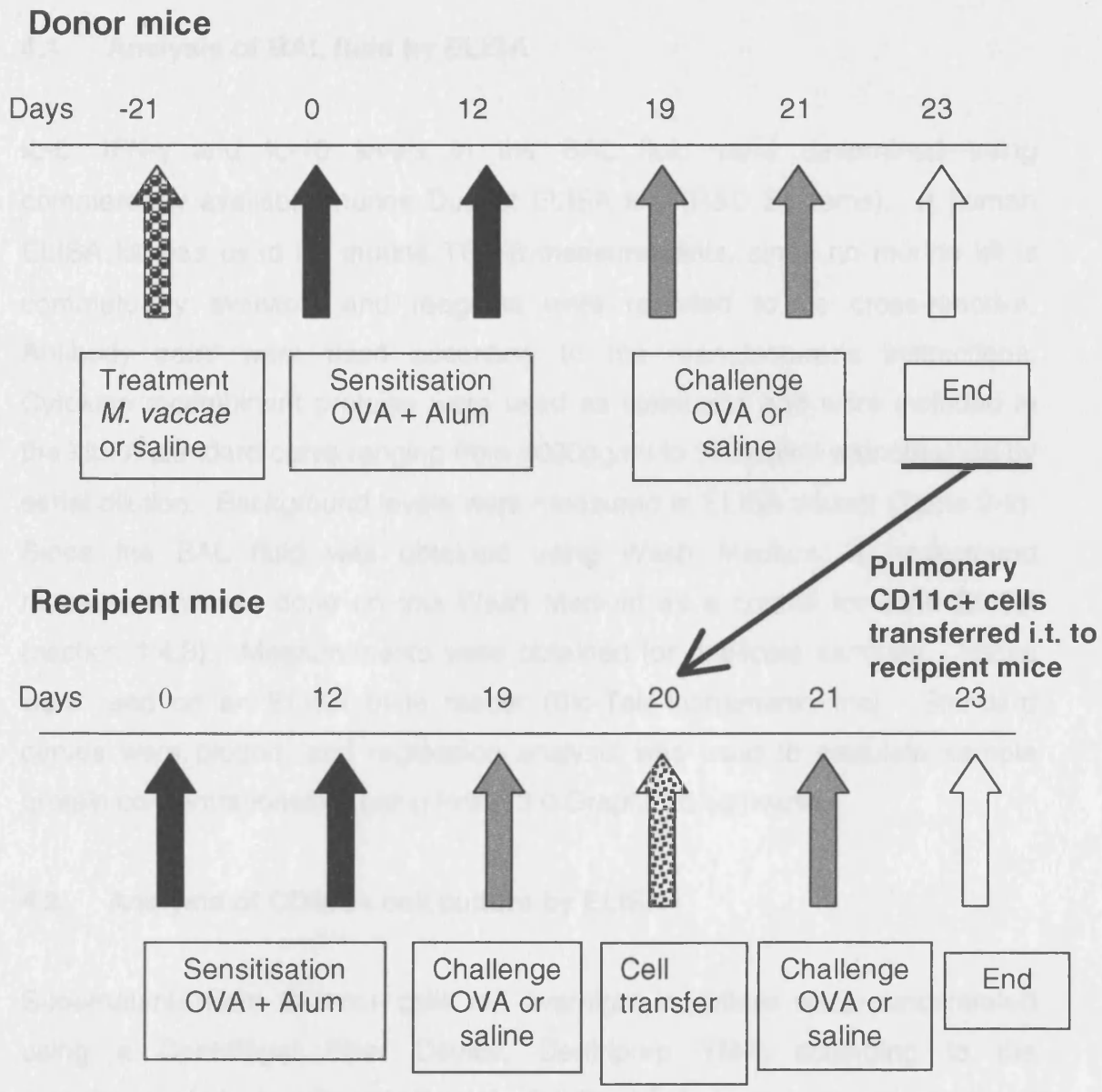


Figure 2-3: Passive transfer experiment. Donor mice were treated with either *M. vaccae* or saline using the preventive protocol as described previously. CD11c+ cells were isolated from the lungs of donor mice and transferred to OVA sensitized recipient animals. Recipients were challenged with OVA a second time the following day and sacrificed on day 23. BAL fluid was obtained for cell counts.

4 Protein analysis

4.1 Analysis of BAL fluid by ELISA

IL-5, IFN-γ and IL-10 levels in the BAL fluid were determined using commercially available murine Duoset ELISA kits (R&D Systems). A human ELISA kit was used for murine TGF-β measurements, since no murine kit is commercially available and reagents were reported to be cross-reactive. Antibody pairs were used according to the manufacturer's instructions. Cytokine recombinant proteins were used as standards and were included in the kit. A standard curve ranging from 4000pg/ml to 32.5pg/ml was obtained by serial dilution. Background levels were measured in ELISA diluent (Table 2-2). Since the BAL fluid was obtained using Wash Medium, a background measurement was done on this Wash Medium as a control for each ELISA (section 1.4.3). Measurements were obtained for duplicate samples. Plates were read on an ELISA plate reader (Bio-Tek Instruments Inc). Standard curves were plotted, and regression analysis was used to calculate sample protein concentrations/ml (using Prism 3.0 GraphPad software).

4.2 Analysis of CD11c+ cell culture by ELISA

Supernatants from CD11c+ cells left overnight in culture were concentrated using a Centrifugal Filter Device, Centriprep YM-3 according to the manufacturer's instructions (Millipore). Briefly, 15ml of supernatant was placed into the Centriprep tube and the filtrate collector was placed on top of the supernatant before the lid was secured. The device was centrifuged at 3000 xg for 95 minutes. The filtrate was then decanted off, and the device centrifuged for a second time, at 3000 xg for 35 minutes. The filtrate was decanted off, and the concentrated supernatant which was left in the collector (approximately 900μl) was aliquoted into eppendorfs and stored at –20°C. ELISA was carried out using commercially available Duoset ELISA kits (R&D Systems) for IL-10 and TGF-β as described in section 4.1, according to the manufacturer's instructions.

4.3 Analysis of *in vitro* culture by ELISA

IL-4, IL-5, IFN-γ, IL-10 and TGF-β levels from *in vitro* cultures (section 7) were determined using commercially available Duoset ELISA kits (R&D Systems) as described in section 4.1, and according to the manufacturer's instructions.

5 mRNA analysis

5.1 RNA extraction

RNA was extracted using a commercially available kit according to the manufacturer's instructions (RNeasy mini kit, Qiagen). Flash frozen lung and spleen tissue was homogenized using a handheld homogenizer and placed through an RNA-binding column. Pulmonary CD11c+ cells and *in vitro* culture cells were placed directly through a shredder column before being passed through an RNA binding column. Between wash steps, the RNA was DNase treated (Qiagen) for 15 minutes to remove any contaminating genomic DNA. RNA was eluted in nuclease free water.

5.2 cDNA conversion

RNA was converted into cDNA using the Omniscript kit according to the manufacturer's instructions (Qiagen) with Oligo dT and RNasin (Promega). Briefly, 15µl RNA was added to 2µg of Oligo dT, 1x RT buffer, 5mM of each dNTP, 10 Units of RNasin, 4 Units of Omniscript Reverse Transcriptase and RNase free water and vortexed. Samples were incubated at 37°C for 1 hour, followed by 5 minutes at 93°C to denature the enzyme. To ensure reagents were not contaminated with exogenous RNA, which would give false positive results, a negative control (sample not containing any RNA) was included in every reverse transcription. cDNA was stored at -20°C before PCR amplification.

5.3 Basic end point PCR

Basic end point PCR is a qualitative method, which amplifies a DNA sequence between two specific primers. In this thesis, it was initially used to determine whether designed primers were amplifying the correct sized PCR product (amplicon). This method of PCR was later used to create standard curves for each primer set, explained in section 6.2 and 6.3.

The 50µl PCR reaction consisted of 25µl of 2 x HotstarTaq Master Mix (Qiagen, Table 2-2), 1µl each of forward and reverse primer (10pmol/µl each; Eurogentec), 22µl RNase free water (Promega) and 1µl cDNA template. Reactions were carried out in a Phoenix thermocycler (Helena Biosciences). Thermocycling conditions were as follows: 95°C for 15 minutes (to activate enzyme), followed by 25-35 cycles of (95°C/20 seconds, Tm°C/20 seconds, 72°C/30 seconds).

Optimal Tm and amplicon size for each primer set can be found in Table 2-3. In order to avoid false positives, negative controls were included in all PCR experiments (sample containing no cDNA) to ensure PCR reagents were not contaminated with exogenous DNA. In order to confirm both presence and size of the PCR amplicon, PCR reactions were run on an agarose gel (Figure 2-7).

5.4 Agarose gel electrophoresis

To visualise the amplicon obtained by end point PCR, the reaction was run on an agarose gel with SYBR® Green I nucleic acid stain, which binds only to double stranded DNA. Amplicons were then visualised using the FLA3000 scanner from Fuji film (Laser 473nm, filter Y520).

Electrophoretic grade agarose (Gibco-BLR) was dissolved in 1 x TAE (Table 2-2) by boiling in a microwave (1.8% final concentration). Stock solution of SYBR® Green I nucleic acid stain (Molecular probes) was added to the agarose solution at a 1:10,000 dilution. The gel was run at 120-150 constant voltage in

an appropriate horizontal gel tank (Amersham Pharmacia Biotec) with 1 x TAE as a running buffer.

PCR products from end point PCR reactions were mixed with 10 x Blue Juice loading dye (1:10 dilution; Invitrogen) and loaded onto the 1.8% agarose gel along with 1µl of appropriate DNA ladder. A 50bp ladder (Gibco-BRL) was used to check primer function. When appropriate, a low DNA Mass Ladder ranging from 500ng to 6.25ng/µl (Invitrogen) was used to establish PCR amplicon copy number of a PCR reaction (referred to in detail in section 6.3). The amount of DNA in each band on the gel was calculated using the AIDA software and the low DNA Mass Ladder. These amplicons were then used to create standards, of known concentration for each specific primer set (see section 6.3). These standards were used to quantify samples using real-time PCR.

5.5 Quantitative real-time PCR

5.5.1 The theory behind real-time PCR

Real-time PCR was used as a quantitative technique. Like basic end point PCR, real-time PCR amplifies a DNA sequence between two specific primers (Figure 2-4). However, instead of having to visualise the PCR product on an agarose gel after amplification, real-time PCR relies on the detection of a fluorescent signal after each cycle by a system of lenses, filters and a camera, set up in the ABI7000. This signal is proportionate to the amount of PCR product that has been amplified.

In the ABI7000 real-time PCR machine, filters separate the light emission based on wavelength, which is picked up by a charge-coupled device camera capable of detecting these wavelengths. The quantity of starting template is established using a threshold cycle. The threshold cycle is defined as the number of cycles that have elapsed before fluorescence levels are detectable. This occurs when the PCR amplification is in its Exponential Phase. Therefore, the greater the quantity of DNA in the sample, the lower its Ct value will be (the cycle number at which the PCR reaction amplifies over the threshold). The ABI software

uses the Ct values against a set of standards with known DNA concentrations to calculate the quantity of unknown samples, using their Ct values.

5.5.2 Real-time Vs end point PCR

Real-time PCR has several advantages over basic end point PCR. Not only is it less time consuming, as there is no need to run an agarose gel, but also real-time PCR has increased sensitivity. Real time PCR can detect differences in DNA presence which end point PCR fails to do. This is mainly because end point PCR is usually analysed after the Plateau Phase has begun. PCR is made up of three stages; the Exponential Phase, where the product is doubling every cycle; the Linear Phase, when the reaction is starting to slow due to the consumption of the reaction components; and the Plateau Phase, when no more product is being amplified. This is the Phase in which most end point PCR products are visualised on gels. Different reactions will reach the Plateau Phase at different rates, depending on the amount of DNA in the sample. However, after a certain number of cycles, all reactions will have reached this phase. The time it has taken to reach the Plateau Phase cannot be determined from the band visualised on an agarose gel. Since all the reactions would have reached the Plateau Phase, the intensity of all the bands will appear the same, even though they reached that phase at different times, depending on the DNA concentration in the sample. Therefore, quantitative analysis of PCR amplification is inaccurate when done by end point PCR. Real-time PCR uses the Exponential Phase of PCR amplification to quantify DNA present in the reaction. A quantitative relationship exists between the amount of DNA in the starting reaction and the PCR cycle at any given time. Real-time PCR detects the accumulation of an amplicon during the reaction, measuring fluorescence during the Exponential Phase. This makes DNA quantification by real-time PCR much more sensitive and accurate than by end point PCR (Figure 2-4).

In these experiments, SYBR® Green I was used as a fluorescence signal. This dye non-specifically binds to double stranded DNA, and therefore will not detect single stranded cDNA or oligonucleotides (like primers), but only amplified PCR product. However, the disadvantage with using SYBR® Green I is the possibility

of non-specific binding. Any double stranded DNA, such as primer dimers, will give off a fluorescent signal. To overcome these problems, after every real-time PCR a dissociation melting curve was carried out. A dissociation-melting curve is obtained from post-PCR product by ramping the PCR reaction from 60°C to 95°C. Fluorescence is recorded after each increase in temperature. At a low temperature, the PCR DNA product is double stranded, so binds SYBR Green, which fluoresces. With increasing temperature, the DNA product denatures, becoming single stranded, releasing SYBR Green and the fluorescent signal decreases. The temperature at which this occurs is dependent on the length of the DNA fragment and its GC% content. The graphs are plotted as a first derivative, so the inflection point in the melting curve then becomes a peak. Single peaks indicate a single product and multiple peaks usually indicate multiple products. These other products can have many sources including primer dimers and genomic DNA contamination. The temperature at which a peak is observed enables the size of the PCR product to be estimated. For example, primer dimers will anneal together at a much lower temperature than a 200bp amplicon. This protocol allows PCR reactions where primer dimers or non-specific binding contribute to the fluorescent signal to be identified (Figure 2-5).

5.6 Real-time PCR protocol

Standard curves for real-time PCR were prepared using PCR amplicons. A basic end point PCR was set up as described (sections 5.3 and 5.4), using the primers for the gene of interest and cDNA of a sample known to express that gene.

PCR reactions were analysed on the ABI7000 real-time PCR machine in PCR microtitre plates, sealed with plastic covers and a compression pad to prevent any evaporation (all Applied Biosystems). Plates were set up to include a standard curve (section 6) for each primer set. Negative controls for both the PCR reaction (with no cDNA added to the PCR), and the cDNA conversion (no RNA added to the RT reaction - section 5.1) were also included on each plate. The standards acted as a positive control for each plate, as they were known to

contain the amplicon. Samples and standards were plated in at least duplicate, and values averaged.

The reaction consisted of 12.5µl of SYBR® Green PCR Master Mix (containing 1.25U of HotStarTaq DNA Polymerase in 1x Qiagen buffer containing 200µM of each dNTP, Tris Cl, KCl, $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, and SYBR® Green I and ROX fluorescent dyes - Qiagen) and 1µl each of the forward and reverse primer (Eurogentec; 10 pmol/µl). Varying amounts of nuclease free water (Promega) were used, depending on how much cDNA was added to the reaction, to make the total volume of each reaction 25µl. Variable amounts of cDNA sample were added to each well, depending on the expression level of the gene of interest. The variable amount of cDNA used was taken into account when calculating copy numbers. A total volume of 25µl was selected. This volume is preferable to 50µl since less time is required to heat the smaller amount of liquid to the correct temperature.

Thermocycling conditions were as follows: 95°C for 15 minutes (to activate enzyme), followed by 35-45 cycles of (95°C/20 seconds, Tm°C/20 seconds, 72°C/30 seconds). A dissociation melting curve was performed at the end of each reaction, to ensure a single amplicon was amplified and to identify any fluorescence due to primer dimers. The cycle number at which each standard, each with a known concentration of DNA, came over the threshold (Ct value) was used to create a standard curve (ABI7000 software). Ct values obtained from samples were used to determined copy number/µl of cDNA sample by extrapolation using linear regression analysis.

The housekeeping gene GAPDH was used as an internal reference. A housekeeping gene is a gene that codes for an essential protein, expressed in all cells. This method of normalising a PCR product against the PCR product for a housekeeping gene assumes that the expression does not change, after treatment or between individual samples. Therefore the amount of housekeeping cDNA detected in a PCR reaction is an indication of the amount of cDNA present in the reaction. In this thesis GAPDH primers were run on every sample.

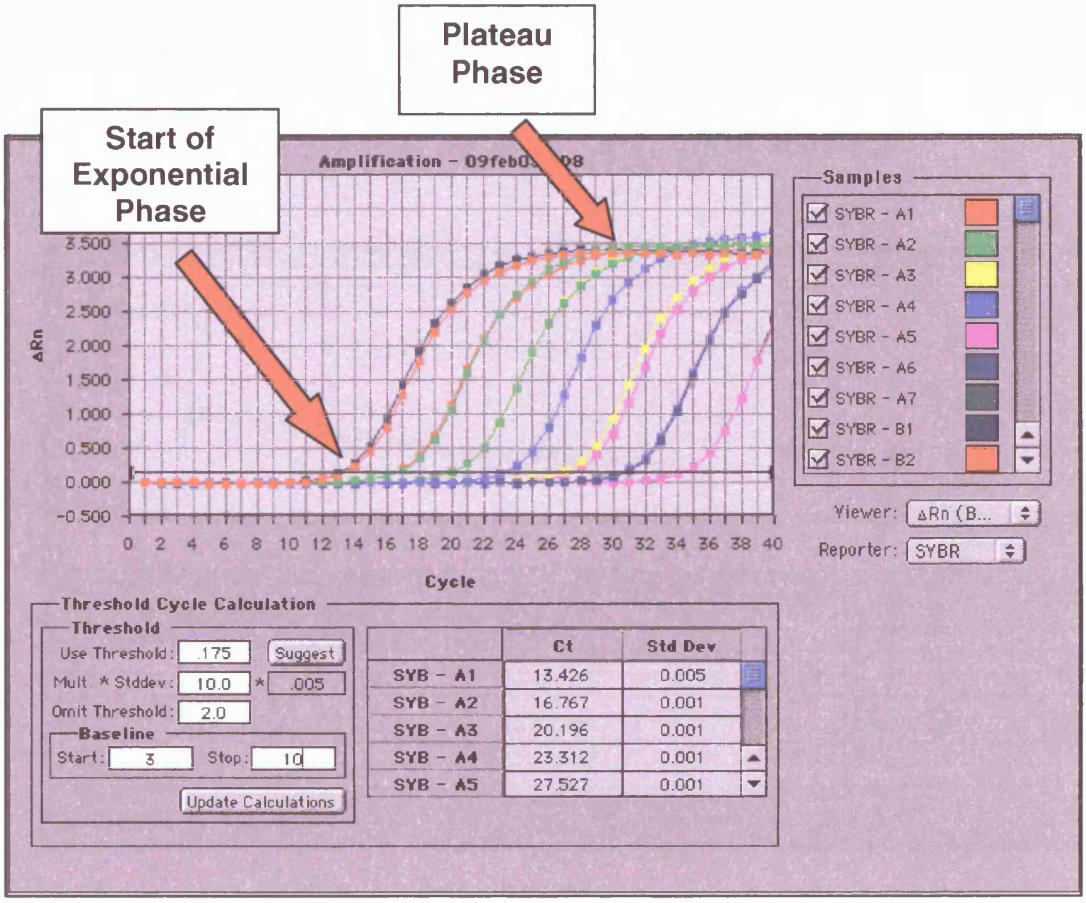


Figure 2-4: The advantage of Real-time PCR. Real-time PCR measures DNA amplification at the Exponential Phase. This makes real-time PCR more sensitive. With end point PCR, the amplicon is visualised on a gel at the end of a number of cycles. Usually the reaction would have reached the Plateau Phase, regardless of how much DNA was present in the sample. For example, in this Figure five out of seven samples emit the same fluorescent signal at cycle 40, even though they obviously reached the Plateau Phase at different cycles. The amount of DNA present in a reaction is proportional to the cycle number that the Exponential Phase starts. Since the cycle when the Exponential Phase occurred cannot be determined from the band on a gel, the amount of DNA present in a sample cannot be determined accurately. All reactions, although having reached the Plateau Phase at different rates, will have similar band intensity when run out on an agarose gel. This can also be seen on a real-time PCR graph, where each plotted line (individual reactions) has a similar fluorescence intensity (y axis) at the Plateau Phase, even though each has a significantly different amplification rate in the Exponential Phase. Visualising the Exponential Phase makes real-time PCR a sensitive and accurate technique, since there is a quantitative relationship between the amount of DNA in the starting reaction and the fluorescent signal detected at any given PCR cycle.

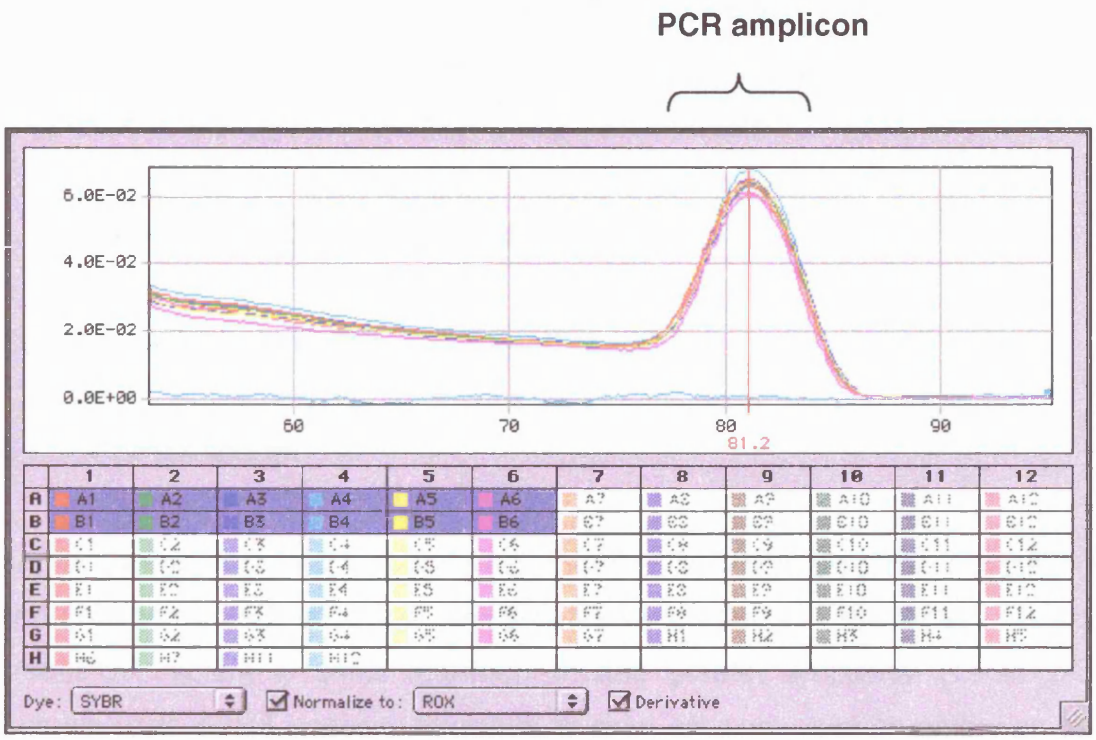


Figure 2-5: Dissociation melting Curve. SYBR® Green I is a dye which non-specifically binds to double stranded DNA. The disadvantage of using a fluorescent dye with non-specific properties is the possible incorporation of primer dimers and non-specific amplification into the fluorescent signal. These are identified by carrying out a dissociation melting curve. The post-PCR reaction is ramped from 60°C to 95°C. A sudden decrease in the fluorescence is observed when two complementary DNA strands denature. The larger the DNA fragment, the higher the temperature it will be when the PCR product denatures. For example, primer dimers will have a lower denaturing temperature than a 200bp amplicon, and their presence can be identified.

In Chapters 3 and 4, the PCR data were generated by creating a standard curve using an initial arbitrary number for the most concentrated point, and then serially diluting it two-fold. This allowed a linear regression equation to be obtained. The Ct values from each sample were then used in this linear regression calculation. Although not representing actual copy numbers, the shape of the final graph is identical using both methods.

6. Primers and DNA quantification

See Table 2-3 for primer sequences and conditions.

6.1 Primer design

Primers were designed using Primer Express software (Applied Biosystems) and obtained from Eurogentec. All primers were designed specifically for real-time PCR, where a small amplicon means greater efficiency (Table 2-3). Primers were cDNA specific, designed to span an exon-exon boundary so that genomic DNA would not be amplified (Figure 2-6). This was an additional precaution, since all RNA extracted had already been treated with DNase to eliminate any genomic DNA contamination in the samples.

6.2 Standard curve for cDNA quantification using real-time PCR

To quantify all PCR reactions, standards for each primer set were made. An amplicon for each gene of interest was obtained from cDNA known to express the gene of interest. After amplification by end point PCR, 5µl of the PCR reaction was run on a 1.8% TAE (Table 2-2) SYBR® Green I stained gel with 1µl of DNA Mass Ladder (Invitrogen) (Figure 2-7 a). Each band on the ladder has a defined weight of DNA in ng, and is of an established bp size. A standard curve was plotted using the known weight from the DNA Mass Ladder (Invitrogen) and the fluorescence units calculated by the AIDA software (Figure 2-7 b). Regression analysis was used to calculate the amount of cDNA amplified in the PCR reaction, in µg/µl.

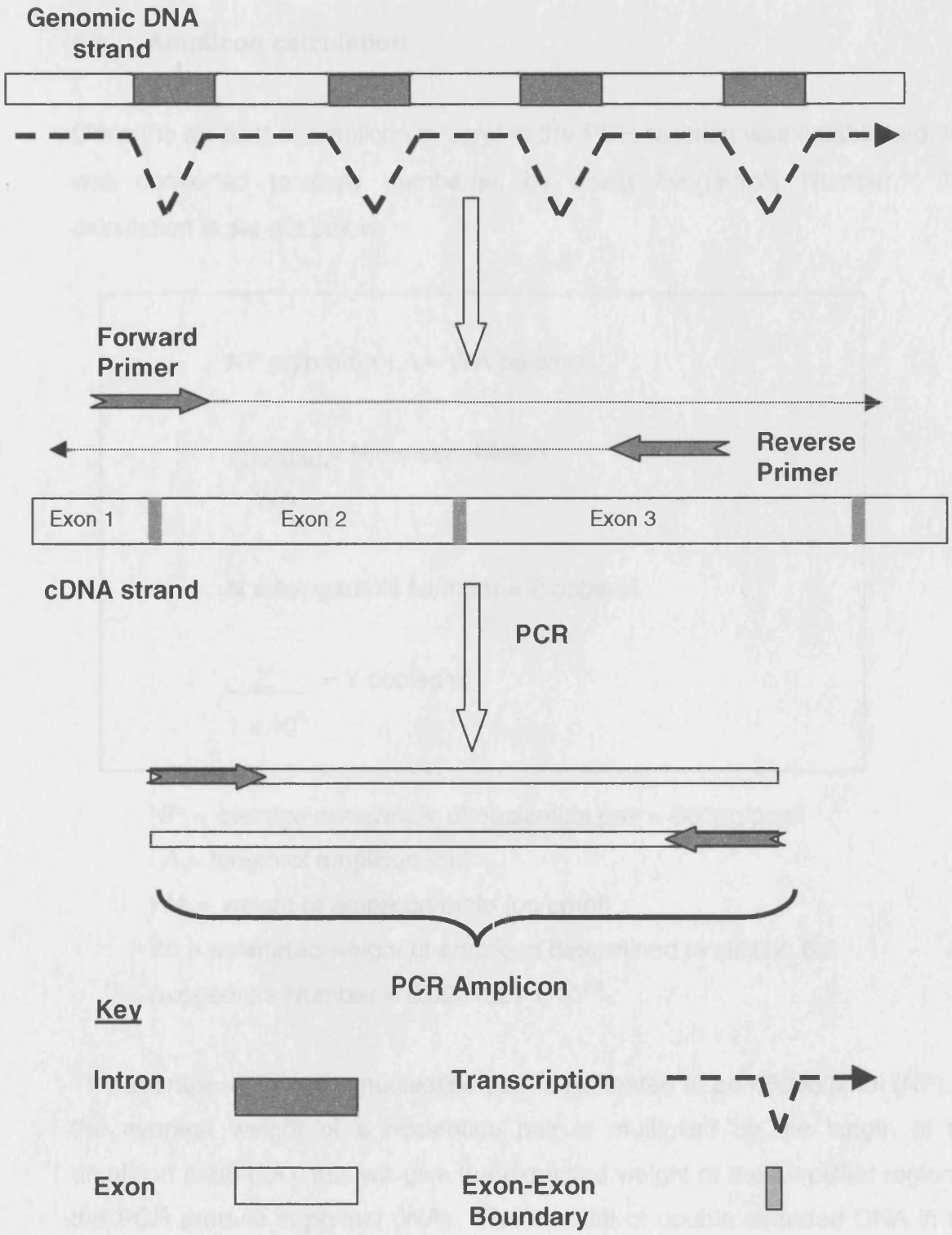


Figure 2-6: cDNA specific primer design. Genomic DNA contains introns and exons. Exons are the "coding" parts of the DNA, which get transcribed into mRNA. Therefore, cDNA is only the exon sequence of DNA. Primers were designed to span the exon-exon boundary to ensure the primers amplified only cDNA obtained from mRNA. This reduces over estimations due to contamination with genomic DNA.

6.3 Amplicon calculation

Once the amount of amplicon in µg/µl in the PCR reaction was established, this was converted to copy number/µl, by using Avogadro's Number. The calculation is set out below:

$$NP\ pg/pmol \times LA = WA\ pg/pmol$$

$$\frac{Wt\ \mu g/\mu l}{WA} = N\ moles/l\ (Molar)$$

$$N \times Avogadro's\ Number = Z\ copies/l$$

$$\frac{Z}{1 \times 10^6} = Y\ copies/\mu l$$

NP = average mass/mole of nucleotide pair = 660pg/pmol
LA = length of amplicon (bp)
WA = weight of amplicon/mole (pg/pmol)
Wt = estimated weight of amplicon determined in section 6.2
Avogadro's Number = 6.0221367×10^{23}

The average weight of a nucleotide pair is estimated to be 660pg/pmol (NP). If the average weight of a nucleotide pair is multiplied by the length of the amplicon in bp (LA), this will give the expected weight of the amplified region in the PCR product in pg/mol (WA). The amount of double stranded DNA in the PCR reaction has already been established using linear regression in µg/µl (Wt - section 6.1). If this is divided by the average weight of the amplicon (WA), this gives moles/l (N). Multiplication of this number by Avogadro's Number, which is the number of molecules in a mole, gives copies/l (Z). This can be divided by 1 $x10^6$ to obtain copies/µl (Y).

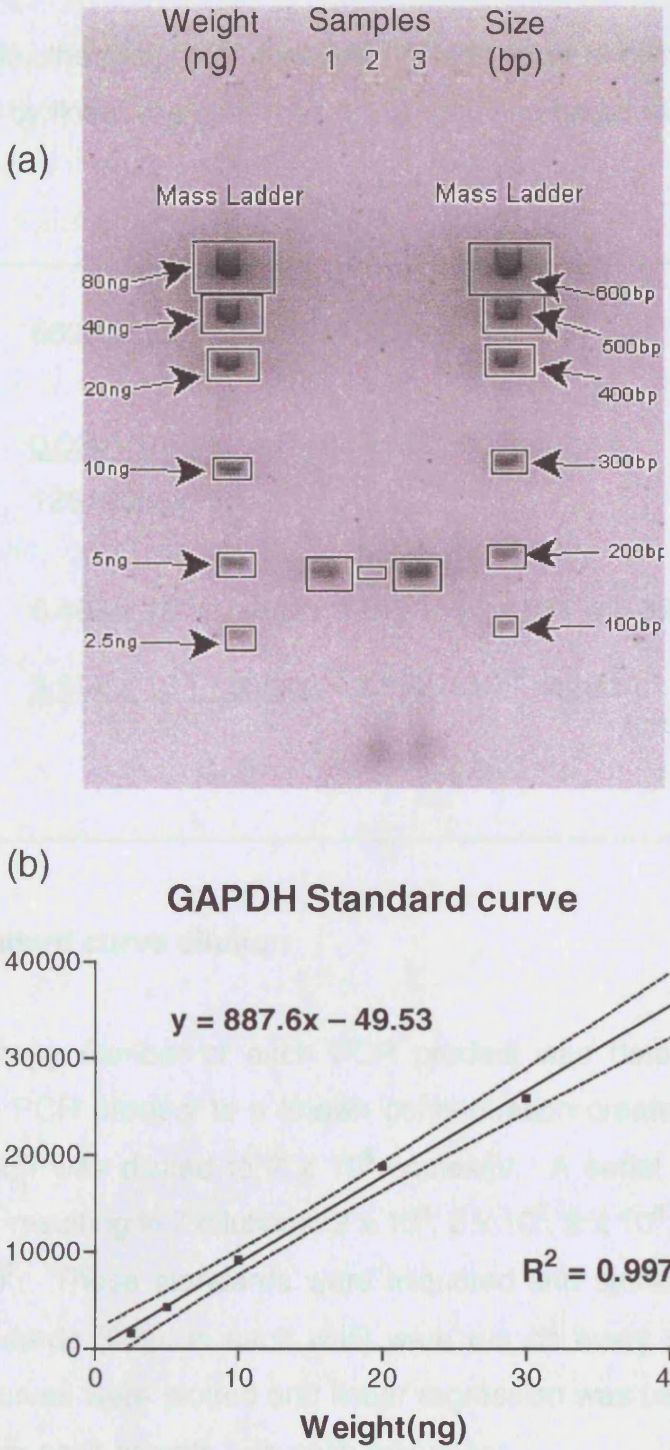


Figure 2-7: Creating a standard curve. A basic end point PCR was run with cDNA known to express the gene of interest. The amplicon was run out on an agarose TAE gel with a DNA Mass Ladder (a). Each band is of a known weight (in ng) and size (in bp). The fluorescence units for each DNA Mass Ladder band were plotted against its corresponding weight to create a standard curve (b). The linear regression equation from the standard curve was used to calculate the amount of amplicon copies in the PCR sample. The amount of DNA in the PCR product is used to serially dilute a standard curve of known concentrations. Sample 1 = GAPDH Amplicon, Sample 2 = Negative Control, Sample 3 = GAPDH Amplicon.

For example, the weight of the GAPDH amplicon shown in Figure 2-7 was established by linear regression as 8.161ng/µl and had a length of 192bp (Table 2-3).

660pg/mol x 192bp = 126720pg/pmol

$$\frac{0.008161\mu g/\mu l}{126720pg/pmol} = 6.463 \times 10^{-8} \text{ moles/l}$$

$$6.463 \times 10^{-8} \text{ moles/l} \times 6.0221367 \times 10^{23} = 3.892 \times 10^{16} \text{ copies/l}$$

$$\frac{3.892 \times 10^{16} \text{ copies/l}}{1 \times 10^{6}} = 3.892 \times 10^{10} \text{ copies/µl}$$

6.4 Standard curve dilution

Once the copy number of each PCR product was determined in copies/µl, diluting the PCR product to a known concentration created a standard curve. The amplicon was diluted to 2 x 10^8 copies/µl. A serial 10 fold dilution was carried out, resulting in 7 dilutions: 2 x 10^8, 2 x 10^7, 2 x 10^6, 2 x 10^5, 2 x 10^4, 2 x 10^3, 2 x 10^2. These standards were aliquoted and stored at -20°C until use. These standards (0.5µl in each well) were run on every real-time PCR plate. Standard curves were plotted and linear regression was used to calculate copy number/µl for each sample with each primer set.

7. Co-culture assay

7.1 CD4 T cell separation

CD4+ T cells were obtained from naïve spleen tissue using R&D Systems negative selection columns following the manufacturer's instructions. Spleens

were processed as described in section 2.2 to obtain a single cell suspension. Splenocytes were then incubated with a cocktail of mAbs for B cells, CD8+ T cells and monocytes. Cells were incubated at room temperature for 15 minutes, before being washed and applied to the CD4 subset column. Cells were left to bind to the column for 10 minutes at room temperature and then washed out with 10ml of 1 x column buffer. Unbound CD4+ cells were eluted from the column in 1 x column buffer and resuspended in Growth Medium. Ab bound cells were retained in the column. Cell purity was determined by FACS (section 8) to be >90%.

7.2 *In vitro* assay method 1

Two methods were used for setting up an *in vitro* assay. The first method did not always give consistent results, so a number of modifications were made. Method 2 was a longer protocol and involved stimulating the cells with IL-2. This method was adapted from a paper by MacDonald and colleagues [MacDonald *et al* 2001].

In the first method, CD11c+ cells obtained from lung tissue were resuspended in 15ml Growth Medium (enough to cover the plastic surface of the flask - Table 2-2), and left to adhere overnight on plastic at 37°C, 5% CO_2 to minimise MΦ contamination. CD11c+ cells were then washed and resuspended at a concentration of 2.5 x 10^5 CD11c+ cells/ml in Growth Medium (Table 2-2). CD4+ T cells isolated from the spleens of naïve mice (section 7.1) were resuspended in Growth Medium at a final concentration of 5 x 10^6 cells/ml (Table 2-2). The *in vitro* assay was set up in a flat bottom 96 well plate (Gibco-BRL). Wells were coated with anti-CD3 (0.5µg/well, Invitrogen) and incubated at 37°C for 2 hours. Plates were then washed with PBS twice to remove unbound Ab. All wells received 5 x 10^5 CD4+ naïve T cells in 100µl. CD11c+ cells were added at different CD11c+:CD4+ cell ratios; 1:100 (5 x 10^3 CD11c+ cells), 1:40 (1.25 x 10^4 cells) and 1:20 (2.5 x 10^4 cells). Total well volumes were made up to 200µl with Growth Medium. Control wells with 5 x 10^5 CD4+ T cells alone in Growth Medium, either stimulated with plate-bound anti-CD3 and stimulated with PBS, were included in a total volume of 200µl Growth Medium.

2.5 x 10^4 CD11c+ cells alone in Growth Medium, stimulated with PBS, were set up alongside. Wells were set up in at least duplicate, and if cell numbers permitted, in triplicate. Cells were left in culture for 96 hours at 37°C. Supernatants were taken for cytokine analysis by ELISA, and cells were snap frozen for PCR analysis.

7.3 *In vitro* assay method 2

CD11c+ cells obtained from lung or spleen tissue and CD4+ cells isolated from spleen were treated as described in section 7.2. CD4+ and CD11c+ cells were rested together in 200µl of Growth Medium for 72 hours, at the same CD11c+:CD4+ cell ratios previously described. After 72 hours, 100µl of Growth Medium was replaced with fresh Growth Medium containing IL-2 (50U/ml – Invitrogen). Cells were left for 48 hours at 37°C, 5% CO_2. Cells were then transferred to a fresh plate pre-coated with plate bound anti-CD3 (0.5µg/well, incubated for 2 hours at 37°C and washed twice with PBS). Plates were left for 48 hours at 37°C, 5% CO_2 before the supernatants were taken for ELISA analysis and stored at –20°C (section 4.3). Cells were snap frozen for PCR analysis (Sections 5). This assay was based on methods previously described [McDonald *et al* 2001].

8 Flow Cytometry

Flow cytometry was carried out on the FACSCalibur™ flow cytometer (Becton Dickinson). Data acquired and analysed using WinList 3.0 software (Verity Software House Inc). The standard 488nm laser was used for up to three colours analysis. Forward and side scatter characteristics were used to set gates. When using two or three colour analysis, compensation for spectral overlaps was performed using single colour fluorescence-labelled beads (Becton Dickinson). Instrument settings were kept consistent for all repeat experiments.

Cells of interest (1 x 10^5 minimum) were resuspended in 100µl of FACS buffer (Table 2-2). Specific Abs (Pharmingen) were added (Table 2-4) and cells were

incubated for 30 minutes at 4°C. The appropriate isotype control was also added to a control tube of cells to ascertain non-specific autofluorescence. One sample was stained with PI to eliminate dead cell populations. After incubation, cells were washed to remove unbound Ab and resuspended in 300µl of FACS buffer. Surface expression was immediately analysed on the FACSCalibur™, acquiring between 5000 and 10,000 events.

Acquired FACS data was analysed using the "Subtract Histogram" feature of WinList 3.0 (Verity Software House Inc). This statistic uses an enhanced normalised subtraction (ENS) method with the Kolmogorov-Smirnov (KS) statistic. This method was developed in the 1930s and quantifies the difference between two frequency histograms with the following design characteristics: 1) no required assumptions about error distributions, 2) easy manual calculations, and 3) robust with noisy data [Overton *et al* 1988, Kolmogorov *et al* 1993]. The KS statistic is a widely used nonparametric test for histogram comparison.

The KS statistic is defined as "the maximum absolute difference (Dmax) between two cumulative probability distributions". A probability distribution is a histogram normalised with an area of one. The ENS is a means to accurately estimate the positive distribution in two overlapping populations, and determine a more accurate percentage of test histogram that is positive or the part that remains after subtraction [Bagwell *et al* 1996]. In this thesis, the control histogram was always subtracted from the experimental histogram. Thus, each resultant histogram represents the fluorescence of the positive cells and the part of the fluorescence from the isotype control that remains after the experimental histogram data are subtracted.

9 Statistical Analysis

Results are expressed as mean ± SEM unless otherwise stated. Data were subjected to a normality test to ensure normal distribution. All graphs are representative of two or more experiments. When appropriate, a one-way ANOVA or Student's *t*-test were used to determine statistically significant differences between the groups. If an ANOVA was used to test for significant

differences, a Bonferroni post test was used to compare the mean values between all data sets. A *p* value of less than 0.05 was considered significant.

Table 2-2 Routinely used Buffers and Tissue Culture Media

Name	Contents	Obtained from
Wash Medium	RPMI Medium Penicillin (5% - 50U/ml) Streptomycin (5% - 50mg/ml)	Gibco-BRL Invitrogen Invitrogen
Hypotonic Lysis Buffer	NH_4Cl (0.15M) $KHCO_3$ (10mM) EDTA (0.1mM)	Sigma Sigma Sigma
Growth Medium	RPMI Medium Penicillin (5% - 50U/ml) Streptomycin (5% - 50mg/ml) Glutamine (1%) Foetal Calf Serum (10%)	Gibco-BRL Invitrogen Invitrogen Invitrogen Gibco-BRL
MACS Buffer	1x PBS BSA (0.5%)	Gibco-BRL Sigma
FACS Buffer	1x PBS BSA (1%) NaN_3 (0.01%)	Gibco-BRL Sigma Sigma
1 x TAE	Tris base (40mM) Acetic Acid (20mM) EDTA (1mM)	Sigma Sigma Sigma
HotstarTaq Master Mix (available ready mixed)	2.5U HotstarTaq DNA polymerase $MgCl_2$ (3mM) ATP (400µM) CTP (400µM) GTP (400µM) TTP (400µM)	Qiagen

Table 2-3 Primer Sequences, Amplicon Length and Melting Temperature (Tm)

PRIMER	FORWARD SEQUENCE	REVERSE SEQUENCE	AMPLICON LENGTH	TM (°C)
GAPDH	5'- CAT-TGT-GGA-AGG-GCT-CAT-GA-3'	5'-GGA-AGG-CCA-TGC-CAG-TGA-GC-3'	192bp	60
IL-4	5'- CGT-CCT-CAC-AGC-AAC-GGA-GA-3'	5'- GCA-GCT-TAT-CGA-TGA-ATC-CAG-G-3'	181bp	60
IL-10	5'- TTT-GAA-TTC-CCT-GGG-TGA-GAA-G-3'	5' – ACA-GGG-GAG-AAA-TCG-ATG-ACA – 3'	52bp	54
TGF-β	5'- ACA-GGG-CTT-TCG-ATT-CAG-CGC-3	5'- CCC-TTG-GGC-TCG-TGG-GGT-CCT-3'	306bp	60
IFN-α	5'- CTC-TGT-GCT-TTC-CTG-ATG-GT-3'	5'- AGA-GAG-GGA-GTC-TCC-TCA-TT-3'	171bp	60
IFN-γ	5'- GGT-GAC-ATG-AAA-ATC-CTG-CAG-A –3'	5'- CCT-CAA-ACT-TGG-CAA-TAC-TCA-TGA- 3'	181bp	59
IL-12	5'-GAC-ATC-ATC-AAA-CCA-GAC-CCG-3'	5'- TTA-CAC-CCC-TCC-TCT-GTC-TCC-TT-3'	182bp	59
ICOS	5'- GCT-GCC-AGA-CTA-CAG-CC-3'	5'- CAG-GCT-GCA-TAG-TTT-CTA-G-3'	196bp	57
ICOS-L	5'- CTT-AAG-TCC-TTC-CCG-AGT-A-3'	5'- CCA-CCT-GAG-TTC-CTG-GG-3'	198bp	57
Foxp3	5'- CAC-AAC-ATG-GAC-TAC-TTC-AAG-TAC-CAC-3'	5'- GAT-GGC-CCA-TCG-GAT-AAG-G-3'	75bp	59

Table 2-4 Cell Surface Markers, Fluorochromes and Isotype Controls

MARKER	FLUOROCHROMES	ISOTYPE CONTROL
CD11c	FITC	Rat IgG1λ
CD11c	PE	Hamster IgG1λ
CD80	PE	Hamster IgG1λ
CD86	FITC	Rat IgG2aκ
I-A^d (MHC II)	FITC	Rat IgG2bκ
CD8α	FITC	Rat IgG2aκ
B220	PerCP	Rat IgG2aκ
CD3	Cy-5	Rat IgG2bκ
CD4	PE	Rat IgG2aκ
CD40	FITC	Rat IgG2bκ
F4/80	PE	Rat IgG2bκ

NB All Abs were from Pharmigen, except F4/80 and its isotype control, which are from Serotec

Chapter 3

Chapter 3

Results 1: Effect of *M. vaccae* on pulmonary inflammation: reducing excessive type-2 response

Data in this chapter have contributed towards a poster presented at the British Society of Immunology meeting, held in Harrogate in 2002.

Adams, V. C., Hunt, J. R. F., Rosa Brunet, L., and Rook, G. A. W.
Effect of *Mycobacterium vaccae* on ICOS expression in a murine model. *Immunology* (2002); 107 [Supplement 1]; 111. **OP141**

1 Introduction

The pathology of allergic asthma is a direct consequence of excessive type 2 responses. Asthma is therefore associated with the secretion of high levels of type 2 cytokines, for instance IL-4, IL-5 and IL-13 [Bradley *et al* 1991, Kay 1991, Umetsu *et al* 2002]. Release of these cytokines, secreted by a number of cells including allergen activated CD4+ T cells, results in eosinophilia, increased mucus production and elevated IgE levels. These are all characteristic of asthma [Yamaguchi *et al* 1988, Whittaker *et al* 2002]. This chapter investigates the effect of *M. vaccae* treatment on the type-2 response and cytokine profile of mice with pulmonary inflammation.

1.2 IL-4

Upon initiation of airway inflammation, IL-4 is primarily secreted by CD4+ T cells. IL-4 is involved in triggering type-2 responses, which results in the elevation of IgE levels, eosinophilia and mucus production [Snapper *et al* 1988, Lebman *et al* 1989, Schleimer *et al* 1992, Whittaker *et al* 2002].

In a murine model of pulmonary inflammation, IL-$4^{-/-}$ mice have fewer eosinophils in the lung tissue and BAL fluid, when challenged with allergens in the airways. However, these mice still have the ability to generate and release eosinophils from the bone marrow [Hamelmann *et al* 2000].

Although IL-4 is necessary for inducing pulmonary inflammation, one other important role of IL-4 is in the induction of B cell secreted IgE. IL-4 causes a switch from IgM to IgE Ab production [Snapper *et al* 1988]. IgE is a major contributor to pulmonary inflammation, since cross-linkage of two IgE Abs, on a mast cell results in degranulation and release of histamine [Kinet *et al* 1990, Umetsu *et al* 2002, Fireman *et al* 2003, Wynn *et al* 2003]. Once activated by IL-4, mast cells, basophils and eosinophils also generate inflammatory cytokines of their own, such as IL-4, IL-5 and IL-13. These cytokines cause more cellular infiltration and perpetuate inflammation [Fireman *et al* 2003]. IL-4 is therefore an essential cytokine for the activation of type-2 cells, and for the development of Th2 mediated diseases.

Human clinical trials using a soluble recombinant human IL-4 receptor (IL-4R; Nuvance; altrakincept) that blocks the function of IL-4, have been carried out. Patients taking inhaled corticosteroids received a single dose of either the IL-4R or a placebo immediately after stopping corticosteroid treatment. Compared to the placebo group, patients that received the IL-4R maintained stable asthma symptom scores despite abrupt withdrawal of corticosteroids [Borish *et al* 1999, Borish *et al* 2001]. This highlights the therapeutic potential of inhibiting IL-4 in asthma patients. However, other cytokines are involved in initiation of asthma symptoms, including IL-5 and IL-13.

1.3 IL-5

Upon initiation of airway inflammation, IL-5 is produced primarily by activated T cells, although secretion by NKs, mast cells and eosinophils is also reported [Tanaka *et al* 1994, Warren *et al* 1995, Okayama *et al* 1995]. IL-5 is essential for the induction of airway eosinophilia, hallmark of allergic asthma [Durham *et al* 1989].

IL-5 stimulates eosinophil expansion and differentiation [Yamaguchi *et al* 1988]. Absence or blockade of IL-5 is reported to significantly reduce eosinophil numbers in experimental models. For example, injection of anti-IL-5 mAb into OVA sensitized mice before OVA challenge significantly reduces eosinophil infiltration into the lungs [Nakajima *et al* 1992, Morokata *et al* 1999]. In addition, IL-$5^{-/-}$ mice sensitized and challenged with OVA have significantly fewer eosinophils in the BAL fluid [Cho *et al* 2004].

Other molecules, which have a direct effect on initiating eosinophilia, include eotaxin and RANTES. These chemotactic chemokines are eosinophil attractants, and even in the absence of IL-5 can activate eosinophils [Lampinen *et al* 2004]. Eotaxin and RANTES, along with their receptor CCR3, have been shown to be significantly increased in the bronchial mucosa in atopic and non-atopic asthma patients when compared to non-asthmatic controls [Ying *et al* 1999]. Blockade of RANTES with mAbs resulted in inhibition of eosinophils in the BAL fluid of pollen allergic patients [Lampinen *et al* 1999]. Blockade of the

eotaxin and RANTES receptor CCR3 with a mAb also suppressed eosinophil activation [Bertrand *et al* 2000]. Although eotaxin and RANTES are important for initiation of eosinophilic inflammation, IL-5 is the predominately essential cytokine whose secretion is induced by IL-4.

1.3.1 IL-5 therapy in animal models

IL-5 has been shown to be essential for the initiation of eosinophilia, which is a hallmark symptom of allergic asthma. The role of IL-5 in the induction and maintenance of airway inflammation has been supported by numerous animal models. For example, sensitisation and aeroallergen-challenge of $IL5^{-/-}$ mice does not induce eosinophilia, indicating a central role for IL-5 in the pathogenesis of allergic airways disease [Foster *et al* 1996, Trifilieff *et al* 2001]. In addition $IL\text{-}5^{-/-}$ mice have significantly less peribronchial fibrosis and less peribronchial smooth muscle (hyperplasia) compared with WT mice when challenged with OVA [Cho *et al* 2004].

Such observations suggest that blocking IL-5 secretion would reduce asthmatic characteristics. Several methods of inhibiting IL-5 have been investigated in animal models. One example is the use of antisense oligonucleotides to block the IL-5 production at the molecular stage, by specifically binding to IL-5 or IL-5 receptor mRNA, and causing RNase H mediated degradation. Karras *et al* demonstrated an antisense oligonucleotide that inhibited the expression of IL-5 *in vitro* also significantly reduced the number of eosinophils in the BAL fluid of OVA sensitized and challenged mice. This correlated with reduced IL-5 protein levels and suppression of AHR, supporting the blocking action of the IL-5 antisense oligonucleotide [Karras *et al* 2000]. Similar results were shown by Lach-Trifilieff *et al,* whose group reported a significant reduction in bone marrow and blood eosinophilia upon treatment with an antisense oligonucleotide to the IL-5 receptor α unit. In addition, i.v. administration of this antisense oligonucleotide inhibited the development of blood and tissue eosinophilia in ragweed-induced allergic peritonitis [Lach-Trifilieff *et al* 2001].

Encouraging results have also been observed by blocking IL-5 protein with a mAb. Injection of anti-IL-5 mAb into OVA sensitized mice before OVA

challenge significantly reduces subsequent eosinophil infiltration into the lungs [Nakajima *et al* 1992, Morokata *et al* 1999]. In addition, treatment with an anti-IL-5 mAb has been shown to have a positive effect on airway remodelling. Blyth *et al* injected the anti-IL-5 mAb TRFK5 i.v 30. minutes before allergen challenge of sensitized mice, and observed a reduction in the amount of subepithelial fibrosis, along with almost total depletion of airway eosinophils [Blyth *et al* 2000].

1.3.2 IL-5 therapy in humans

Since pre-clinical work has shown promise, a number of clinical trials have been conducted in human patients. SCH55700 (Schering-Plough Research Institute) and mepolizumab (SB-240563) (GlaxoSmithKline) were both used in a trial on patients with asthma [Leckie *et al* 2000, Flood-Page 2003, Menzies-Gow *et al* 2003, Kay *et al* 2004]. SCH55700 is a humanized mAb derived from the rat anti-human IL-5, 39D10 [Zhang *et al* 1999], whereas mepolizumab is a humanized mAb derived from the murine mAb anti-human IL-5 Ab, 2B6 [Zia-Amirhosseini *et al* 1999].

Patients with severe asthma treated with SCH55700 had reduced numbers of eosinophils circulating in their peripheral blood. At the highest dose, SCH55700 offered a long lasting reduction without any significant side affects. However, there was no significant clinical benefit, even though there was a non-significant trend towards improvement at day 30 in the Forced Expiratory Volume in 1 second (FEV_1) [Kips *et al* 2003].

A trial in which patients suffering with mild asthma were treated with a single dose of mepolizumab resulted in a significant reduction in blood and sputum eosinophil numbers. Unfortunately, no significant changes in late asthmatic reaction or AHR were observed [Leckie *et al* 2000]. Subsequent trials using mepolizumab reported drastically decreased numbers of eosinophils in the bone marrow (70% reduction compared to placebo treated patients), BAL fluid (79%) and the peripheral blood (100%) 4 weeks after treatment. This decrease is maintained for at least 10 weeks after mepolizumab injection. In spite of this, there was no significant change between the placebo and treatment groups in

clinical measures of asthma, including FEV_1, AHR or peak flow readings [Flood-Page *et al* 2003, Menzies-Gow *et al* 2003].

1.4 IL-13

IL-13 is secreted primarily by T cells and binds to the IL-4 receptor [de Waal Malefyt *et al* 1993]. This cytokine plays an essential role in mucus production in the airways [Wills-Karp *et al* 1999]. Blockade of IL-13 by soluble IL-13R inhibits mucus production in the lungs of OVA sensitized mice [Wills-Karp *et al* 1998]. In the complete absence of IL-13, there is no mucus secretion in the airways even when pulmonary inflammation is induced [Whittaker *et al* 2002]. In addition to mucus production, IL-13 is also essential in the development of AHR. AHR is a short term narrowing of the airways, which limits breathing. In a murine model of OVA-induced pulmonary inflammation, blockade of IL-13 with the soluble fusion protein IL-13αc-IgGFc before OVA challenge resulted in a complete reversal of AHR development in the lungs [Wills-Karp *et al* 1998]. However, blockade did not completely inhibit IgE levels or eosinophilic inflammation in the lung. This suggests that AHR is dependent on IL-13, but IgE production and eosinophilia are dependent upon other cytokines, such as previously described IL-4 and IL-5. IL-13 also plays a major role in fibrosis is the lungs. Fibrosis is a characteristic of chronic asthma. IL-$13^{-/-}$ mice infected with *Schistosoma mansoni* showed reduced liver fibrosis, compared to wild type mice [Kaviratne *et al* 2004]. Together, all these data suggest that secretion of IL-4, IL-5 and IL-13 by CD4+ T cells initiate mast cell and eosinophil activation, and cellular recruitment into the respiratory tract.

1.5 Th1 Vs Th2

In the past it was hypothesised that pathology caused by excessive Th2 responses may be reversed by inducing a type-1 response in the host [Surs *et al* 1996] Cells secreting IFN-γ, a Th1 cytokine, are known to cross regulate Th2 cells, and, some researchers believed them to be ideal candidates to downregulate Th2-induced AHR [Mosmann *et al* 1989]. *In vitro* assays have promoted these views as Th1 and Th2 cytokines have antagonistic effects on each other. For example when the supernatant from cultures of Th2 clones was

added into the culture medium of Th1 clones, type-1 cytokine production was suppressed [Fiorentino *et al* 1989]. In addition, IFN-γ has been observed to inhibit both Th2 cell proliferation and secretion of IL-4 [Fitch *et al* 1993]. From this cumulative evidence, the hypothesis that treatment aimed at inducing a type-1 response would be helpful in reducing the excessive Th2 response associated with allergic inflammation was put forward [Mosmann *et al* 1989].

A number of observations however were soon made which added some confusion. IFN-γ is an abundant cytokine in lung tissue of asthmatics [Krug *et al* 1996] and it is now evident that IFN-γ may actually contribute to the severity of the disease. For example, the introduction of Th1 cells to the lungs of mice with allergic pulmonary inflammation actually worsens symptoms instead of counterbalancing the type-2 response, by aggravating airway inflammation and cellular infiltration [Hansen *et al* 1999]. It is implicit in these results that the Th1/Th2 paradigm, in this context, may be too simplistic a hypothesis and other mechanisms may be involved. Therefore, the first objective of this thesis was to analyse the production of type-1 and type-2 cytokines in the lungs of mice with allergic pulmonary inflammation, and compare them to that of mice treated with *M. vaccae*. This work would confirm published papers reporting a decrease in type-2 mediated eosinophilia, unaccompanied by changes in type-1 cytokine levels in the BAL fluid [Zuany-Amorim *et al* 2002a]. Despite being the site of inflammation the lung tissue itself has received surprisingly little attention. To determine if *M. vaccae* has an effect on the cytokine profile of treated mice, analysis of cytokines in the whole lung tissue was therefore undertaken, in parallel with type-1 and type-2 levels in the BAL fluid.

1.6 Immunoregulation

Although type-1 and type-2 cytokines antagonise each other, they generally have a positive effect on inducing immune responses. Other cytokines are essential in inhibiting the immune response. Recently, it has been suggested that suppressive cytokines, such as IL-10 and TGF-β, are associated with regulation of excessive immune responses in a number of allergic conditions, in autoimmune disorders and during transplant rejection [Arkwright *et al* 2001, Alansari *et al* 2002, Bickerstaff *et al* 2002, Sharif *et al* 2002, von Bubnoff *et al*

2004]. These cytokines are also associated with the induction of Tregs, which are responsible for suppression of immune responses, and immunoregulation [Chen *et al* 1994, Ronocarolo *et al* 2000, Read *et al* 2001, Ling *et al* 2004].

Generally, a lack of immunoregulation during an infection can have fatal consequences. Death is not always caused by the infectious pathogen itself, but rather as a consequence of immunopathology. An immune response mounted against a pathogen can cause irreversible tissue damage if not inhibited once the pathogen has been destroyed. Suppressive cytokines, such as IL-10, are essential in inhibiting immune response. Several examples from animal models can be cited to support this. For example IL-$10^{-/-}$ mice have been observed to mount an over exuberant response when infected with the malaria parasite *Plasmodium chabaudi chabaudi*. This excessive response is damaging to the hosts tissue, leading to hypoglycemia, hypothermia, and loss of weight, when compared to IL-10 sufficient animals [Li *et al* 1999]. IL-$10^{-/-}$ mice infected with *Escherichia coli* clear the bacteria significantly faster than wild type animals. In spite of this, the mortality rate and incidence of peritonitis in these mice is significantly higher. Although IL-$10^{-/-}$ mice can mount a vigorous response to the pathogen, they cannot reverse it once the infection is cleared. Death is a consequence of the aggressive immune response mounted [Sewnath *et al* 2001]. The exaggerated immune response observed in these mice, and the inability to switch this response off, illustrates that IL-10 is an essential regulator of the immune response.

1.7 IL-10

IL-10 has the ability to suppress both type-1 and type-2 cytokine production [Hoffmann *et al* 1999, Hoffmann *et al* 2000]. Single (IL-$10^{-/-}$) and double (IL-10/IL-$4^{-/-}$ or IL-10/IL-$12^{-/-}$) knock out mice were infected with *Schistosoma mansoni* and the progression of infection monitored. The IL-$10^{-/-}$ and double (IL-10/IL-$4^{-/-}$ or IL-10/IL-$12^{-/-}$) cytokine-deficient mice developed the most extreme infection-related tissue pathology. Interestingly, pathology in IL-10/IL-$4^{-/-}$ (Th1 polarized) differed from that observed in IL-10/IL-$12^{-/-}$ (Th2-polarized). IL-10/IL-$4^{-/-}$ mice rapidly succumbed to infection, developing fibrotic hepatic granulomas in response to the parasite eggs. In contrast, the IL-10/IL-$12^{-/-}$

(Th2-polarized) mice developed a slow and prolonged wasting disease that resulted in significant morbidity during chronic stages of infection, with much larger granulomas than the Th1-polarized mice. This study further offers evidence that IL-10 production during *Schistosoma mansoni* infection is not only important for suppressing type-1 inflammatory cytokines, but is also critical for the dampening of pathogenic type-2 immune responses during infection [Hoffmann *et al* 1999, Hoffmann *et al* 2000]. IL-10 is now considered as an essential cytokine in immunoregulation.

1.7.1 IL-10-like family

Cytokines have generally been placed in one of seven protein fold-based superfamilies, depending on their protein structure. A number of molecules are defined as being "α-helical", and within this grouping are at least three subtypes: those with short α-helices, α-helices and those with multiple repeat α-helices. The IL-10 family of proteins includes IL-10 and other cytokines with structural similarity such as IL-19 [Gallagher *et al* 2000], IL-20 [Blumberg et al 2001], IL-22 [Dumoutier *et al* 2000], IL-24 [Jiang *et al* 1995, Caudell *et al* 2002] and IL-26 [Knappe *et al* 2000]. Functionally, these molecules share little similarity. The structure common to all mature IL-10 family members is the α-helix. Amino acid identity of family members to IL-10 ranges from 20% (IL-19) to 28% (IL-20) [Fickenscher *et al* 2002, Dumouter *et al* 2002]. The IL-10 family share a combination of 5 receptors subunits: IL-10R1, IL-20R1, IL-22R, IL-10R2 and IL-22R2. The receptors for the IL-10 family are all members of the class II cytokine receptor family (CRF2) [Soo *et al* 1999].

All functional receptors are heterodimers composed of an α or type 1 (R1) chain and a β or type 2 (R2) chain. At a minimum, it would appear that there are at least two α and two β chains involved in each signalling receptor complex [Kotenko *et al* 2000]. Traditional nomenclature has often defined the a subunit as being the ligand-binding molecule and the β subunit as being the signal transducing subunit. For at least one member of the IL-10 family, both subunits can serve as ligand-binding moieties [Xie *et al* 2000, Kotenko *et al* 2001] and the α-subunit in this case generally serves as a STAT3 docking port (i.e. the first step in signal transduction) [Kotenko *et al* 2000]. Class II cytokine

receptors are characterized by the presence of two fibronectin type III (FNIII) domains in their extracellular region, which are typically associated with cell surface adhesion molecules.

1.7.2 Function of IL-10-like family members

The functions of IL-10-like cytokines are only partially understood. IL-10 was first described as a Th2 cytokine in mice that inhibited IFN-γ and GM-CSF cytokine production by Th1 cells [Moore *et al* 1990, Fiorentino *et al* 1989]. Cells known to express IL-10 include CD8+ T cells [Varney *et al* 1999, Daftarian *et al* 1996], CD14+ monocytes [Frankenberger *et al* 1996, de Saint Vis *et al* 1998], Th2 CD4+ cells [Schmidt-Weber *et al* 1999], Th1 CD4+ T cells [Del Prete *et al* 1993], activated macrophages [Panuska *et al* 1995, Delgado *et al* 1999], NK cells [Mehrotra *et al* 1998], DCs [Iwasaki *et al* 1999], B cells [O'Garra *et al* 1990, Spencer *et al* 1997] and eosinophils [Nakajima *et al* 1996]. IL-10 binds to the IL-10R1/IL-10R2 receptors. Secreted by many cell types, IL-10 plays an essential role in many aspects of the immune system, including Ig class switching [reviewed in Stavnezer *et al* 1996] and as an immunoregulatory cytokine, responsible for inhibiting and downregulating immune responses [Read *et al* 2001, Ronocarolo *et al* 2000]. Although structurally similar, the biological activities of the rest of the IL-10-like family appear to be different from IL-10. In addition, the sites of expression are distinct for each cytokine. Whereas IL-10 is secreted by a variety of cells, the expression pattern of the other family members is more restricted [Fickenscher *et al* 2002].

IL-19 was originally isolated from an Epstein-Barr virus-transformed human B-cell library [Gallagher *et al* 2000]. IL-19 shares approximately 20% amino acid identity with IL-10. More than 80% of the 50 amino acids that generate IL-10s dimeric structure are conserved in IL-19. Cells known to secrete IL-19 include monocytes and B cells [Gallagher *et al* 2000, Wolk *et al* 2002]. It is known that IL-19 binds the IL-22R1/IL-22R2 receptors. The function of IL-19, however, is still poorly understood. It has been demonstrated that treatment of monocytes with LPS strongly induces the production of IL-19 [Liao *et al* 2002]. When monocyte cultures are treated with IL-19, proinflammatory cytokines, such as

IL-1 and TNF-a, are released, along with the production of reactive oxygen intermediates, which eventually induce apoptosis [Parrish-Novak *et al* 2002].

IL-20 was initially identified in a human keratinocyte library during a bioinformatics search for molecules expressing α-helices. In humans, there is 26% amino acid identity between IL-20 and IL-10, and 41% amino acid identity between IL-20 and IL-19 [Gallagher *et al* 2000, Blumberg *et al* 2001]. Cells known to produce IL-20 are monocytes [Wolk *et al* 2002] and keratinocytes [Blumberg *et al* 2001, Xie *et al* 2000]. The only suggested function for IL-20 involves keratinocytes. Partly based on overexpression studies, it is proposed that IL-20 induces keratinocyte differentiation and proliferation [Blumberg *et al* 2001, Xie *et al* 2000]. IL-20R is expressed on keratinocytes and endothelial cells [Blumberg *et al* 2001] and keratinocytes likely express the IL-20 cytokine [Grone *et al* 2002].

IL-22 is a 179 amino acid polypeptide that contains one 33 amino acid signal sequence and a 146 amino acid mature region [Xie *et al* 2000, Dumoutier *et al* 2000]. The mature molecule contains three potential N-linked glycosylation sites plus four cysteines, only two of which are conserved in IL-10 [Fickenscher *et al* 2002]. There is 24% amino acid identity between mature human IL-22 and IL-10 [Dumoutier *et al* 2000]. Cells known to express IL-22 include NK cells and CD4+ Th1 cells [Wolk *et al* 2002]. The functions associated with IL-22 are still unclear. IL-22 induces acute phase proteins in hepatocytes (i.e. haptoglobin, α1-antichymotrypsin) [Dumoutier *et al* 2002, Dumoutier *et al* 2000] and may reduce IL-4 production by certain Th2 cells [Xie *et al* 2000]. In addition, pancreatic acinar cells, which synthesize inactive precursor forms of digestive enzymes, are now known to express PAP1 (pancreatitis-associated protein-1) and osteopontin in response to IL-22 [Lenhoff *et al* 1998]. These are two molecules that may have protective or enhancing effects during inflammation.

IL-24 was originally isolated from actively proliferating melanoma cells [Jiang *et al* 1995]. IL-24 consists of a 207 amino acid precursor, with an extended 47 amino acid signal sequence and a 160 amino acid mature segment [Zhang *et al* 2000]. Within the mature segment, there are three potential N-linked glycosylation sites and two cysteines. Thus, it differs markedly from all other

known IL-10 family members in the potential for disulfide-bonding [Jiang *et al* 1995]. In its mature segment, it shares 20% amino acid identity with IL-10 [Zhang *et al* 2000]. Cells known to express IL-24 include murine Th2 cells [Wang *et al* 2002], melanocytes [Jiang *et al* 1995, Soo *et al* 1999, Huang *et al* 2001], breast epithelium [Huang *et al* 2001], fibroblasts (in rat) [Soo *et al* 1999], monocytes [Wang *et al* 2002], vascular smooth muscle [Ekmekcioglu *et al* 2001], NK cells [Caudell *et al* 2002], B cells [Caudell *et al* 2002] and CD4+ CD45RA+ (naïve) T cells [Wolk *et al* 2002]. IL-24 was initially believed to induce melanocyte/melanoma cell differentiation. Melanoma cells lack IL-24 expression, while melanocytes express IL-24. In the presence of IL-24, melanoma cell growth was inhibited [Jiang *et al* 1995, Ekmekcioglu *et al* 2001]. Thus, it appeared that IL-24 might be a tumour suppressor molecule [Huang *et al* 2001, Su *et al* 1998]. Subsequent studies have shown that IL-24 can inhibit tumour growth in cells other than melanoma, such as non-small cell lung carcinoma, breast cancer, prostate cancer [Su *et al* 1998, Jiang *et al* 1996 Saeki *et al* 2000], and that apoptosis can be induced in transformed cells without negatively impacting any normal cell types [Ekmekcioglu *et al* 2001, Su *et al* 1998, Saeki *et al* 2000]. IL-24 has also been shown to induce IL-6 and TNF-a secretion by monocytes, demonstrating an activity antagonistic to that of IL-10 [Caudell *et al* 2002].

IL-26 is a 36 kDa homodimer that was initially identified in the supernatant of cultured herpesvirus-transformed T cells [Knappe *et al* 2000]. Its open reading frame encodes a 171 amino acid precursor that contains a 21 amino acid signal sequence and a 150 amino acid mature segment. Its mature segment shares 25% amino acid identity with human IL-10 and retains the typical six a-helix pattern seen in IL-10. Remarkably, there is no mouse counterpart to IL-26 [Dumoutier *et al* 2002]. Cells known to express IL-26 include CD4+ CD45RO+ T cells and NK cells [Wolk *et al* 2002 Fickenscher *et al* 2002]. To date, almost nothing is known about IL-26 activity. It thought to play a role as an autocrine factor in lymphocyte transformation by HVS or in the natural γ-herpesvirus infection, since IL-26 is over-expressed by HVS-transformed human T cells, a rare phenotypic differences between HVS-transformed T cells and their parental cells [Fickenscher *et al* 2002].

1.7.3 Type III IFNs

In addition to these structurally similar cytokines, IFN-λ1 (IL-29), -λ2 (IL-28A), and -λ3 (IL-28B) or type III IFNs, are also part of the IL-10-like family, since they share the IL-10R2 subunit (which IL-10, IL-22 and IL-26 also signal through) [Acharya *et al* 2001, Conti *et al* 2001]. These IFNs share limited sequence similarity with the type-I IFNs (IFN-α and IFN-β), but like the type-I IFNs are expressed by human PBMCs and DCs upon infection with viruses or double stranded RNA [Acharya *et al* 2001, Conforti *et al* 2001]. IFN-λ1, -λ2, and -λ3 induce tyrosine phosphorylation of the signal transducers and activators of transcription (STAT) factors, which is a typical feature of the type-I IFNs [Luzio *et al* 2001, Stefano *et al* 2001]. Hence, although these type-III IFNs share a receptor with some members of the IL-10-like family, their function appears to be similar to that of the type-I IFNs.

1.8 TGF-β

In addition to IL-10, TGF-β has also shown immunosuppressive characteristics [Ludviksson *et al* 00]. TGF-$\beta^{-/-}$ mice have not been extensively used in experimental models, as although they do not show any abnormalities at birth, they die within 5 weeks of birth. These mice succumb to a wasting syndrome, and die from multiple organ failure caused by excessive inflammation and tissue necrosis [Shull et *al* 1992]. More recent studies of TGF-$\beta^{-/-}$ mice have shown that TGF-β supports regulatory function of peripheral Tregs. At 8-10 days old, TGF-$\beta^{-/-}$ animals show a significant reduction in CD4+CD25+ T cells in the spleen [Marie *et al* 2005]. These data suggest that TGF-β may be essential for immunosuppression.

1.8.1 TGF-β processing and activation

The TGF-β family of cytokines consists of three closely related isoforms (TGF-β1–3) with a number of functions ranging from regulation of cell growth and differentiation, inflammatory responses and extracellular matrix (ECM) production. TGF-β is secreted as a latent precursor into the ECM, and activity

is regulated by the conversion of the latent to the active form. Secretion of latent TGF-β prevents uncontrolled activation of the cognate receptors [Annes *et al* 2003]. The importance of TGF-β regulation is supported by the observation that TGF-$\beta^{-/-}$ mice die within 5 weeks of birth from multiple organ failure caused by excessive inflammation and tissue necrosis [Shull et *al* 1992].

There are two types of latent precursors, the small and the large latent complex. The small latent complex (TGF-β-LAP) consists of a mature TGF-β dimer, noncovalently associated with a latency-associated protein (LAP) [Munger *et al* 1998]. The large latent complex (LLC) is formed by the TGF-β-LAP, and a latent-TGF-β binding protein (LTBP). The LAP and LTBP are bonded together by disulphide linkages [Saharinen *et al* 1996, Gleizes *et al* 1996]. LTBPs comprise a central stack of epidermal growth factor (EGF)-like repeats and interspersed cysteine repeats, which binds the ECM or to TGF-β-LAP [Saharinen *et al* 2000]. These features allow LTBPs to target latent TGF-β to the ECM, creating localised deposits of TGF-β that can be accessed easily and rapidly by surrounding cells [Taipale *et al* 1998]. As part of the LCC, TGF-β cannot interact with its receptors. The LAPs function as inhibitors due to their noncovalent, high affinity association with mature TGF-β [Lawrence *et al* 1984, Dubois *et al* 1995]. The process by which latent TGF-β is activated is still relatively unclear. The term "TGF-β activation" refers to the release of mature TGF-β from the latent complex. There are several known activators of TGF-β, including proteases, thrombosin-1 and low pH.

1.8.2 TGF-β activators

Proteases, including plasmin, matrix metallopeptidase (MMP)-2 and MMP-9, have been identified as TGF-β activators *in vitro*. Proteases may activate TGF-β in several possible ways. A protease-sensitive hinge region on the LTBP may be a potential target for the release of the LAP [Taipale *et al* 1994]. In addition, proteases may be involved in proteolytic cleavage of LAP, to destabilise the LAP-TGF-β bonds, releasing active TGF-β from its latent complex [Lyons *et al* 1988]. Thrombosin-1 is known to activate latent TGF-β by direct interaction between the matricellular protein (a non-structural extracellular matrix protein

which is involved in adhesion) and LAP, probably by disrupting the noncovalent bond interactions between LAP and TGF-β [Schultz-Cherry *et al* 1993, Murphy-Ullrich *et al* 2000, Crawford *et al* 1998]. Latent TGF-β present in conditioned medium can also be activated by mild acid treatment (pH 4.5). It is thought that acid treatment denatures LAP, thereby disrupting the interaction between LAP and TGF-β [Lyons *et al* 1988].

1.8.3 Measuring active TGF-β

The fact that TGF-β requires activation for the cytokine to effect other cells could explain the discrepancies often observed between measurements of TGF-β message and protein levels. Often an increase in TGF-β mRNA fails to translate in an increase at the protein level when TGF-β ELISA is carried out. This discrepancy suggests that TGF-β has been made, but is being held in the latent form as only the active form is measured by ELISA. The most common method used by ELISA kits for releasing TGF-β from the latent complex is by treating the supernatants with acid (usually HCl), which is thought to disrupt the bonds between LAP and the LTBP [Danielpour *et al* 1993, Lyons *et al* 1988]. However, measuring the amount of TGF-β after treatment with acid may not be accurately measuring the level of TGF-β cytokine that is active *in vivo.* Although the latent TGF-β may be present, it may not necessarily have an effect on cells. Measurement of active TGF-β protein following by treatment with acid therefore may not correlate with cytokine bioavailability.

The activation of latent TGF-β to mature TGF-β ensures that the action of TGF-β is tightly regulated and localised. TGF-β has been shown to be secreted by a number of cells, and to have many varied functions. For example, TGF-β is involved in remodelling of the airways during asthma and in the regulation of immune responses [Redington *et al* 1997, Chen *et al* 1996]. The tight control of TGF-β activity is therefore a necessity as release of active TGF-β could lead to an inappropriate reaction.

TGF-β has been shown as important for the function of Tregs [Zuany-Amorim *et al* 2002, Chen *et al* 2003], and is even secreted by a certain subtype, the Th3

inducible Treg [Chen *et al* 1996]. Induction of Treg activity has been shown as advantageous for animals suffering from allergic disease, such as asthma [Zuany-Amorim *et al* 2002, Kearley *et al* 2005, Wilson *et al* 2005].

1.8.4 Tregs and TGF-β

Tregs have been observed to express surface membrane-bound TGF-β in both the latent and active form [Nakamura *et al* 2001, Chen *et al* 2002]. Interestingly, a population of Tregs have been shown to also express the TGF-β type II receptor (TGF-βRII) in addition to TGF-β [Chen *et al* 2003]. In addition, TCR interaction with the MHC on APCs upregulates the expression of active cell surface TGF-β and TGF-βRII. In comparison, naïve CD4+CD25- T cells, which are negative for both TGF-β and TGF-βRII, upregulate only TGF-βRII after TCR stimulation [Chen *et al* 2003]. Engagement of CTLA-4 with CD28 on the surface of the Treg also results in upregulation of active TGF-β and TGF-βRII expression [Nakamura *et al* 2001]. Active TGF-β signals through the heretomeric complex consisting of TGF-βRI and TGF-βRII. TGF-βRI and TGF-βRII are serine-threonine kinases, which phosphorylate downstream signaling proteins upon TGF-β binding. This then propagates the signal through phosphorylation of cytosolic proteins, including the Smad proteins, Smad2 and Smad3 [Massague *et al* 1998]. Once activated Smad2 and Smad3 form a hetero-oligomeric complex and translocate to the nucleus where they can regulate transcription of specific genes [Roberts *et al* 2002]. This results in inhibition of proliferation and cytokine production.

The coexpression of this active TGF-β and its receptor may explain why CD4+CD25+ Tregs are functionally anergic and lack IL-2 expression upon TCR stimulation, since TGF-β has been shown to block IL-2 production [Kehrl *et al* 1986, Seder *et al* 1998, Chen *et al* 2001]. This coexpression of latent and active TGF-β on the cell surface of certain populations of Tregs suggests that activation TGF-β is tightly regulated locally, at the Treg surface.

Tregs inhibit the immune response, which may also inhibit responses, which are necessary to eliminate pathogens [Boussiotis *et al* 2000, Trinchieri *et al* 2001,

Gerosa *et al* 1999] or detect and destroy tumour cells [Liyanage *et al* 2002, Morse *et al* 2002, Noma *et al* 2005]. This fine balance between excessive and insufficient immunoregulation suggests that the regulation of Treg induction and function needs to be very tightly controlled and localised. In terms of Treg function, the regulation of mature TGF-β secretion may be essential in maintaining immunotolerance.

1.9 Mechanisms of immunoregulation mediated by IL-10 and TGF-β

A variety of mechanisms have been described to explain the regulatory effect that IL-10 and TGF-β have on both type-1 and type-2 mediated responses. For example, IL-10 has been shown to prevent CD28 co-stimulation. The CD28-B7 pathway is a major co-stimulatory pathway, necessary along with the TCR-MHC complex for rapid activation of T cells. IL-10 has been observed to inhibit tyrosine phosphorylation of CD28, which is the initial step in the CD28 signal pathway [Moore *et al* 1993, Akdis *et al* 2001]. This would inhibit activation of naïve T cells, resulting in diminished immune responses. Mechanisms of TGF-β mediated regulation include inhibition of IL-2 production [Seder *et al* 1998, Chen *et al* 2001] and downregulation of IL-12Rβ_2 expression on T cells [Ludviksson *et al* 2000]. This affects the proliferative capacity of naïve T cells. TGF-β has also been shown to induce the production of IL-10 by APCs, thereby diminishing immune responses further [Maeda *et al* 1995].

Another possible mechanism for which IL-10 and TGF-β may be necessary is the induction of Tregs. IL-10 and TGF-β have been described as essential for the activation and development of these cells [Zuany-Amorim *et al* 2002b, Cobbold *et al* 2004, Huber *et al* 2004]. These T cells have the ability to regulate immune cells, such as other T cells, either through cytokine-mediated mechanisms or via cell-contact [Groux *et al* 1997, Shevach *et al* 2002]. Indeed, treatment of allergic mice with *M. vaccae* has been observed to induce IL-10 and TGF-β dependent Tregs. Since these immunosuppressive cytokines play such an important role in regulation of the immune system, one objective of this thesis is to analyse the production of IL-10 and TGF-β in the lungs of mice with

pulmonary inflammation, and to compare the results to those obtained from the lungs of mice treated with *M. vaccae*.

1.10 Hypothesis

Treatment of allergic asthma with *M. vaccae* has been studied using a murine model of OVA-induced pulmonary inflammation. Treatment of mice with *M. vaccae* results in Treg induction. These Tregs are responsible for suppressing type-2 responses and consequently alleviating airway inflammation. These Tregs transfer protection against OVA specific pulmonary inflammation, when transferred i.v. to OVA sensitized animals. In addition, IL-10 secretion by these *M. vaccae*-induced Tregs was observed. Moreover, blockade of IL-10 and TGF-β *in vivo* with neutralising mAbs inhibited the function of these Treg, and as a consequence airway inflammation developed. These results suggest either that these *M. vaccae*-induced Tregs may be dependent on these cytokines for further development *in vivo,* or that they must release (or cause other cells to release) these cytokines in order to be suppressive [Zuany-Amorim *et al* 2002b]. The effect of treatment with *M. vaccae* on IL-10 and TGF-β has not been studied extensively at the site of inflammation, the lungs. In this chapter, the type-1 and type-2 profile of the lungs is investigated to establish the effect *M. vaccae* is having.

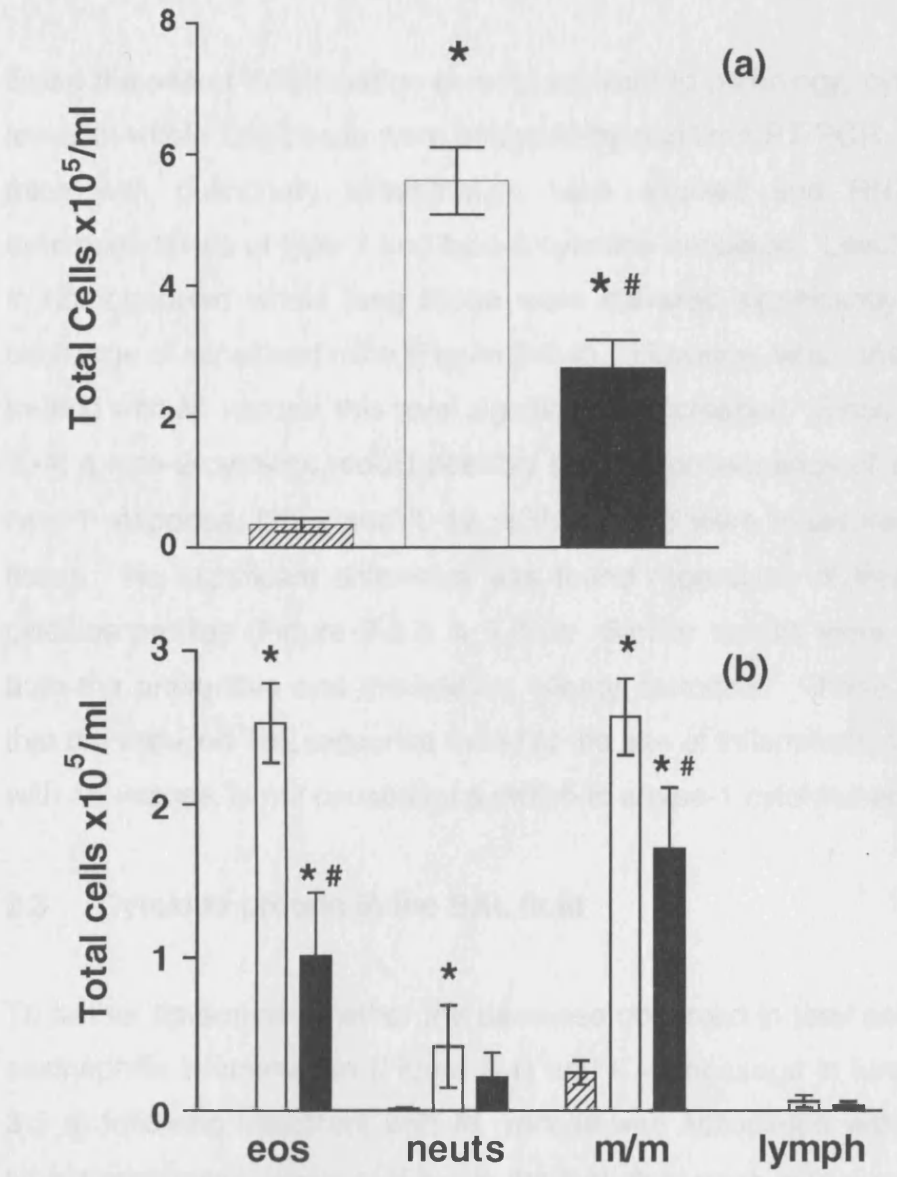
This chapter analyses pulmonary inflammation during allergen challenge. Whole lung tissue and BAL fluid were investigated for cytokines essential for both the development and alleviation of asthma, such as IL-4, IL-5, IFN-γ, IL-12, TGF-β and IL-10, to establish whether the mechanism behind the abrogation of allergic symptoms observed following *M. vaccae* treatment is due to a type-2 to type-1 switch, or whether immunoregulatory cytokines are involved.

2 Results

2.1 Cellular infiltrate in the BAL fluid

To confirm a suitable model of pulmonary inflammation, characterized by an excessive type-2 response, the lung tissue from mice were investigated after OVA sensitisation and challenge. To determine the extent of pulmonary inflammation, the total number and the specific type of cells recruited in the BAL fluid were established. Since allergic inflammation is characterized by an excessive type-2 immune response, it was determined whether treatment with *M. vaccae* alters cytokine production in the lungs. The following results were observed when both preventive and therapeutic allergy protocols were carried out.

Following OVA challenge, mice previously sensitized with OVA in Alum presented with a dramatic increase in total cell numbers in the BAL fluid (Figure 3-1 a). This cellular infiltrate was a result of allergen challenge, since challenge of OVA sensitized animals with saline alone did not result in cellular influx in the BAL fluid (Figure 3-1 a). However, OVA challenged animals treated with *M. vaccae* were observed to have a significantly decreased number of cells in the BAL fluid, compared to those mice treated with saline (Figure 3-1 a). The cellular infiltrate that characterizes allergic pulmonary inflammation consists for the most part of eosinophils, neutrophils and monocyte/MΦs. An elevation in the number of these cells was observed in the BAL fluid of mice challenged with OVA. However, mice treated with *M. vaccae* had significantly lower numbers of eosinophils and monocyte/MΦs in their BAL fluid (Figure 3-1 b). The decrease in eosinophilia did not result in an increase of neutrophilia at the site of inflammation. These results suggest that in OVA sensitized and challenged mice, treatment with *M. vaccae* significantly reduces the severity of pulmonary inflammation that follows allergen challenge.



Positive Control - saline treated and OVA challenged

Treatment Group - *M. vaccae* treated and OVA challenged

Negative Control – saline treated and saline challenged

Figure 3-1: Murine Allergic Pulmonary Inflammation Model – Cellular infiltrate into the lungs. Mice were treated with *M. vaccae* (black bar) or saline (white bar, shaded bar), sensitized with OVA/Alum, and challenged with either OVA (black bar, white bar) or saline (shaded bar). Total cell numbers (a) and specific cell types (b) were quantified in the BAL fluid (n=6-8 per group, * $p< 0.05$ compared to negative control, # $p<0.05$ compared to saline treated animals, data expressed as mean ± SEM, ANOVA used to test for significant differences, with a Bonferroni post test which compares all data groups). eos = eosinophils, neuts = neutrophils, m/m = monocytes/MΦs, lymph = lymphocytes

2.2 Cytokine mRNA in whole lung tissue

Since the site of inflammation is most relevant to pathology, cytokine message levels in whole lung tissue were analysed by real time RT-PCR. The lungs from mice with pulmonary inflammation were excised and RNA extracted to determine levels of type-1 and type-2 cytokine message. Levels of IL-4 mRNA in homogenised whole lung tissue were elevated significantly after the OVA challenge of sensitized mice (Figure 3-2 a). However, when the mice had been treated with *M. vaccae*, this level significantly decreased. Since the reduction of IL-4, a type-2 cytokine, could possibly be the consequence of an increase in a type-1 response, IFN-γ and IL-12 mRNA levels were measured in whole lung tissue. No significant difference was found regardless of treatment in either cytokine profiles (Figure 3-2 b & 3-2 c). Similar results were observed using both the preventive and therapeutic allergy protocols. These results suggest that the reduced Th2 response found at the site of inflammation of mice treated with *M. vaccae*, is not caused by a switch to a type-1 cytokine environment.

2.3 Cytokine protein in the BAL fluid

To further determine whether the decrease observed in total cellular infiltration, eosinophilic inflammation (Figure 3-1) and IL-4 message in lung tissue (Figure 3-2 a) following treatment with *M. vaccae* was associated with an increase in type-1 responses, levels of IFN-γ in the BAL fluid were measured. Levels of IL-5, IL-10 and TGF-β in the BAL fluid were also determined.

Upon OVA challenge of OVA sensitized mice, levels of IFN-γ, IL-5 and TGF-β in the BAL fluid of mice treated with *M. vaccae* remained unchanged, compared to levels observed in animals treated with saline (Figure 3-3 a & 3-3 b and 3-4 a). However, there was a significant increase in IL-10 protein (Fig 3- 4 b). These results were observed using both the preventive and the therapeutic protocols.

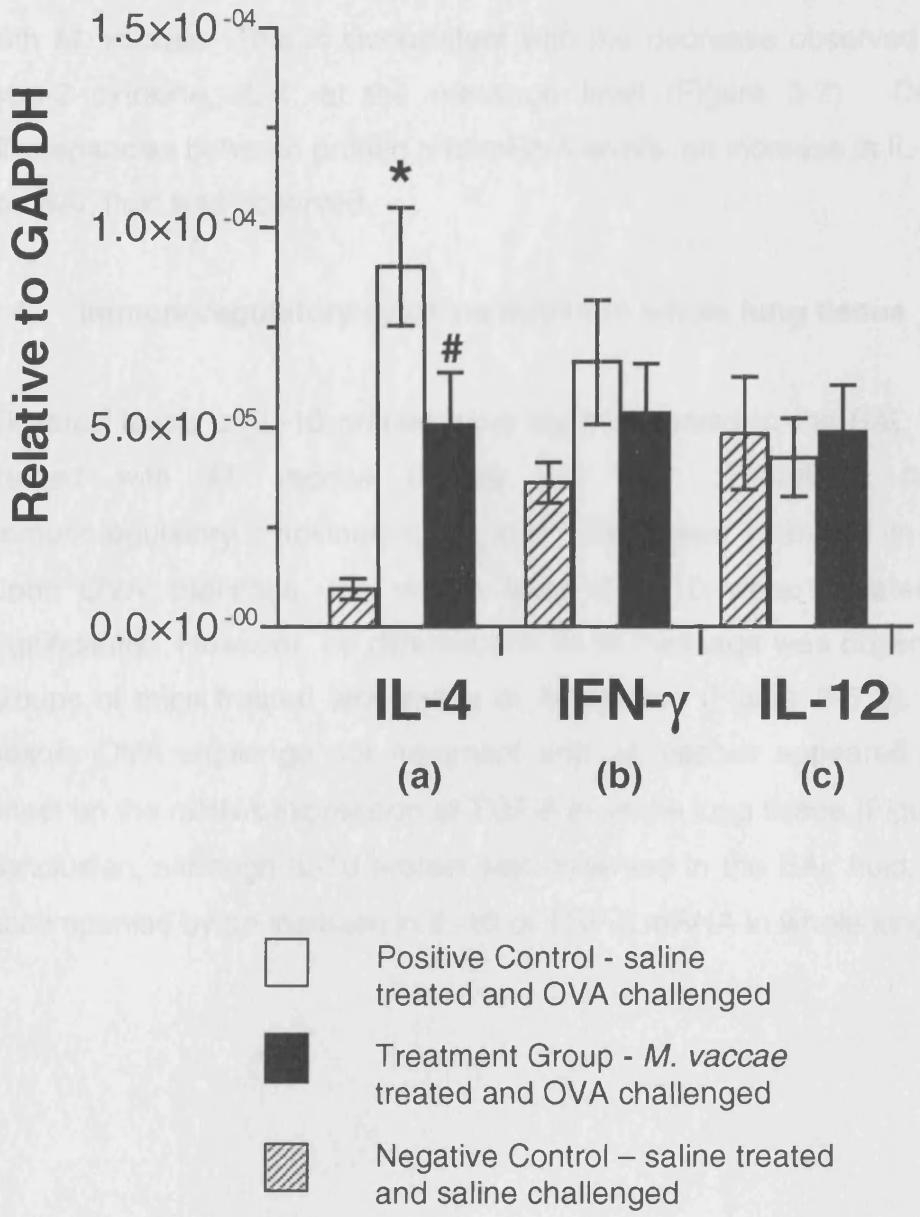


Figure 3-2: Murine Allergic Pulmonary Inflammation Model – IL-4, IFN-γ and IL-12 expression. Mice were treated with *M. vaccae* (black bar) or saline (white bar, shaded bar), sensitized with OVA/Alum, and challenged with either OVA (black bar, white bar) or saline (shaded bar). IL-4 (a), IFN-γ (b) and IL-12 (c) message in lung tissue was analysed using real time RT-PCR (n=4-6 per group, data expressed as mean ± SEM, * $p<0.01$ compared to negative control, # $p<0.05$ compared to saline treated animals, ANOVA used to test for significant differences, with a Bonferroni post test which compares all data groups).

Type-1 cytokine protein data support the mRNA profile observed in whole lung tissue, where no changes in IFN-γ or IL-12 are observed. However, no reduction was detected in IL-5 protein levels in the BAL fluid from mice treated with *M. vaccae.* This is inconsistent with the decrease observed in the other type-2 cytokine, IL-4, at the message level (Figure 3-2). Despite these discrepancies between protein and mRNA levels, an increase in IL-10 protein in the BAL fluid was observed.

2.4 Immunoregulatory cytokine mRNA in whole lung tissue

Elevated levels of IL-10 protein have been observed in the BAL fluid of mice treated with *M. vaccae* (Figure 3-4 b). Therefore, message for immunoregulatory cytokines IL-10 and TGF-β was analysed in whole lung. Upon OVA challenge, the mRNA level of IL-10 were elevated, albeit not significantly. However, no difference in IL-10 message was observed between groups of mice treated with saline or *M. vaccae* (Figure 3-5 b). In addition, neither OVA challenge nor treatment with *M. vaccae* appeared to have any effect on the mRNA expression of TGF-β in whole lung tissue (Figure 3-5 a). In conclusion, although IL-10 protein was observed in the BAL fluid, this was not accompanied by an increase in IL-10 or TGF-β mRNA in whole lung tissue.

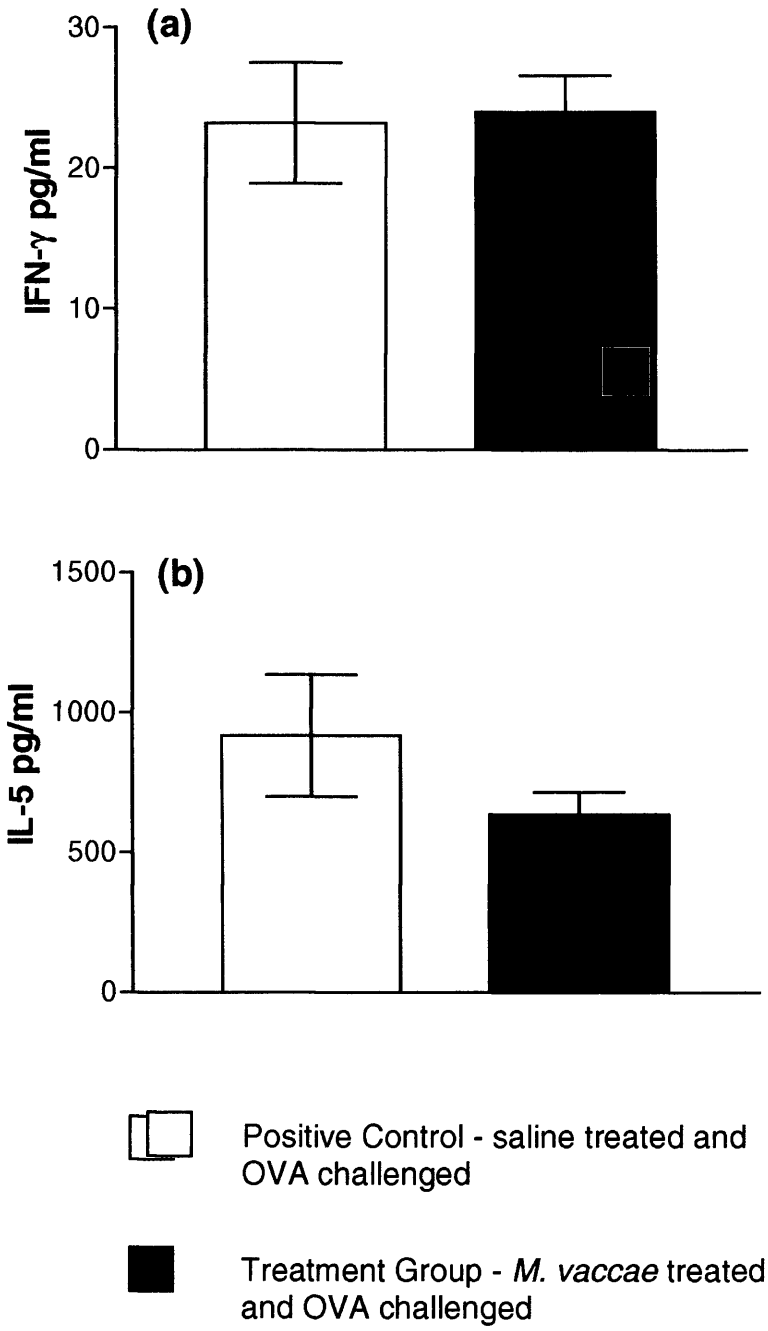


Figure 3-3: Effect of *M. vaccae* on the levels of IFN-γ and IL-5 in the BAL fluid of allergic mice. Mice were treated with *M. vaccae* (black bar) or saline (white bar), sensitized with OVA/Alum, and challenged with OVA. IFN-γ (a) and IL-5 (b) protein levels in the BAL fluid were analysed by ELISA (n=4-6 per group, data expressed as mean ± SEM, Student's t-test used to test for significant differences).

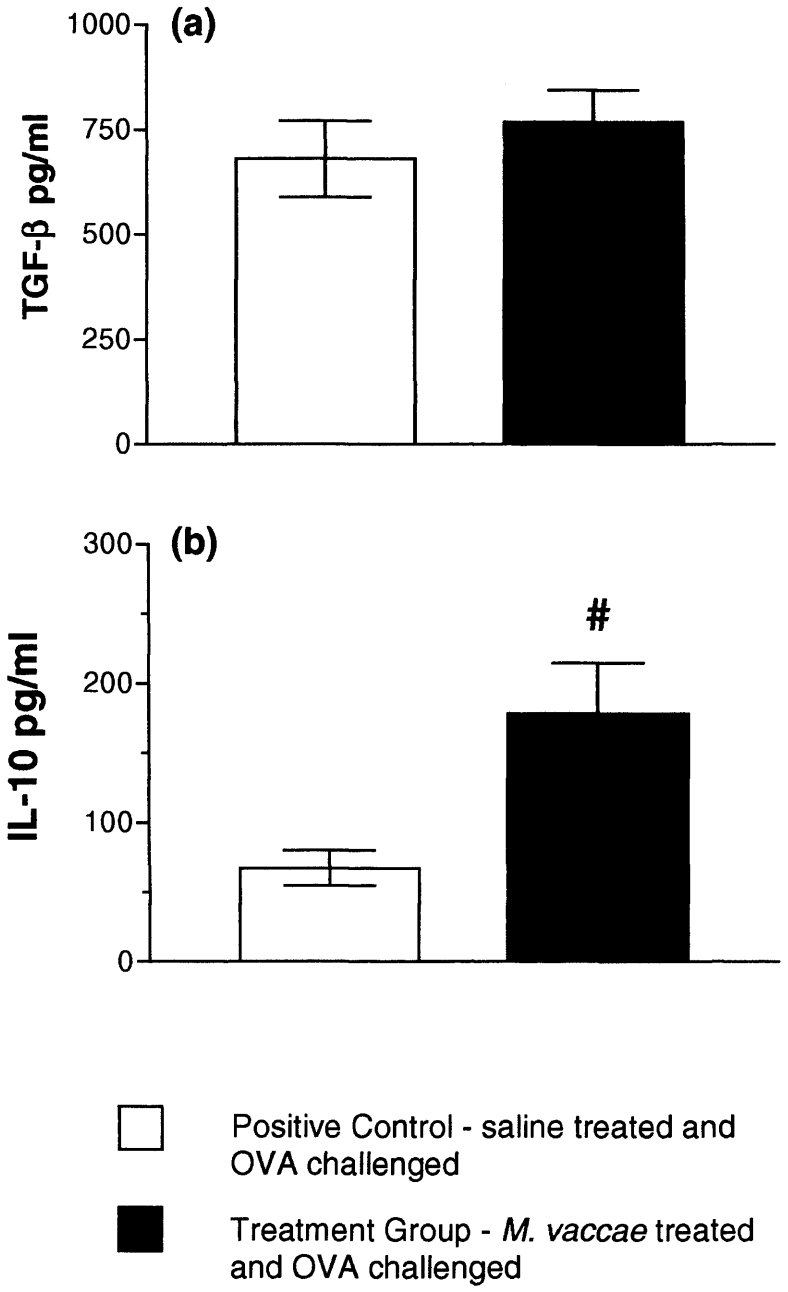


Figure 3-4: Effect of *M. vaccae* on the levels of TGF-β and IL-10 in the BAL fluid of allergic mice. Mice were treated with *M. vaccae* (black bar) or saline (white bar), sensitized with OVA/Alum, and challenged with OVA. TGF-β (a) and IL-10 (b) protein levels in the BAL fluid were analysed by ELISA (n=4-8 per group, # $p<0.05$ compared to saline treated animals, data expressed as mean ± SEM, Student's t-test used to test for significant differences).

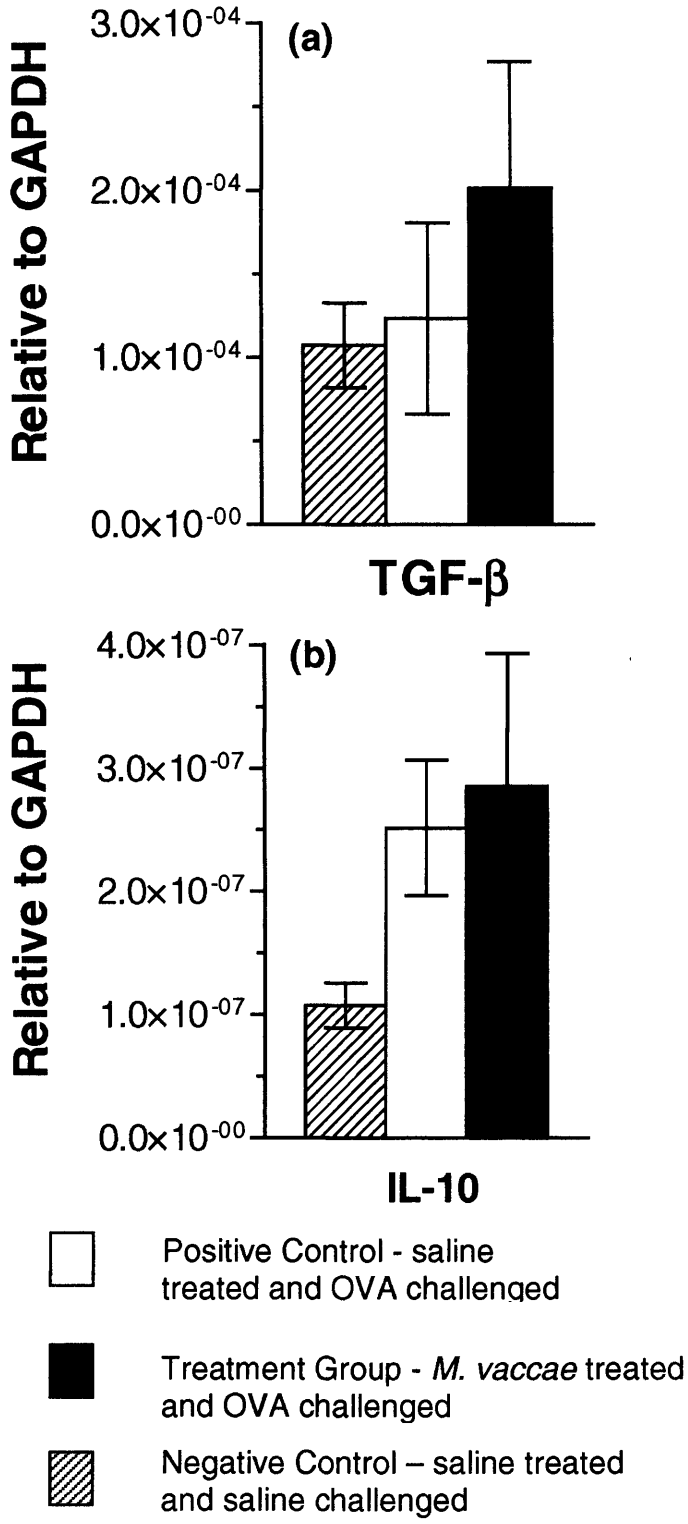


Figure 3-5: Murine Allergic Pulmonary Inflammation Model – TGF-β and IL-10 expression. Mice were treated with *M. vaccae* (black bar) or saline (white bar, shaded bar), sensitized with OVA/Alum, and challenged with either OVA (black bar, white bar) or saline (shaded bar). TGF-β (a) and IL-10 (b) message in whole lung tissue was analysed using real time RT-PCR (n=4-6 per group, data expressed mean ± SEM, ANOVA used to test for significant differences, with a Bonferroni post test which compares all data groups).

3 Discussion

The lung, as the site of inflammation, is most relevant to pathology and forms the focus of this chapter. Both the extent of the cellular infiltration and type-1, type-2 and immunoregulatory cytokine production in the lung were studied. Establishing cell number and cell type in the BAL fluid of mice sensitized to and challenged with OVA determined the extent of pulmonary inflammation. Cytokine production was established by carrying out an ELISA on the BAL fluid, and by RT-PCR on whole lung tissue. *M. vaccae* treatment significantly ameliorates pulmonary inflammation and eosinophilia, and decreases type-2 cytokine production without inducing a type-1 response. These observations confirm those reported by other groups [Hopfenspirger *et al* 2001, Zuany-Amorim *et al* 2002a, Smit *et al* 2003a, Smit *et al* 2003b,]. This chapter indicates that *M. vaccae* treatment significantly ameliorates classic inflammatory symptoms.

3.1 *M. vaccae* treatment induces a decrease in type-2 responses

Results in this chapter suggest that *M. vaccae* treatment significantly ameliorates inflammatory charcateristics, such as cellular influx and eosinophilia in the lungs of allergic animals. This has also been observed by several other collaborating groups [Zuany-Amorim *et al* 2002a, Smit *et al* 2003a]. The other results presented in this thesis show that *M. vaccae* affects the IL-4 levels in the lung. Upon OVA challenge of sensitized mice, levels of IL-4 mRNA were significantly elevated in whole lung tissue. In contrast, levels of IL-4 message were decreased in the animals that had been treated with *M. vaccae.* This suggests that *M. vaccae* treatment decreases the excessive type-2 response associated with asthma.

To establish whether the reduction observed in IL-4 message correlated with a type-2 cytokine protein level, IL-5 was measured in the BAL fluid. In spite of a decrease in IL-4 mRNA, no difference was detected in the levels of IL-5 protein in the BAL fluid. *M. vaccae* treatment resulted in reduced eosinophilia in the BAL fluid and IL-4 message in the lung tissue, which are both components of

the Th2 response. IL-5 levels were therefore expected to decrease, IL-5 being the type-2 cytokine primarily responsible for eosinophilic inflammation.

There are several possible explanations as to why IL-5 protein was not reduced in the BAL fluid. One may be the site at which this analysis was carried out. Although treatment with *M. vaccae* has been shown to reduce type-2-mediated pulmonary inflammation, the failure of IL-5 protein production in the BAL fluid to fall in parallel has been observed before, both by colleagues at SR Pharma [Rosa Brunet, Hunt *et al* – unpublished observations] and a collaborating group [Zuany-Amorim *et al* 2002a]. However, a reduction in IL-5 has been observed in the splenocytes of mice suffering from pulmonary inflammation and treated with *M. vaccae,* compared to levels from mice treated with saline [Wang *et al* 1998]. This suggests that *M. vaccae* may not be effecting IL-5 production at the site of inflammation, the lungs, but may influencing effector T cell function in lymphoid organs, such as the spleen.

Another possibility is that treatment with *M. vaccae* is affecting IL-5 production by effector T cells and eosinophils in the lung, but other cells are triggered to induce IL-5 instead. For example, inducible Tregs (termed Tr1 cells) have been observed to secrete IL-5 in addition to IL-10 [Groux *et al* 1997]. When CD4+ T cells isolated from DO11.10 mice were culture with splenic APCs and IL-10, they produced high levels of both IL-10 and IL-5, with little or no IL-2 and IL-4. These Tr1 cells also proliferated poorly in response to Ag stimulation. Since *M. vaccae* has been shown to induce a population of IL-10 secreting Tregs [Zuany-Amorim *et al* 2002b], it may be possible that these are another source of IL-5, masking any reductions that do occur as a result of treatment with *M. vaccae.* This may explain the inconsistent results observed between type-2 cytokine protein and message levels.

M. vaccae treatment of mice with allergic pulmonary inflammation generally results in a decrease in Th2-mediated inflammation. One possible mechanism for a decrease in type-2 responses is an induction of type-1 responses, already shown to occur *in vitro* [Mosmann *et al* 1989, Fitch *et al* 1993]. If this were the mechanism induced by *M. vaccae*, an increase in type-1 cytokines in the lungs would be expected.

3.2 *M. vaccae* does not induce an elevated type-1 response

Although a decrease was observed in the Th2 cytokine IL-4, along with the reduced allergen-induced eosinophilia, this was not associated with an increase in type-1 responses. IL-12 and IFN-γ message in whole lung tissue were investigated, but no difference was observed between allergic mice treated with *M. vaccae* and those treated with saline. In addition, no difference was observed in IFN-γ protein levels in the BAL fluid. Increased neutrophilia, which characterizes Th1-induced inflammation, was not detected in mice treated with *M. vaccae.* This confirms previous work, which reported in a similar model that the beneficial effects of *M. vaccae* were independent of IFN-γ. Upon OVA challenge, IFN-γ levels in the BAL fluid significantly decreased, when compared to levels in the BAL fluid of sensitized mice challenged only with saline. Treatment with *M. vaccae* did not induce any increase in IFN-γ production, compared to levels in the BAL fluid of animals treated with saline, as also observed in this chapter. In addition, transfer of splenocytes from donor mice treated with *M. vaccae* to recipient allergic mice was shown to inhibit airway eosinophilia. Transfer of splenocytes together with IFN-γ mAb did not reverse this suppression [Zuany-Amorim *et al* 2002a]. If *M. vaccae* inhibited excessive type-2 responses by inducing a Th1 response, blocking the action of IFN-γ would have suppressed Th1 responses and prevented the beneficial effect of *M. vaccae.* These results suggest that *M. vaccae* is not inducing a decrease in Th2-mediated inflammation by initiating a type-1 response.

3.3 *M. vaccae* induces regulatory cytokine IL-10 protein

Rather than affecting the Th1/Th2 balance, treatment of OVA sensitized and challenged mice with *M. vaccae* induced production of IL-10 protein in the BAL fluid. This increase in IL-10 protein the BAL fluid of mice treated with *M. vaccae* has been observed previously in other studies [Zuany-Amorim *et al* 2002a, Zuany-Amorim *et al* 2002b, Adams *et al* 2004]. This is important, since IL-10 has been described to have potent anti-inflammatory and immunoregulatory properties [Stampfli *et al* 1999, Umetsu *et al* 1999]. For example, in a murine model of pulmonary inflammation, administration of IL-10 directly into the lungs, using an adenoviral vector, results in an abrogation of airway eosinophilia and

decreased IL-4 and IL-5 protein in the BAL fluid of mice sensitized and challenged with OVA. In this model, no increase in IFN-γ protein was observed in the BAL fluid [Stampfli *et al* 1999]. Since IL-10 is induced by treatment with *M. vaccae* in the absence of IFN-γ, this suggests the possibility of immunoregulation, perhaps by Tregs [Zuany-Amorim *et al* 2002b].

3.4 *M. vaccae* does not induce regulatory cytokine TGF-β protein

Another cytokine with potential to initiate such a regulatory response is TGF-β [Shull *et al* 1992]. TGF-β has been observed to have an inhibitory effect on T cell proliferation [Ludviksson *et al* 2000]. In addition, TGF-β is sometimes secreted by inducible Tregs (termed Th3 cells) [Chen *et al* 1994]. In this thesis, TGF-β levels did not differ between treatment groups when measured in the BAL fluid. This cytokine is secreted, amongst other cell types, by eosinophils and bronchial epithelial cells, stimulating fibrosis and airway remodelling [Redington *et al* 1997]. Therefore, although it is described as important for suppression of allergic inflammation [Nakao *et al* 2001] TGF-β is also involved in the immunopathology of the disease. This duality means that measuring levels in the BAL fluid may not identify the actual cell source of TGF-β. *M. vaccae* treatment may be inhibiting one cell source of TGF-β whilst showing no effect on another. Hence no overall difference may be seen in the BAL fluid. This may account for the similar levels of TGF-β protein seen in the BAL fluids of animals treated with saline and those treated with *M. vaccae*. This suggests that investigation of individual cell sources may be necessary before differences in TGF-β levels can be detected.

3.5 *M. vaccae* does not affect the levels of immunoregulatory cytokines in whole lung tissue

There is a surprising contrast between results relating to mRNA and those relating to proteins. Elevated IL-10 protein was detected in the BAL fluid of OVA sensitized and challenged mice treated with *M. vaccae.* However, animals treated with *M. vaccae* did not have significantly elevated levels of IL-10 mRNA in whole lung tissue compared to animals treated with saline. Since *M. vaccae*-

induced IL-10 protein production, an increase in IL-10 mRNA at the site of inflammation was anticipated.

The discrepancy in the IL-10 data may be in part due to differences between the heterogeneity of the cell populations present in the lung tissue and those present in the BAL fluid. Compared to BAL fluid, lung tissue comprises a heterogeneous number of cell types, which may act as sources of cytokine. Many cells produce IL-10, not just T cells and the effect *M. vaccae* is having on IL-10 secretion of individual cell populations is so far relatively unknown. Hence, cytokine levels detected from all the different tissue resident cells may mask any differences that exist in individual cell populations. This may cause some of the discrepancy observed in the data between cytokine protein and mRNA levels. Indeed, a large difference was observed in both IL-10 and TGF-β mRNA levels between individual animals. The presence of IL-10 protein at the site of inflammation does suggest that it plays an important role in the decrease Th2 responses observed.

4 Conclusion

In a murine model of allergic pulmonary inflammation, treatment of mice with *M. vaccae* significantly reduced the excessive Th2 response induced by OVA challenge. Levels of IL-4 mRNA were reduced in the lungs, as well as cellular infiltration and eosinophilia in the BAL fluid. This reduction of type-2 mediated inflammation was not a consequence of a type-1 response in the lungs. No difference in IL-12 or IFN-γ expression was observed in whole lung tissue of mice treated with *M. vaccae*. In addition, no increase in IFN-γ protein in the BAL fluid was detected. Therefore *M. vaccae* treatment does not induce a type-2 to type-1 response switch. Interestingly, IL-10 protein was elevated in the BAL fluid, suggesting an intriguing role for *M. vaccae* in immunoregulation. To identify the possible source of IL-10, a specific cell subtype was chosen based on the literature, for further study and will be discussed in Chapter 4.

Chapter 4

Chapter 4

Results 2: Pulmonary CD11c+ APCs induced by treatment with *M. vaccae:* Analysis of cytokine profile and phenotype

Data in this chapter have contributed towards two posters presented at the British Society of Immunology meetings in Harrogate in 2002 and 2003, and a manuscript published in the European Journal of Immunology.

Adams, V. C., Hunt, J. R. F., Rosa Brunet, L., and Rook, G. A. W.
Effect of *Mycobacterium vaccae* on ICOS expression in a murine model. *Immunology* (2002); 107 [Supplement 1]; 111. **OP141**

Adams, V. C., Hunt, J. R. F., Martinelli, R., Palmer, R., Rook, G. A. W. and Rosa Brunet, L.
Mycobacterium vaccae induces a CD11c+ population that produces regulatory cytokines in the lungs of allergic mice. *Immunology* (2003); 110 [Supplement 1]; 50. **13.14**

Adams V. C., Hunt J. R. F., Martinelli R., Palmer R., Rook G. A. W., and Rosa Brunet L. *Mycobacterium vaccae* induces a population of pulmonary CD11c+ cells with regulatory potential in allergic mice *European Journal of Immunology.* (2004); **34**, 631-638.

1 Introduction

Asthma is described as a type-2 mediated disease. However, to initiate Th2 cell activation, Ag presentation by APCs is required before any response can be mounted. The group of cells collectively termed APCs, generally consist of DCs, MΦs and B cells. They are all capable of capturing and presenting Ag to T cells, consequently initiating T cell activation. The important role of APCs in allergic asthma is indicated by the increased numbers of APCs which infiltrate the lungs after allergen sensitisation and challenge [Lambrecht *et al* 1999]. In addition, APCs have also been shown to play a regulatory role in asthma [Spiteri *et al* 1991, Stumbles *et al* 1998]. Of the APCs, DCs are the most efficient at processing and presenting Ag to T cells, and have been shown to be the APCs responsible for initiation of asthma [van Rijt *et al* 2005]. This chapter investigates the possible role *M. vaccae* plays in inducing APCs with a regulatory phenotype.

1.1 APCs initiation of asthma

APCs are essential for both the induction of allergic asthma [Holt *et al* 2000, van Rijt *et al* 2005] and immunoregulation [Terrazas *et al* 2001]. Evidence for initiation of asthma will be presented below, and evidence for immunoregulation will be described later (sections 1.2 and 1.3). Much of the evidence for APC initiation of asthma has been obtained using DCs, but MΦs and B cells are also capable of presenting Ag and playing a role in allergy. In this chapter, APCs will only be referred to as DCs if the reference being discussed has characterized them as DCs. Otherwise the term APCs will be used to refer to all cells capable of presenting Ag to T cells.

Upon inhalation, APCs capture Ag in the peripheral lung tissue. These cells then migrate to the LNs where T cells are continuously circulating [Vermaelen *et al* 2001]. Migration directly to the T cell areas of the LN is a specialised DC function, although MΦs have also been observed to transport Ag to the LNs [Havenith *et al* 1993]. Once in the LNs, APCs express high

levels of activation and co-stimulatory molecules, such as MHC II, CD80 and CD86 [Roitt *et al* 2001]. Ag is presented to the T cell via the interaction of the TCR with the peptide-MHC complex, and a co-stimulatory signal, such as the interaction of CD28 and the CD80 or CD86 ligand [Lenschow *et al* 1996]. Without APCs, allergen would never be presented to T cells, and a type-2 response would not be initiated.

Interaction of the TCR and MHC II receptors on APCs and T cells, in conjunction with CD28 and B7 ligation, leads to T cell proliferation via production of IL-2 from T cells. This cytokine binds to low affinity IL-2 receptors on the T cell surface, which consist of the β and γ chains. Interactions between the MHC II-TCR and co-stimulatory molecules also result in upregulation of the IL-2 receptor α chain. Together these chains form a high affinity receptor [Roitt *et al* 2001]. This means that once activated, T cells can be self-propagating.

Once activated, the CD4+ T cells are influenced by cytokine production to develop either a Th1 or Th2 phenotype. IL-12 secreted by MΦs and DCs promotes IFN-γ producing Th1 cells [Hsieh *et al* 1993, Macatonia *et al* 1995]. In contrast, IL-4 secretion induces Th2 development. The primary source of IL-4 remains an area of debate, but possibly is secreted by mast cells or basophils [Swain *et al* 1990, Seder *et al* 1992].

Upon activation, Th2 CD4+ T cells secrete Th2 inflammatory cytokines, such as IL-4 and IL-5 [Caux *et al* 1994]. This cytokine release results in increased IgE secretion, histamine release and eosinophil activation (described in more detail in Chapter 3). These inflammatory symptoms are all induced by type-2 cytokines, and allergic asthma is therefore termed a type-2 mediated disease. APCs are responsible for activating CD4+ T cell cytokine secretion, and therefore APCs are essential for initiating type-2 mediated inflammation.

Experimental animal models have shown that APCs are essential for inducing type-2 mediated airway inflammation. DCs in particular are

responsible for sensitisation to inhaled Ag, are essential for initiation of eosinophilia and are important for the maintenance of inflammation. For example, when injected in the airways, FITC-conjugated OVA was observed to be taken up by DCs, which migrated to the DLNs and induced T cell proliferation of naive OVA-specific T cells [Vermaelen *et al* 2001], suggesting DCs play a role in T cell sensitisation to OVA. In addition, eosinophilic airway inflammation can be initiated in naïve rats by simply transferring OVA-pulsed BM-DCs directly into the lungs [Lambrecht *et al* 2000a, Lambrecht *et al* 2000b]. Furthermore, depletion of airway DCs during a secondary immune response to OVA resulted in a decrease of cellular infiltrate into the BAL fluid, reduced eosinophilia and impaired Th2 cytokine secretion. These suggest that DCs are involved both in the initiation and maintenance of airway inflammation.

MΦs have also been observed as important in airway inflammation. In sensitized mice, depletion of alveolar MΦs resulted in significantly increased eosinophil numbers in the BAL fluid and IgE levels in the peripheral blood, when compared to animals where alveolar MΦs were not depleted [McWilliam *et al* 1996, Lambrecht *et al* 1998, Lambrecht *et al* 1999]. In conclusion, APCs in general are a potential target for therapeutic intervention.

1.2 Suppression of T cell proliferation by APCs

APCs have also been associated with mucosal tolerance, and play an important role in regulating immune responses. There are several possible mechanisms that both DCs and MΦs can adopt to regulate the immune system. One is by the secretion of suppressive molecules. One example of such a suppressive product is NO. When secreted from alveolar MΦs, NO suppresses the function of local DCs, keeping them in an immature Ag capturing mode expressing low levels of MHC II and B7 [Holt *et al* 2000]. Lower expression of activation and co-stimulatory markers results in decreased T cell activation. Another example is the expression of

indoleamine 2,3 dioxygenase (IDO). IDO depletes tryptophan, and results in release of toxic catabolites, such as L-kynurenine and picolinic acid. These can lead to inhibition of T cell proliferation [Frumento *et al* 2002, Swanson *et al* 2003].

APCs secreting regulatory cytokines have also been observed. For instance two poorly characterised monoclonal antibodies, designated RFD1 and RFD7, that bind to "MΦ"-like cells appear to define a regulatory macrophage subset. RFD1 is an interdigitating cell marker, which recognises an MHC II associated epitope. RFD7 is a mature tissue MΦ cell marker, which recognises a predominantly cytoplasmic Ag [Allison *et al* 1991, Taams *et al* 1999]. "Suppressive" MΦs have been observed as RFD1+ RFD7+ and can be obtained *in vitro* following exposure of human blood derived monocytes to IL-10. These cells secrete IL-10 and can suppress T cell proliferation *in vitro* when cultured with allogeneic T cells in a Mixed Lymphocyte Reaction (MLR) assay [Tormey *et al* 1998]. These observations suggest that MΦs may play a pivotal role in APC mediated immunoregulation through secretion of regulatory cytokines.

Similar secretion of regulatory cytokines by other APCs has also been observed. APCs that mediate regulatory responses have been observed following injection of mice with oligosaccharides derived from schistosome, lacto-*N*-fucopentaose III (LNFPIII) and lacto-*N*-neotetraose (LNnT). These modified APCs secreted low levels of proinflammatory cytokines, such as IL-12 and IFN-γ, and increased levels of the regulatory cytokines IL-10 and TGF-β. These APCs also suppressed T cell proliferative responses *in vitro,* when added to CD4+ T cells previously stimulated with schistosome derived oligosaccharide [Terrazas *et al* 2001].

Secretion of regulatory cytokines by APCs plays an essential role in the induction of Tregs [Akbari *et al* 2001, Mahnke *et al* 2003]. For example, DCs isolated from LNs of mice 24 hours after exposure to OVA produced IL-10 and induced OVA-specific T cells to also produce IL-10. These IL-10-

secreting T cells blocked development of AHR and eosinophilia [Akbari *et al* 2001]. IL-10-secreting DCs expressed high levels of co-stimulatory markers, such as CD80, CD86 and ICOS-L [Akbari *et al* 2001, Akbari *et al* 2002]. CD80 (B7.1) and CD86 (B7.2), as well as being ligands for CD28, also bind the receptor CTLA-4 [Greenwald *et al* 2002]. CTLA-4 is found on T cells and engagement of CTLA-4 with CD80 or CD86 results in suppressed T cell proliferation. Due to its negative regulatory properties, CTLA-4 is highly, although not exclusively, associated with Tregs [Grohmann *et al* 2002].

The ICOS-ICOS-L pathway is a co-stimulatory pathway, similar to the CD28-B7 pathway. Engagement between ICOS and ICOS-L results in augmented T cell proliferation [Coyle *et al* 2000]. Although blockade of this co-stimulatory pathway has suggested that it is essential for the initiation of type-2 mediated airway inflammation [Gonzalo *et al* 2001, Tescubia *et al* 2001], it has also been observed to be involved in DC-mediated induction of Tregs. The development of OVA-specific IL-10 secreting Tregs and reduction of airway inflammation can be inhibited by blockade of ICOS-L with neutralising Abs. This suggests that the induction of Tregs may be dependent on the ICOS-ICOS-L pathway [Akbari *et al* 2002].

1.3 Immunoregulatory cytokine secretion by APCs

The cytokines IL-10, TGF-β and IFN-α, have been reported to have immunoregulatory properties [Umetsu *et al* 1999, Levings *et al* 2001, Ito *et al* 2001]. Treg secretion of IL-10 and TGF-β is believed to mediate T cell tolerance [Akbari *et al* 2002, Zuany-Amorim *et al* 2002b]. APCs have also been observed to secrete these cytokines. Secretion of IL-10 in particular by APCs has been observed to suppress immune responses both in humans and murine models [Tormey *et al* 1998, Terrezas *et al* 2001]. For example, monocytes isolated from the peripheral blood of asthmatic patients are more efficient at stimulating an MLR when cultured with allogeneic T cells than monocytes isolated from healthy people. Treatment of these monocytes from

asthmatic patients with exogenous IL-10 inhibited this T cell proliferation [Tormey *et al* 1998].

Even though the majority of the literature published on the regulatory properties of IL-10 and TGF-β focuses on secretion by Treg [Chen *et al* 1994, Groux *et al* 1997, Levings *et al* 2002], the development of Tregs themselves also appears to be dependent upon the presence of these two cytokines [Nakao *et al* 2000, Zuany-Amorim *et al* 2002b, Akbari *et al* 2002, Oh *et al* 2002, Dao Nguyen *et al* 2004]. Hence other cell sources of these cytokines are involved in Treg induction and Treg function. APCs have been observed to secrete IL-10 and under specific conditions TGF-β [Dhadopkar *et al* 2001, Mahnke *et al* 2001, Lutz *et al* 2002, Adams *et al* 2004]. There is now a growing body of evidence that a particular subset of APCs initiates Treg development from non-committed T cell or memory T cells. These APCs, referred to as "regulatory" APCs [Smits *et al* 2005], are DCs which tend to secrete low levels of IL-12 and high levels of IL-10 [Jonuleit *et al* 2000, Gasche *et al* 2000, de Heer *et al* 2004].

IFN-α is another cytokine described as having immunoregulatory properties. This cytokine has long been considered an important mediator of the innate immune response, and has stimulatory effects on NK cells and MΦs. IFN-α is generally secreted by APCs in response to viral infections [Colonna *et al* 2002]. Recombinant IFN-α has been used as a short term Hepatitis C (HCV) treatment for several years, with 40-60% of patients responding to this treatment in combination with ribavirin (a broad spectrum antiviral). There is an improved level of response, when compared to the response of patients treated with ribavirin alone [Davis *et al* 1997, Hoffman *et al* 2005]. Evidence has also highlighted the ability of IFN-α to support proliferation and survival of specific T cell subsets [Tough *et al* 1996, Akbar *et al* 2000]. For example, survival of anergic T cells is inhibited by the addition of IFN-α neutralising mAb to the *in vitro* culture [Lombardi *et al* 2000]. A proposed mechanism of this suppression is the ability of IFN-α to delay entry of cells into the G_1

phase of the cell cycle, resulting in anergy of the effected cell [Dondi *et al* 2004].

In addition to maintaining anergy, exogenous IFN-α, together with IL-10 has also been observed to induce Tregs. Culture of CD4+ T cells with IL-10 and IFN-α resulted in T cells that secreted IL-10 and had a low proliferative capability. When co-cultured *in vitro* with APCs and naïve T cells, these Tregs significantly inhibited CD4+ T cell proliferation [Levings *et al* 2001]. IFN-α also upregulates the secretion of IL-10 by DCs. IL-10 in turn promotes the induction of T cells with regulatory properties [Bilsborough *et al* 2003]. These data suggest that IFN-α plays a role in the maintenance of immune tolerance, which is mediated by APCs.

1.4 Subsets of DCs

Of all APCs, DCs are the most efficient at presenting Ag to T cells. Early studies identified two main DC subsets, classified mainly by their cell surface phenotypes [Shortman *et al* 2002]. Some of these subsets of DCs have been observed to play immunoregulatory roles at different stages of maturity. The "myeloid" DCs were described as CD11c+CD11b+MHC II+CD8α- [Inaba *et al* 1993] and are derived from monocytes or myeloid precursors that retain the ability to generate MΦs [Ardavin *et al* 2001]. "Lymphoid" DCs were described as CD11c+CD11b-MHC II+CD8α+ [Ardavin *et al* 1993] and originate from lymphoid-committed precursors [Ardavin *et al* 2001].

More recently a third DC subset was described in humans [Cella *et al* 1999, Jarrossay *et al* 2001]. These cells initially have plasma like morphology, but upon activation acquired DC morphology [Liu *et al* 2001, Shortman *et al* 2002]. Termed "plasmacytoid" DCs, they were observed to be CD11c+ Gr-1+ B220+ and CD8α+. Whether these cells are of myeloid or lymphoid origin remains unclear. These pDCs have the ability to suppress the generation of effector T cells. Stimulation of pDCs with CpG resulted in IL-10 secretion, and incubation of pDCs with naïve CD4+ T cells resulted in Treg induction

[Nakano *et al* 2001, Martin *et al* 2002]. They also secrete large amounts of IFN-α [Cella *et al* 1999, Siegal *et al* 1999, Dalod *et al* 2002].

1.5 Phenotype of Immunoregulatory DCs

It was originally believed that Tregs could only be induced by immature DCs. The current hypothesis is that immature, semi-mature and mature DCs can all play a role in Treg development. Immature DCs induce development of naïve T cells into Tregs by lacking expression of co-stimulatory molecules, such as CD80 and CD86. CD80 and CD86 have two receptors to which they bind, CD28 and CTLA-4. Ligation of CD80 or CD86 with CD28 induces T cell activation and proliferation, whilst ligation with CTLA-4 inhibits T cell proliferation. In addition, CTLA-4 also has a stronger binding avidity to CD80 and CD86 compared to CD28, so it is more likely to bind these ligands. With less CD80 and CD86 available, the CD28 receptor is out competed by the other co-stimulatory receptor CTLA-4 [Jonuleit *et al* 2000].

Semi-mature DCs acquire the phenotype of mature DCs, for example high expression of co-stimulatory molecules and the ability to migrate to the LNs. However these DCs lack the ability to secrete proinflammatory cytokines, such as IL-12. They are however, producing IL-10 hence they are capable of triggering Treg development [Lutz *et al* 2002]. Mature DCs have also been observed to induce tolerance by release of IL-10. These DCs express high levels of MHC II, CD80 and CD86, and high levels of the chemokine CCR7 and are capable of secreting IL-12 [Gasche *et al* 2000, Jump *et al* 2002, Wilson *et al* 2003, Kobayashi *et al* 2004]. DCs of all levels of maturity have been observed to induce tolerance. In addition, whether the DCs are from lymphoid or myeloid origin appears to make no difference to the probability that they will acquire a regulatory phenotype.

Under homeostatic conditions, three subsets of DCs have been observed to exhibit distinct regulatory features. $CD11c^{Hi}$ mDCs that continuously migrate to the LNs as immature or semi-immature DCs have been shown to drive the

development of Ag-specific Tregs. These Tregs are specific for ubiquitous or self-Ag [Geissmann *et al* 2002, Dhodapkar *et al* 2002]. Other studies show that immature pDCs can drive the development of both IL-10 producing CD8+ Tregs [Gilliet *et al* 2002] and CD4+ Tregs [Martin *et al* 2002]. These pDCs induce tolerance to harmless inhaled Ags in the lungs and pDCs have been observed in murine models of pulmonary inflammation [Kadowaki *et al* 2000].

Although the level of maturity does not appear to be a limiting factor, cytokine secretion of IL-10 is a feature of all "regulatory" DCs. DCs isolated from mucosal sites, such as the lung and PP, and in immune privileged sites, like the anterior chamber of the eyes, have a unique cytokine phenotype, secreting low levels of IL-12 and high levels of IL-10. These DCs also drive Treg development [Wilbanks *et al* 1990, Jump *et al* 2002, de Heer *et al* 2004]. Interestingly, mDCs found at these mucosal surfaces appear to be mature, expressing high levels of MHC II, CD80 and CD86 [Akbari *et al* 2002, Kobayashi *et al* 2004, Goddard *et al* 2004].

In the lungs of OVA-sensitized mice, pDCs have been observed to take up inhaled Ag. When pDCs are depleted from the lungs, enhanced eosinophilic inflammation is observed after OVA challenge compared to control mice, which had not undergone pDC depletion. This suggests that pDCs may have the ability to inhibit eosinophilic inflammation in response to an otherwise harmless Ag. Depletion of pDCs also led to increased IgE and Th2 cytokine production, exacerbating pulmonary inflammation. Furthermore, the suppressive effects pDCs have on the initiation of airway inflammation are transferable. Adoptive transfer of OVA-pulsed pDCs to OVA-sensitized mice did not induce T cell division after OVA challenge, but suppressed it [de Heer *et al* 2004]. A possible mode of action that pDCs may adopt is to induce Tregs. T cells stimulated with pDCs obtained from the MLN of OVA-exposed mice developed the ability to suppress naïve T cell proliferation [de Heer *et al* 2004]. Similar observations have been made from studying pDCs isolated from human peripheral blood. When pDCs were cultured together with CD4+ T cells, they adopted an anergic state and secreted increase amounts of IL-

10 [Kuwana *et al* 2001]. These data suggest that pDCs may possibly be involved in protection against inflammatory responses to harmless Ag, maybe through Treg development.

1.6 Pathogen induction of "regulatory" DCs

A role for "regulatory" DCs fits well with the revised version of the hygiene hypothesis. "Regulatory" DCs may be important for homeostasis at mucosal surfaces, but these same properties can easily be manipulated by invading pathogens to evade immunity. Several pathogens have been observed to induce "regulatory" DCs as a way of preventing immune responses. One mechanism adopted involves blocking the ability of the APCs to mature, thereby preventing T cell activation. Examples of pathogens that exploit this mechanism include the malarial parasite *Plasmodium falciparum* and HCV. *Plasmodium falciparum* infected erythrocytes have been observed to bind to the scavenger receptor CD26 on DCs. This binding inhibits LPS-induced maturation. The capacity for the DC to activate naïve T cells is therefore inhibited [Urban *et al* 1999]. In patients chronically infected with HCV, elevated numbers of IL-10 secreting CD4+ T cells have been observed [MacDonald *et al* 2002]. *In vitro* studies using human DCs suggest that DC maturation, upregulation of co-stimulatory molecules and cytokine production are severely inhibited upon exposure to the HCV core protein [Dolganiuc *et al* 2003]. This suggests that *Plasmodium falciparum* and HCV may induce "regulatory" APCs to evade immunity.

An alternative mechanism used by pathogens to evade immunity is through the manipulation of the cytokine secretion of DCs. These pathogens induce the production of variable quantities of IL-10, a cytokine previously described as instrumental for the development of Tregs. Examples of such pathogens include the helminth *Schistosoma mansoni* and the bacterium *Bordetella pertussis*. *In vitro* studies with human monocyte derived DCs have shown that *Schistosoma mansoni* derived lysophosphatidylserine (which is a glycerophospholipid involved in mast cell activation and hence histamine release) triggers maturation of DCs. These DCs are characterised by

decreased secretion of IL-12, and increased production of IL-10 [van der Kleij *et al* 2002]. *Bordetella pertussis,* which causes whooping cough, which is often complicated by severe pneumonia, has been observed to induce IL-10 secreting T cells. These T cells were isolated from the lungs of infected mice, and were specific for the *Bordetella pertussis* derived virulence factor filamentous haemagglutinin (FHA). FHA enhances maturation of DCs, inhibits DC secretion of IL-12 and induces DC production of IL-10. These mature DCs drive development of Tregs, which are capable of blocking Th1 responses in secondary, unrelated infections such as Influenza A virus [McGuirk *et al* 2002]. These data suggest that pathogens avoid host detection by suppressing the immune system using "regulatory" DCs. It is also possible that pathogenic microbes mimic the mechanisms already adopted by non-pathogenic organisms, such as *M. vaccae,* to avoid activation of the immune system.

1.7 Hypothesis

In a murine model of OVA-induced pulmonary inflammation treatment with *M. vaccae* results in the induction of Tregs, which played a role in alleviating symptoms of disease. IL-10 and TGF-β have been shown as essential for the function of these Tregs, since blockade *in vivo* with mAbs prevented the therapeutic affect of *M. vaccae.* This suggests that the Tregs which *M. vaccae* induces are dependent on these cytokines for their function [Zuany-Amorim *et al* 2002b]. An alternative source of IL-10 and TGF-β, besides from Tregs themselves, may therefore exist. Treg induction by DCs which secrete regulatory cytokines, such as IL-10 has been observed by several groups [Jump *et al* 2002, Akbari *et al* 2002, de Heer *et al* 2004, Jonuleit *et al* 2000, Lutz *et al* 2002, Gasche *et al* 2000]. For this reason, the effect of treatment with *M. vaccae* on pulmonary CD11c+ APCs phenotype was investigated.

CD11c is a marker that is expressed by lymphoid DCs, mDCs and pDCs [Metlay *et al* 1990]. It is also known as the integrin αX subunit, and combines with CD18 (integrin β2 subunit) to form the integrin p150,95 (αXβ2). Abs for CD11c are often used for isolation of DCs. In spite of this,

the existence of cells that express both CD11c and the MΦ markers F4/80 or CD14 has been described [Ancuta *et al* 2000, Hogg *et al* 2003]. This leaves doubts on the identity of a CD11c+ cell as a DC or MΦ. Therefore, in this thesis CD11c+ cells will be referred to as APCs.

This chapter investigates pulmonary CD11c+ APCs isolated from allergic mice, treated with or without *M. vaccae,* to establish whether these APCs have regulatory potential. Production of regulatory cytokines, such as IL-10, TGF-β and IFN-α by pulmonary CD11c+ APCs was measured. These cells were also characterized for cell surface marker expression to establish their phenotype.

2 Results

2.1 Cytokine message of pulmonary CD11c+ APCs

The role of pulmonary CD11c+ APCs in a murine model of pulmonary inflammation was investigated. It was determined whether treatment with *M. vaccae* induced IL-10-producing pulmonary CD11c+ APCs. CD11c+ APCs have previously been observed to produce IL-10 following i.n. exposure to OVA, and have been reported to be critical for the development of Tregs [Akbari *et al* 2001, Akbari *et al* 2002]. The following results were observed when both preventive and therapeutic allergy protocols were carried out.

To determine whether treatment with *M. vaccae* induced IL-10 expression in CD11c+ APCs, these cells were isolated from the lungs of mice with allergic pulmonary inflammation by positive selection with microbeads. RNA was extracted and the cytokine profile analysed. Cytokines IL-10, TGF-β and IFN-α were observed to be significantly elevated in the CD11c+ APCs isolated from the lungs of mice treated with *M. vaccae,* when compared to cells from animals treated with saline (Figures 4-1 a, Figure 4-1 b & Figure 4-1 c). This effect was not seen in the CD11c- fraction collected. These results suggest that *M. vaccae* treatment induces CD11c+ APCs to express elevated levels of IL-10, TGF-β and IFN-α message.

To confirm that the observed increase in mRNA is restricted to immunoregulatory cytokines, IL-12 and IFN-γ message were also analysed. Expression levels of IL-12 and IFN-γ were found to be unchanged after treatment with *M. vaccae*, in both the pulmonary CD11c+ and CD11c- cell fractions (Figure 4-2 a & Figure 4-2 b). These observations suggest that in a model of allergic pulmonary inflammation, treatment with *M. vaccae* induces a population of CD11c+ APCs in the lung tissue, which preferentially produce immunoregulatory cytokines IL-10, TGF-β and IFN-α mRNA, but not message levels of type-1 cytokines IL-12 and IFN-γ.

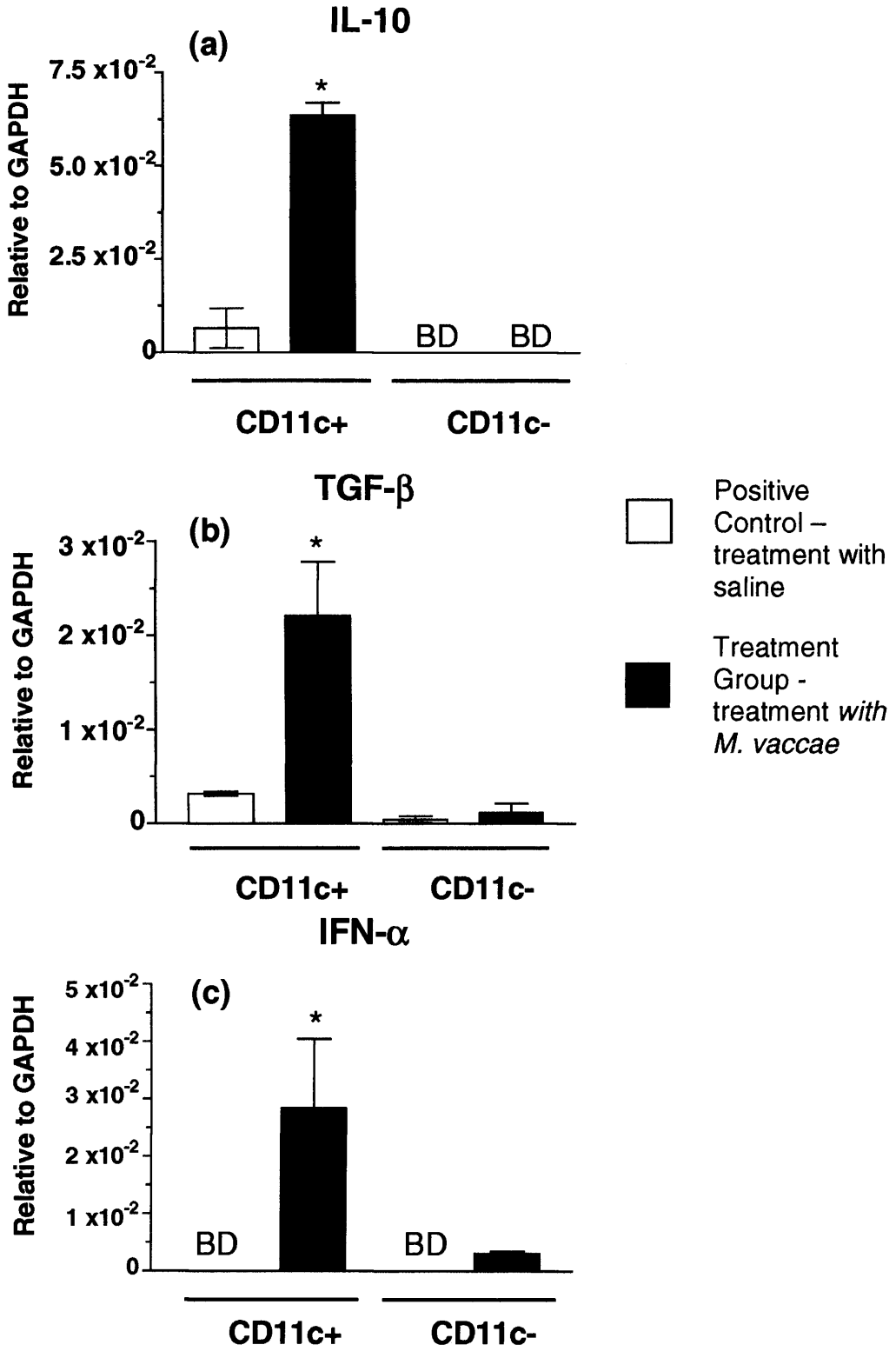


Figure 4-1: Expression of IL-10, TGF-β and IFN-α message by Pulmonary CD11c+ and CD11c- cells. Mice were treated with *M. vaccae* (black bar) or saline (white bar), sensitized with OVA/Alum, and challenged with OVA. Cells were isolated from the lungs and separated into CD11c+ and CD11c- fractions. IL-10 (a), TGF-β (b) and IFN-α (c) message levels were analysed using real time RT-PCR (samples from each treatment group were pooled, n=6-8 mice per group. Data expressed as mean ± SD, BD = below detection, * $p<0.05$ compared to positive control one-tailed Student's *t*-test used to determine significant differences).

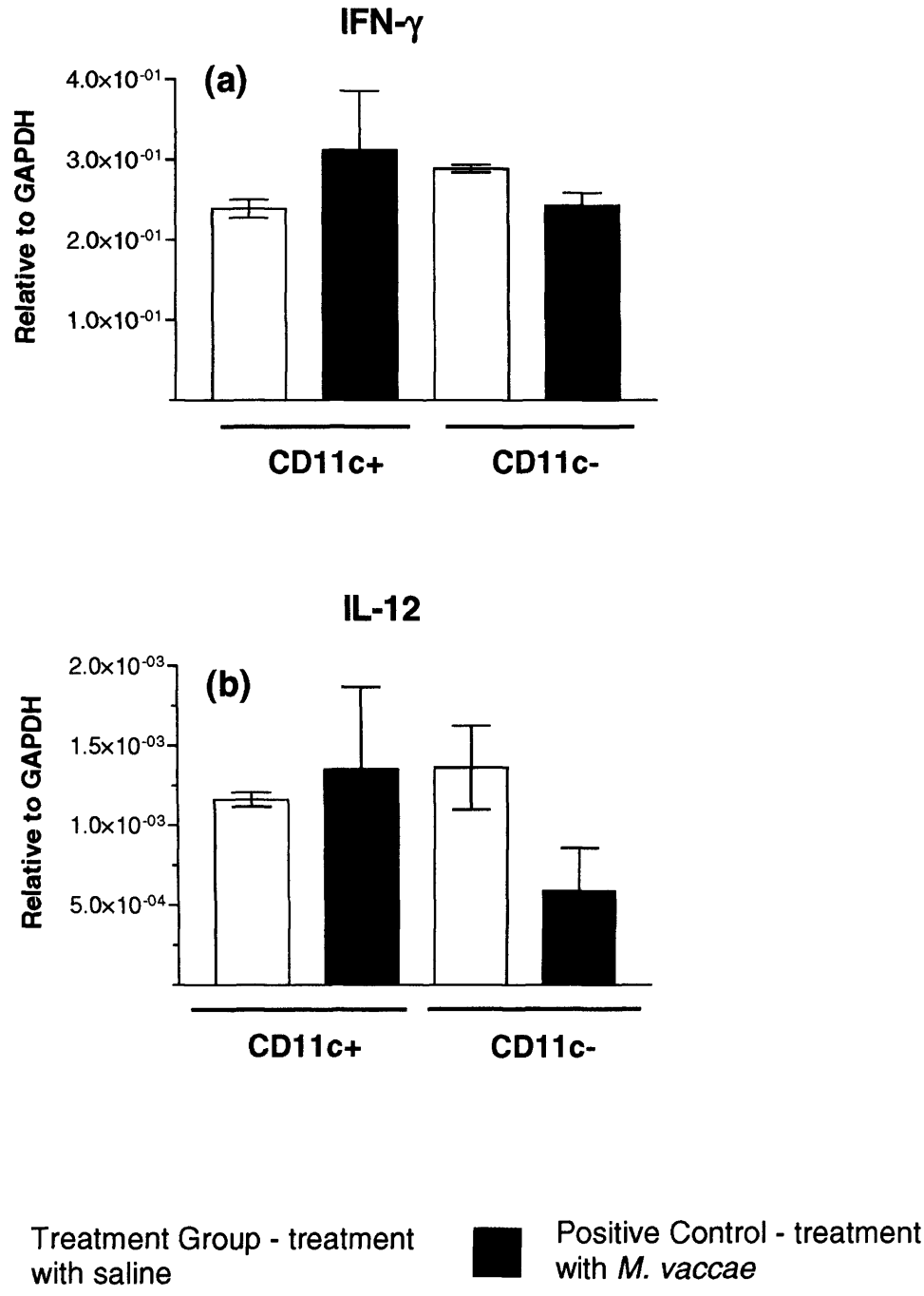


Figure 4-2: Expression of IFN-γ and IL-12 by Pulmonary CD11c+ and CD11c- cells. Mice were treated with *M. vaccae* (black bar) or saline (white bar), sensitized with OVA/Alum, and challenged with OVA. Cells were isolated from the lung and separated into CD11c+ and CD11c- fractions. IFN-γ (a) and IL-12 (b) message from cells was analysed using real time RT-PCR (samples from each treatment group were pooled, n=6-8 mice per group. Data expressed as mean, one-tailed Student's *t*-test used to determine significant differences).

2.2 Cytokine protein levels of pulmonary CD11c+ APCs

To confirm that the increase observed in the levels of cytokine mRNA is translated into increased protein levels, ELISA measurements for IL-10 and TGF-β were done. CD11c+ cells isolated from the lungs of mice with pulmonary inflammation were left to incubate overnight in Growth Medium at 37°C, 5% CO_2. Supernatants were then collected and concentrated as described in Chapter 2. Protein analysis revealed elevated IL-10 and TGF-β levels in the supernatants from the cultures containing CD11c+ cells isolated from mice treated with *M. vaccae*, when compared to levels in the supernatant of cultures of CD11c+ cells isolated from animals treated with saline (Figure 4-3 a & Figure 4-3 b). In summary, both protein and message data suggest that CD11c+ APC cells isolated from the lungs of mice with allergic pulmonary inflammation secrete elevated levels of IL-10 and TGF-β protein following treatment with *M. vaccae*.

2.3 Effect of *M. vaccae* on the ICOS-ICOS-L pathway in naïve mice

Several research groups have investigated the role of the ICOS-ICOS-L pathway in the initiation of allergic asthma. For example, blockade of the ICOS-ICOS-L pathway has been observed to ameliorate pulmonary inflammation [Gonzalo *et al* 2001, Tesciuba *et al* 2001, Akbari *et al* 2002]. These data suggest that ICOS may play a role in the initiation of allergic inflammation. The potential role of ICOS in allergic inflammation led to the investigation of the effect of *M. vaccae* treatment on the ICOS-ICOS-L pathway.

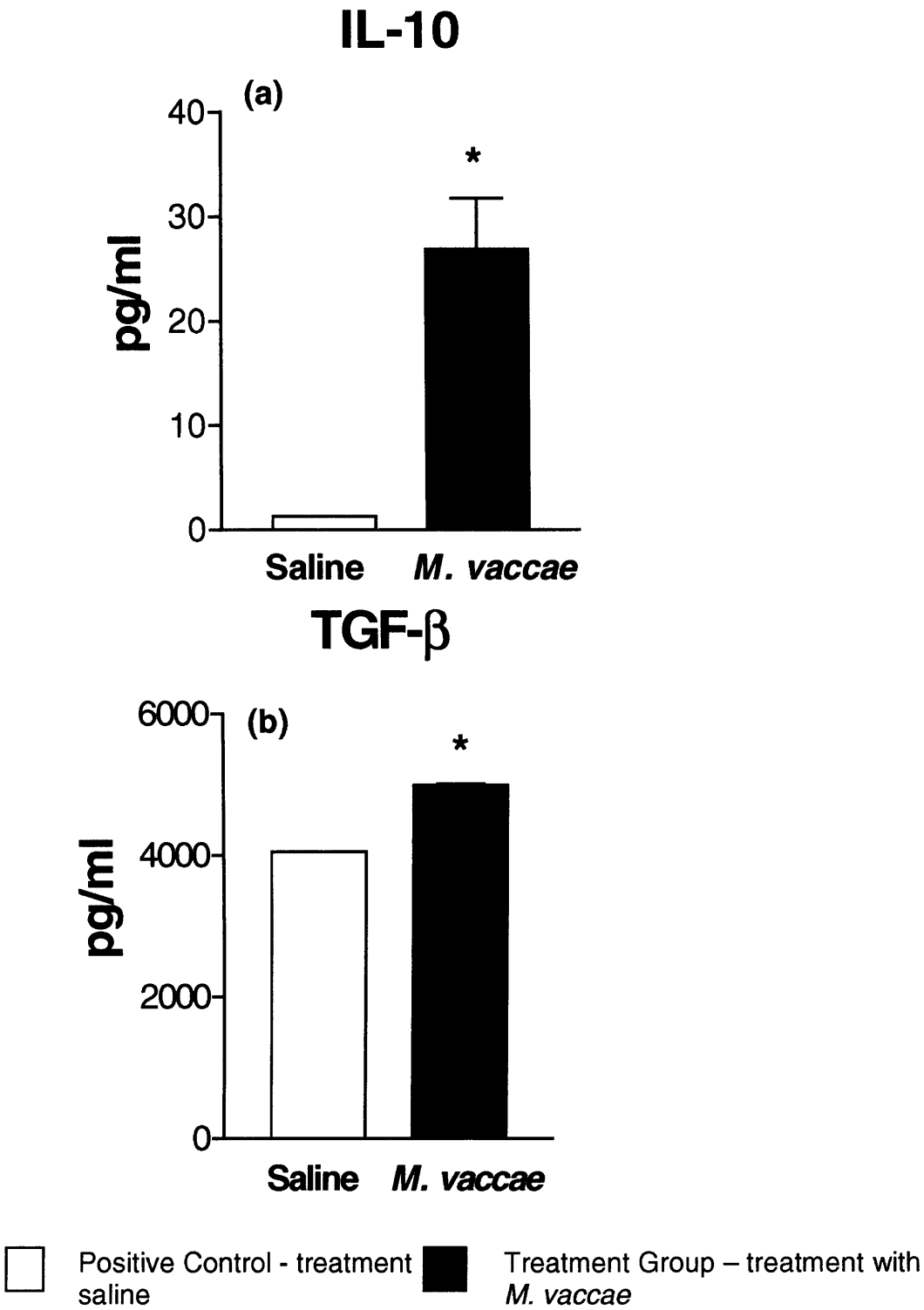


Figure 4-3: Production of IL-10 and TGF-β Protein by Pulmonary CD11c+ cells. Mice were treated with *M. vaccae* (black bar) or saline (white bar), sensitized with OVA/Alum, and challenged with OVA. CD11c+ cells were isolated from the lungs, pooled and cultured overnight. IL-10 (a) and TGF-β (b) protein production was analysed using ELISA (samples from each treatment group were pooled, n=6-8 per group, data expressed as mean ± SD, * $p<0.05$ compared to negative control one-tailed Student's *t*-test used to determine significant differences).

To determine whether *M. vaccae* treatment did indeed influence the expression of ICOS or ICOS-L, preliminary experiments were carried out using naïve mice. Naïve BALB/c mice were injected s.c. with either *M. vaccae* or saline. Animals were sacrificed on days 0 (before treatment), and days 1, 8 and 21 after treatment, when the spleens were harvested. RNA was extracted from the homogenised tissue and real time RT-PCR analysis of ICOS and ICOS-L expression carried out on the samples.

Following *M. vaccae* treatment, ICOS expression in the spleen of naïve mice was observed to increase on day 1, albeit not significantly. By day 21 it had decreased, to below the level expressed by untreated animals (Figure 4-4 b). When Student's t-test was used to test significance between Day 0 and Day 21, there was a significant difference ($p<0.05$). However, a Student's t-test can only be used if there are two groups to compare. In this time course, there were four different time points, so an ANOVA was used. This reduction was not significant ($p=0.372$) when tested by one-way ANOVA, but since the Student's t-test showed a significant difference between day 0 and day 21, this suggested *M. vaccae* may have a possible effect on ICOS expression, especially since the ICOS expression in the spleens of animals treated with saline did not alter over time (Figure 4-4 a). No significant differences were observed in the expression of ICOS-L, neither over time nor between treatment groups (Figure 4-5 a & Figure 4-5 b). In summary, although treatment of naïve mice with *M. vaccae* had no effect on the expression of ICOS-L in whole spleen tissue, a small decrease in ICOS expression was observed 3 weeks after treatment, albeit not a significant one. These initial observations were encouraging enough to warrant analysis of the expression in the lung tissue of animals with allergic pulmonary inflammation.

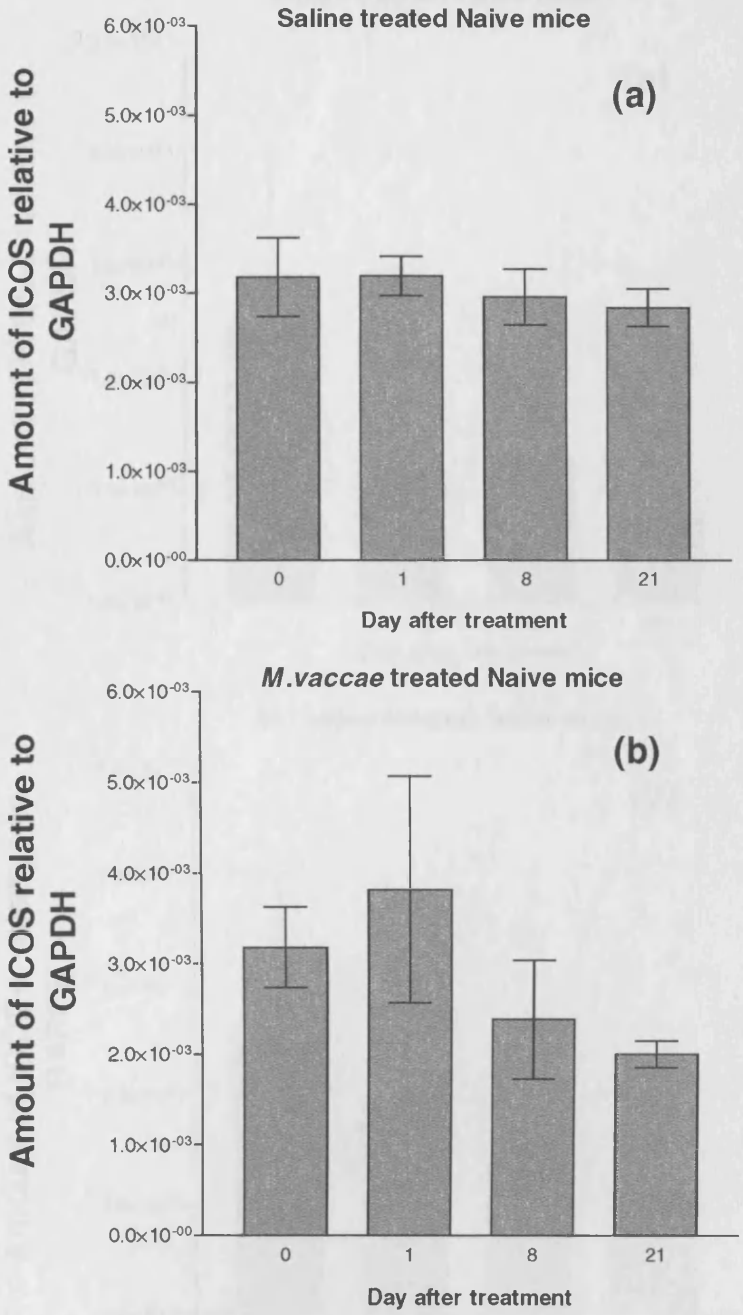


Figure 4-4: Time Course Experiment. Naïve mice, injected s.c. with either saline (a) or *M. vaccae* (b) on day 0, were sacrificed on days 1, 8 and 21. ICOS message in spleen tissue was then analysed using real-time RT-PCR (n=4 per group, data expressed a mean ± SEM, ANOVA used to test for significant differences).

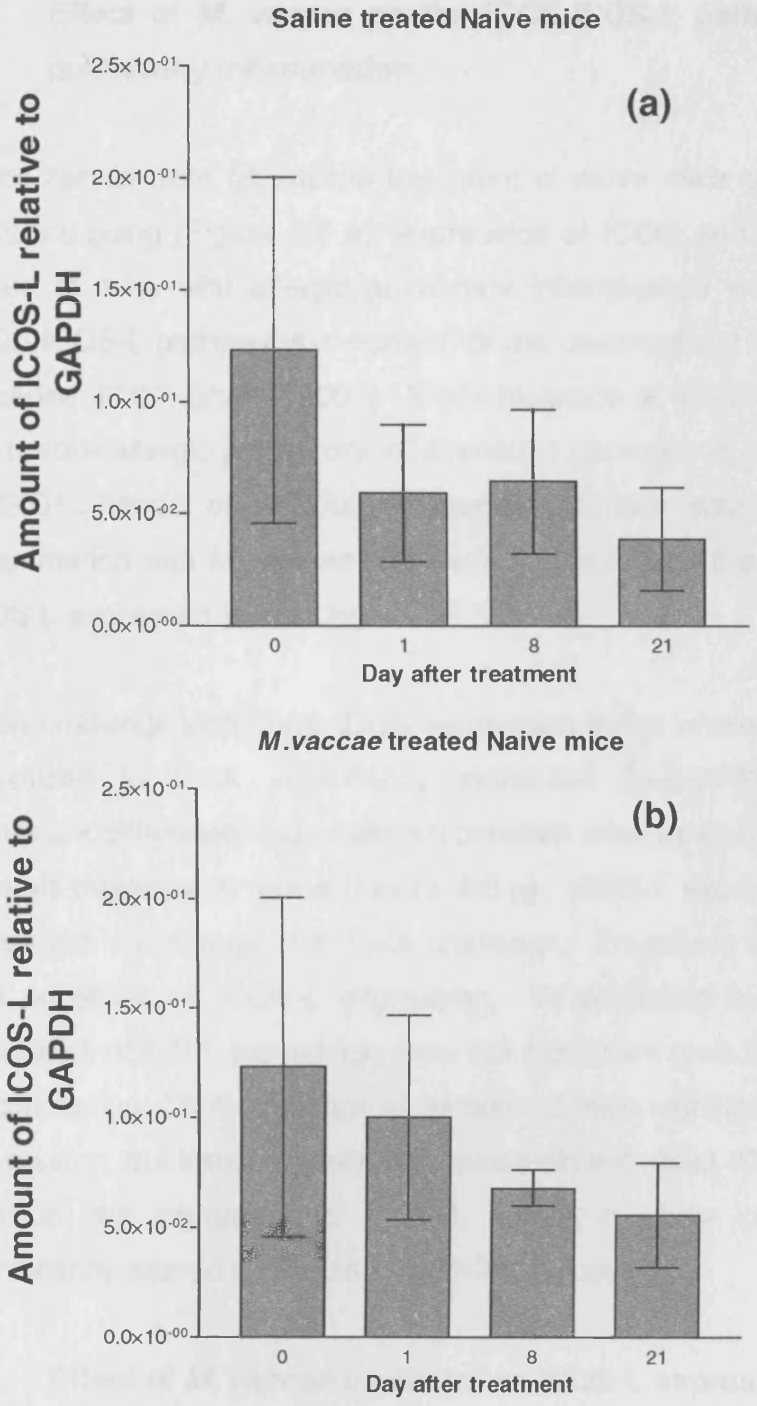


Figure 4-5: Time Course Experiment. Naïve mice, injected s.c. with either saline (a) or *M. vaccae* (b) on day 0, were sacrificed on days 1, 8 and 21. ICOS-L message in spleen tissue was then analysed using real-time RT-PCR (n=4 per group, data expressed a mean ± SEM, ANOVA used to test for significant differences).

2.4 Effect of *M. vaccae* on the ICOS-ICOS-L pathway in mice with pulmonary inflammation

Since results from *M. vaccae* treatment of naïve mice on ICOS expression were intriguing (Figure 4-6 a), expression of ICOS and ICOS-L in the lung tissue of mice with allergic pulmonary inflammation was examined. The ICOS-ICOS-L pathway is important for the development of type-2 responses [McAdam 2000, Sharpe 2002]. Since blockade of ICOS has been observed to improve allergic pulmonary inflammation [Gonzalo *et al* 2001, Tesciuba *et al* 2001, Akbari *et al* 2002], treatment of mice with allergic pulmonary inflammation with *M. vaccae* was expected to reduce the levels of ICOS and ICOS-L expressed in the lung.

Upon challenge with OVA, ICOS expression in the whole lung tissue of mice sensitized to OVA significantly increased (p=0.0103). However, no significant difference was observed between mice treated with *M. vaccae* and animals treated with saline (Figure 4-6 a). ICOS-L expression in whole lung tissue did not change after OVA challenge. Treatment with *M. vaccae* also had no effect on ICOS-L expression. When tested by one-way ANOVA, changes in ICOS-L expression were not significant (p=0.1015) (Figure 4-6 b). In conclusion, OVA challenge of sensitized mice significantly elevates ICOS expression, but treatment with *M. vaccae* did not affect ICOS expression. In addition, the expression of ICOS-L mRNA in whole lung tissue was not significantly altered by treatment with *M. vaccae*.

2.5 Effect of *M. vaccae* treatment on ICOS-L expression of pulmonary CD11c+ APCs

Although no significant differences were seen in ICOS or ICOS-L expression in whole spleen tissue, it was still deemed worthwhile to look at the expression of ICOS and ICOS-L in the pulmonary CD11c+ APCs, since the induction of Tregs has been shown to be dependent upon the ICOS-ICOS-L pathway [Akbari *et al* 2001, Akbari *et al* 2002]. The effect that *M. vaccae*

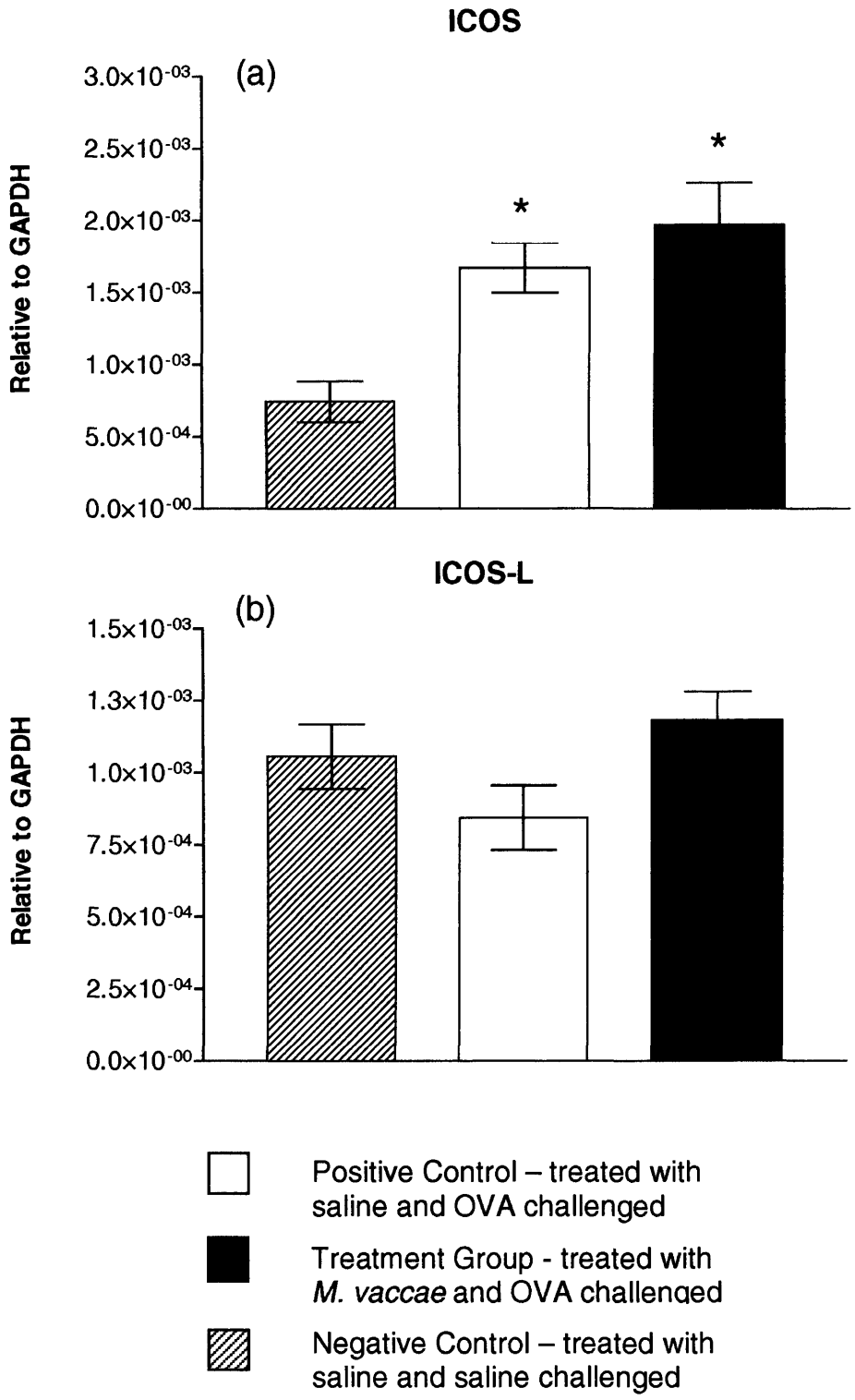


Figure 4-6: Expression of ICOS and ICOS-L in the Lung Tissue of a Murine Model of Allergic Pulmonary Inflammation. Mice were treated with nothing (shaded bar), *M. vaccae* (black bar) or saline (white bar). They were sensitized with OVA/Alum, and challenged with either OVA (black and white bar) or saline (shaded). ICOS (a) and ICOS-L (b) message in lung tissue was analysed using real-time RT-PCR (n=4-6 per group, * $p<0.05$ compared to negative control, data expressed as mean ± SEM, ANOVA used to test for significant differences, with a Bonferroni post test which compares all data groups).

treatment had on ICOS-L expression by these pulmonary APCs was investigated. Initially, levels of ICOS-L mRNA were elevated. However, no difference was detected between the ICOS-L mRNA extracted from the cells of both treatment groups in subsequent experiments. No significant changes were observed in the expression of ICOS-L by the pulmonary CD11c+ APCs isolated from the lungs of animals treated with *M. vaccae.*

2.6 Phenotype of CD11c+ APCs

As previously mentioned, the term APCs covers a relatively large group of cells with the ability to present Ag to T cells. CD11c is a marker universally used for DC isolation, but has been detected also on MΦs [Ancuta *et al* 2000, Hogg *et al* 2003]. Therefore, to further characterise these pulmonary APCs, isolated CD11c+ cells were stained for a selection of cell surface markers and analysed using FACS. Staining for CD3, B220 and F4/80 confirmed limited T cell, B cell or MΦ contamination. MHC II ($I\text{-}A^d$), CD80 and CD86 were detected on cells isolated from the lungs of both the animals treated with *M. vaccae* and saline, although no significant difference was seen between them (Figure 4-7). Surprisingly CD40 staining appeared to be negative when analysed. In addition, CD8-α was also undetected on the CD11c+ APCs (data not shown). In conclusion, the pulmonary CD11c+ APCs isolated stained negative for CD3, B220 and F4/80, which suggests they are DCs. Analysis of activation and co-stimulatory markers suggests that there is no significant difference between CD11c+ APCs isolated from the lungs of mice treated with *M. vaccae* and those isolated from the lungs of animals treated with saline.

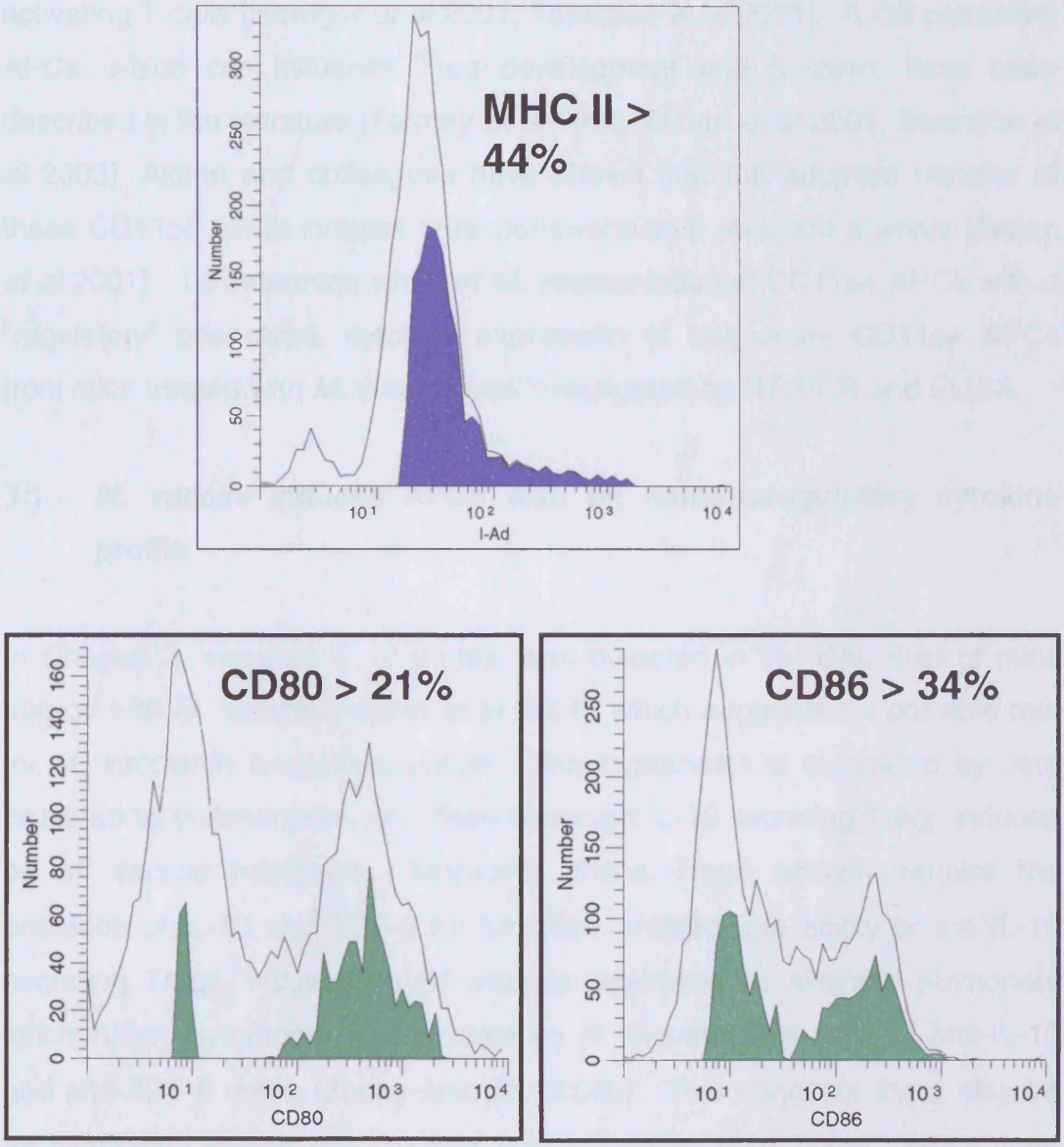


Figure 4-7: Examples of MHC II, CD80 and C86 positive staining. Pulmonary CD11c+ cells stained positive for MHC II, CD80 and CD86 from allergic mice treated with *M. vaccae*. Saline treated animals showed similar staining. Staining for MHC II ranged from between 44-50%, CD80 between 21-52% and CD86 between 34-40%. All data was analysed using WinList.

3 Discussion

APCs have the ability to influence T cell development, and are essential for activating T cells [Hawiger *et al* 2001, Terazzas *et al* 2001]. IL-10 producing APCs, which can influence Treg development and function, have been described in the literature [Tormey *et al* 1998, Akbari *et al* 2001, Swanson *et al* 2003]. Akbari and colleagues have shown that the adoptive transfer of these CD11c+ APCs induces unresponsiveness in recipient animals [Akbari *et al* 2001]. To determine whether *M. vaccae*-induced CD11c+ APCs with a "regulatory" phenotype, cytokine expression of pulmonary CD11c+ APCs from mice treated with *M. vaccae* was investigated by RT-PCR and ELISA.

3.1 *M. vaccae* induces APCs with an immunoregulatory cytokine profile

In Chapter 3, elevated IL-10 protein was detected in the BAL fluid of mice treated with *M. vaccae* [Adams *et al* 2004], which suggested a possible role for *M. vaccae* in immunoregulation. This hypothesis is supported by data collected by collaborators, who have observed IL-10 secreting Tregs induced by *M. vaccae* treatment. Moreover, these Tregs actually require the presence of IL-10 and TGF-β for function. Indeed, the ability of the IL-10 secreting Tregs, induced by *M. vaccae* treatment, to alleviate pulmonary inflammatory symptoms was blocked by *in vivo* administration of anti-IL-10 and anti-TGF-β mAbs [Zuany-Amorim 2002b]. This suggests there may be an alternative source of IL-10 and TGF-β besides from Tregs. APCs were chosen for study, since a review of the literature suggested that APCs with regulatory properties existed.

Previous studies have shown that following i.n. exposure to OVA, CD11c+ APCs express high levels of IL-10 mRNA. The presence of IL-10 influences T cell development, as Ag specific $CD4^+$ T cells cultured *in vitro* in the presence of these CD11c+ cells acquire a Treg phenotype. Further work has also showed that adoptive transfer of IL-10 producing CD11c+ cells induces

unresponsiveness to OVA sensitization and challenge in recipient mice. Thus, there is convincing evidence to suggest that CD11c+ APCs play a critical role in the regulation of the immune responses in the lungs [Akbari *et al* 2002, Swanson et al 2003]. For these reasons, CD11c+ APCs were focussed on for further investigation and results from this chapter suggest that *M. vaccae* induces pulmonary CD11c+ APCs that secrete both the cytokines IL-10 and TGF-β, as well as IFN-α.

IL-10, TGF-β and IFN-α have all been reported by many to play a significant role in the differentiation and effector function of Tregs [Levings *et al* 2001, Read *et al* 2001, Akbari *et al* 2001, Akbari *et al* 2002, Levings *et al* 2002, Zuany-Amorim *et al* 2002b]. However, the mechanisms by which they affect T cells are still largely unclear. It is likely that these cytokines have work in conjunction with each other. For example, TGF-β and IL-10 have been described as modulators of one another. TGF-β has been observed to influence both production and function of IL-10. A single i.n. dose of a plasmid encoding active TGF-β1 (pCMV-TGF-β1) prevented the development of Th1 mediated experimental colitis induced by the haptenating reagent, 2, 4, 6-trinitrobenzene sulfonic acid (TNBS). This treatment led to the expression of TGF-β1 mRNA in the intestinal lamina propria and spleen, as well as the appearance of TGF-β1- producing T cells and MΦs in these tissues. Enhancement of IL-10 production was also observed in these cells. If pCMV-TGF-β1 was co-administrated with anti-IL-10, IL-10 production was inhibited and the suppression of IL-12 reversed. This suggests that TGF-β promotes IL-10 production, which suppresses IL-12 secretion and hence inhibits development of colitis [Kitani *et al* 2000].

IL-10 has also been shown to modulate TGF-β production. Addition of IL-10 *in vitro* enhanced CD4+ T cell production of TGF-β in response to stimulation by anti-CD3, anti-CD28 and IL-2 [Seder *et al* 1998]. In the presence of TGF-β and IL-2, T cells have been reported to become non proliferative after activation, and acquire the ability to suppress the responses of other T cells [Levings *et al* 2002, Steinbrink *et al* 2002, Ludviksson *et al* 2000].

IFN-α also has immunoregulatory properties. It has been observed to upregulate IL-10 production by DCs [Levings *et al* 2001, Ito *et al* 2001, Stordeur *et al* 2002, Bilsborough *et al* 2003] and prevent anergic T cell death [Lombardi *et al* 2000]. Together, all these cytokines derived from APCs may be acting in synergy to promote Treg development, which are responsible for inhibition of pulmonary inflammation. Therefore, the presence of these cytokines in protein and message form is an important observation and suggests a role for these *M. vaccae*-induced CD11c+ APCs in immunoregulation.

3.2 *M. vaccae* does not affect type-1 cytokine profile of CD11c+ APCs

Although IFN-γ is usually thought to be produced mainly by T cells, APCs secretion of IFN-γ has been described in the literature [Le Page *et al* 2000, Fukao *et al* 2000]. For this reason, expression of IFN-γ message by pulmonary CD11c+ APCs was investigated alongside IL-12, which initiates T cell production of IFN-γ production and hence a Th1 response [Alzona *et al* 1995]. The increases observed in the immunoregulatory cytokines IL-10, TGF-β and IFN-α from in CD11c+ APCs following *M. vaccae* treatment was not mirrored by an increase in the proinflammatory cytokines L-12 and IFN-γ. These data further support the earlier observations in Chapter 3, that *M. vaccae* does not induce a type-1 response.

3.3 Treatment with *M. vaccae* affects the ICOS-ICOS-L pathway in naïve mice

In this chapter, *M. vaccae* is reported to induce CD11c+ APCs, which secrete immunoregulatory cytokines. The ICOS-ICOS-L pathway has been described as important in the induction of Tregs [Akbari *et al* 2002] so the effect that *M. vaccae* has on ICOS and ICOS-L expression became an area of interest. No difference was seen in the ICOS-L expression after *M. vaccae* treatment. A large number of cells express ICOS-L, such as DCs, B cells and T cells [Ling *et al* 2000], particularly in the spleen where numerous

heterogeneous populations of cells reside. This would make differences difficult to detect when looking at whole tissue, since *M. vaccae* may affect the activation of individual cell types differently. Any difference in ICOS-L expression present in any one population may be masked by the expression of ICOS-L by other cells. Treatment with *M. vaccae* may affect ICOS-L expression on one cell subtype differently from another. In contrast, ICOS expression is almost uniquely limited to activated T cells [Hutloff *et al* 1999]. Although the groups treated with *M. vaccae* had slightly less ICOS-L mRNA in the spleen on days 1, 8 and 21 compared to day 0, but this general decrease is also observed in the group treated with saline. This may be an artefact of the injection, for example LPS on the animals' coat being injected along with the saline or *M. vaccae.*

3.4 Treatment with *M. vaccae* does not affect the expression of ICOS or ICOS-L in the lungs of mice with pulmonary inflammation

The decision to analyse ICOS and ICOS-L expression in the lung tissue of allergic animals was taken because of published data, suggesting that ICOS and ICOS-L are important for the induction of Tregs [Akbari *et al* 2002]. It was difficult to predict whether expression of ICOS and ICOS-L would increase or decrease after treatment with *M. vaccae*, because of conflicting published reports. Blockade of ICOS in murine models of pulmonary inflammation inhibits airway eosinophilia [Gonzalo *et al* 2001, Tesciuba *et al* 2001], suggesting treatment with *M. vaccae* should decrease ICOS expression. On the other hand, blockade of ICOS-L was shown to inhibit development and function of Tregs [Akbari *et al* 2002], which suggests treatment with *M. vaccae* should increase expression of ICOS and ICOS-L. In this thesis no significant difference in ICOS expression was observed between mice treated with saline and animals treated with *M. vaccae*.

Along with being important for Treg induction, ICOS is also involved in initiating Th2 responses [Coyle *et al* 2000]. Data in this thesis supports this observation. Upon OVA challenge of sensitized mice, ICOS expression significantly increased. This was expected, since ICOS is upregulated after

naïve T cell activation by the CD28-B7 co-stimulatory pathway [Hutloff *et al* 1999, Mueller *et al* 2000, Chambers *et al* 2001]. Several other groups have observed the importance of the ICOS-ICOS-L pathway in the onset of allergic airway inflammation. Blockade of ICOS at various stages of sensitisation and challenge with both OVA and *Schistosoma mansoni* egg Ag, inhibits airway inflammation and eosinophilia [Gonzalo *et al* 2001, Tesciuba *et al* 2001]. In view of these data, it would be expected that ICOS and ICOS-L expression should decrease in the lung tissue, since both blockade of ICOS and treatment with *M. vaccae* result in ameliorated inflammatory symptoms.

The finding of no difference in ICOS or ICOS-L expression in whole lung tissue may be explained by the fact that whole tissue was used to analyse ICOS and ICOS-L expression. Expression of ICOS by effector T cells and Tregs could be detected altogether. The same could have happened with ICOS-L expression by different APC subsets. Any changes in expression on particular cell subsets could be masked by the changes in expression by other cells. Separation of different T cell types, or APC subsets, may be required to observe any differences. *M. vaccae* may be affecting ICOS expression by Tregs, which may not be picked up in whole tissue. This was the reason for investigating the expression of ICOS-L in the pulmonary CD11c+ APCs isolated from mice with allergic pulmonary inflammation.

3.5 Treatment with *M. vaccae* does not affect ICOS-L expression in pulmonary CD11c+ APCs

In this thesis, treatment with *M. vaccae* was found to enhance IL-10, TGF-β and IFN-α expression by pulmonary APCs. Therefore, the expression of ICOS-L in the same APCs was expected to increase similarly. Surprisingly, the expression levels of ICOS-L on CD11c+ APCs did not differ between the cells isolated from the lungs of mice treated with *M. vaccae* and those from mice treated with saline. This result may be due to a technical limitation. The observation of an increased ICOS-L expression in the first experiment could have been due to a contaminating population, such as B cells, which also express ICOS-L. ICOS is known to be important in T cell-B cell

interactions [McAdam *et al* 2000]. *In vivo*, blockade of ICOS results in deficient T-cell-dependent B-cell responses. Germinal centre formation is impaired and Ig class switching is defective, including production of IgE [Tafuri *et al* 2001, McAdam *et al* 2001].

B cell contamination remains a possibility, as these particular samples were not checked by FACS staining for CD11c+ purity or B cell contamination. All subsequent CD11c+ samples were B220-, suggesting no B cell contamination. Treatment with *M. vaccae* may have effected the ICOS-L expression of the B cells, which may have been contaminating the CD11c cell separation from the first experiment, and not have effected the expression on CD11c+ APCs. The differences picked up in the first experiment could therefore have been coming from a contaminating population, which was not present in subsequent experiments. IL-4 has been shown to play a major role in downregulating B cell expression of ICOS-L. Treatment of B cells with IL-4, when in culture together with CD4+ T cells, resulted in rapid reduction in ICOS-L expression by the B cells [Liang *et al* 02]. It is a possibility that since *M. vaccae* is reducing IL-4 production in the lungs, downregulation of B cell ICOS-L expression is not occurring. Therefore, animals treated with *M. vaccae*, which have less IL-4 in the lungs, would have B cells which express more ICOS-L, when compared to animals treated with saline, which have elevated IL-4 levels in the lungs.

An increase in ICOS-L expression on the pulmonary CD11c+ cells was anticipated, since T cell tolerance by DCs is dependent on the ICOS-ICOS-L pathway. Blocking ICOS-L abrogated the inhibitory capacity of DCs [Akbari *et al* 2002]. This may be explained by the fact that these cells were isolated from the lungs and not from a lymphoid organ. APCs may need to migrate to the LNs before maturation occurs. Expression of ICOS has been observed to upregulate 24-48 hours after activation. Expression of ICOS and ICOS-L is also abundant in places like the spleen, tonsils and thymus. Since these are all lymphoid organs, this suggests that migration of APCs to the LNs may be necessary before these APCs are capable of influencing T cell development. It may therefore have been more appropriate to test for ICOS-

L expression on APCs isolated from one of the LNs [Coyle *et al* 2004]. The timing of the experiment may also explain why no difference was observed. A longer period between final challenge and cell isolation may be needed before differences between treatment groups can be identified.

3.6 Phenotype of CD11c+ APCs

The pulmonary CD11c+ cells isolated from mice with pulmonary inflammation in this thesis were further characterized by FACS analysis. Pulmonary CD11c+ cells were stained for a variety of cell surface markers, to establish firstly if any contaminating populations existed. All CD11c+ cell fractions stained negative for lymphocyte markers CD3 and B220. This eliminated both T cells and B cells populations from the CD11c+ cell fractions. CD3 and B220 were both detected in the CD11c- cell fractions obtained from the separation. These data suggest that the positive selection process did not non-specifically bind any T cell or B cell populations, hence the CD11c+ cells subsequently used in all further experiment were not significantly contaminated with lymphocyte populations which may have affected results.

Although contamination of the CD11c+ cells with lymphocyte populations was limited, the actual classification of these cells was still unclear. CD11c was used to isolate these cells, which is a commonly used marker for DCs isolation. The existence of cells that express both CD11c and F4/80 or CD14, which are common MΦ markers have also been described [Ancuta *et al* 2000, Hogg *et al* 2003]. To try and establish if these CD11c+ cells isolated from the lungs of mice with pulmonary inflammation were indeed DCs, they were also stained for F4/80. F4/80 was not detected on the CD11c+ cells from either the mice treated with *M. vaccae* or the ones treated with saline. This result suggests that the contamination of the CD11c+ cells with MΦs was limited. However, there is some confusion as to whether alveolar MΦs express F4/80. Some groups have observed that alveolar MΦs do not express F4/80 [Tedjo *et al* 2003], whilst other groups have observed that they

do [Maus *et al* 2001]. It would therefore have been useful to stain CD11c+ cells with another MΦ marker, such as CD14.

Three main types of DC exist, lymphoid DCs, mDCs and pDCs. The pDC subset is generally associated with immunoregulation [Martin *et al* 2002, Lambrecht *et al* 2005] and has been shown to secrete large amounts of IFN-α [Cella *et al* 1999, Dalod *et al* 2002]. Since IFN-α mRNA was observed in *M. vaccae*-induced pulmonary APCs, CD11c+ cells were stained with CD8α, which along with B220 have been described as pDC markers [Nakano *et al* 2001]. CD8α was not detected on the CD11c+ cells isolated from the lungs of allergic mice. The lack of CD8α suggests that these DCs are not plasmacytoid, although there is evidence for a subset of pDCs that are CD8α- [de Heer *et al* 2004]. Incidently, the cells also stained negative for B220, another marker expressed by some pDCs [Nikolic *et al* 2002, Bilsborough *et al* 2003, Okada *et al* 2003]. This is surprising as the properties of *M. vaccae*-induced CD11c+ cells mirror those of pDCs, which have been shown to provide protection against inflammatory responses to harmless inhaled Ags [Colonna *et al* 2002, Lambrecht *et al* 2003]. More staining needs to be done, possibly Gr-1 and CD11b to establish that the CD11c+ cells are not pDCs.

The difference in cytokine profile between CD11c+ cells isolated from the lungs of mice treated with saline versus mice treated with *M. vaccae* suggests that *M. vaccae* is inducing a population of "regulatory" DCs. Therefore, the differences in cell surface marker expression were investigated. Although these CD11c+ APCs were probably DCs, their state of maturity was not known. The co-stimulatory markers MHC II, CD80, CD86 and CD40 were chosen since their expression was enhanced on the IL-10 secreting DCs described by Akbari *et al* as tolerogenic DCs [Akbari *et al* 2001, Akbari *et al* 2002]. CD11c+ cells isolated from both the saline and *M. vaccae* treatment groups stained positive for MHC II (I-A^d), CD80 and CD86. This was expected, since these molecules are upregulated after cell activation [Mahanonda *et al* 2002, Sahoo *et al* 2002, Cheng *et al* 2003].

However, no significant differences in CD11c+ expression levels were detected between the two groups.

The surprising observation was the lack of CD40 staining detected on the CD11c+ cells isolated from mice treated with either saline or *M. vaccae*. This was unexpected since the CD40-CD40-L pathway is involved in T cell activation by DCs. CD40 has been observed to upregulate upon OVA challenge [Vermaelen *et al* 2003]. Also, when DCs are exposed to *M. tuberculosis,* another member of the mycobacterial genus, CD40 expression increases [Gonzalez-Juarrero *et al* 2001]. It may be possible that *M. vaccae* activates APCs through a different pathway to the CD40 pathway. CD40 signalling is associated with IL-4 production [Flynn *et al* 1998]. Since IL-4 has been shown to decrease in the lungs of mice treated with *M. vaccae*, it is possible that another co-stimulatory pathway is activated and the CD40 pathway is not involved. However, this does not explain the lack of CD40 on CD11c+ APCs isolated from mice treated with saline, where IL-4 is in abundance.

Another possibility is that these observations are due to technical problems. During characterization of pulmonary CD11c+ APCs by FACS, extremely high autofluorescence of the cells was detected. Gating specific populations did not significantly alter this. Autofluorescence of APCs has been observed before and often hampers attempts to characterize APCs, since they emit within the wavelengths of the two most commonly used flurochromes, FITC and PE. This significantly interferes with the detection of weakly expressed cell surface markers [Keping *et al* 2000]. It is possible that this occurred with CD40 and CD8α staining, which in this thesis were not detected on the pulmonary CD11c+ APCs isolated from the allergic mice. CD40 is unlikely to be completely unexpressed by all APCs in the lungs, so it is possible that CD40 and, maybe CD8α, was weakly expressed, and was indistinguishable from the APCs autofluorescence.

One method that may help reduce this autofluorescence, is the treatment of cells with crystal violet. Crystal violet quenches both cell surface and intracellular fluorescence. The problem with this method is that the crystal violet alters the FSC/SSC, but does decrease background noise by up to 80%. Detection of low level Ab staining is possible after treatment with crystal violet [Keping *et al* 2000]. Any further FACS staining of pulmonary APCs should use the crystal violet method to maximize cell surface marker staining.

4 Conclusion

This chapter describes the finding of *M. vaccae*-induced pulmonary CD11c+ APCs, expressing increased levels of both IL-10 and TGF-β mRNA and protein. In addition, elevated IFN-α message, another cytokine described to have immunoregulatory potential, was detected. Pulmonary CD11c+ cells from mice treated with either saline or *M. vaccae* showed no significant differences in cell surface marker expression of MHC II, CD80 or CD86. The effect of *M. vaccae* on the ICOS-ICOS-L pathway was also addressed albeit without significant findings. CD11c+ cells may play a pivotal role in immunoregulation of responses in the lung, and may be a potential target of *M. vaccae*. Therefore, these cells were further investigated in the next chapter to establish what immunoregulatory role they played in a mouse model of allergic pulmonary inflammation.

Chapter 5

Chapter 5

Results 3: Immunoregulatory role of *M. vaccae*-induced pulmonary CD11c+ cells

1 Introduction

APCs, in particular DCs, have been shown to play a regulatory role in asthma [Spiteri *et al* 1991, Stumbles *et al* 1998]. This thesis proposes that pulmonary CD11c+ APCs, that secrete IL-10, TGF-β and IFN-α, can be isolated from the lungs of mice with allergic inflammation that have been treated with *M. vaccae*. The mechanism by which these APCs induce tolerance is unclear. It is not yet determined whether the *M. vaccae*-induced CD11c+ cells are "suppressor" APCs in their own right, or whether they induce suppressive responses via other cells. This chapter examines two of the possible mechanisms that CD11c+ APCs have been observed to adopt to inhibit inflammatory responses in the lungs.

1.1 Mechanisms of action adopted by "regulatory" APCs

There are several mechanisms, which "regulatory" APCs are involved in, and which result in inhibition of immune responses. One such mechanism is the failure of APCs to fully mature in response to certain foreign, non-pathogenic Ags. It has been observed that upon exposure to these Ags, such as OVA, T cell proliferation *in vivo* is initiated in the MLNs [Lambrecht *et al* 2000, Hammad *et al* 2003]. Despite the activation of effector T cells, the functional outcome for the immune response is tolerance [Lambrecht *et al* 2000]. This may explain the failure of harmless Ags to fully activate airway DCs, leading to immature or semi-mature DCs, which induce an abortive T cell response. The T cells fail to become effector cells and undergo apoptosis [Gett *et al* 2003]. This hypothesis is supported by the observation that stimulation of T cells with immature DCs results in T cell death [Brimnes *et al* 2003]. Immature APCs express low levels of MHC II, CD80 and CD86, so activation of T cells will not occur and apoptosis would occur. This is known as deletion tolerance.

APCs can also inhibit immune responses by secreting proteins that have a suppressive effect. These are termed "suppressor" APCs. For example, a subset of DCs which express IDO has recently been described both in human peripheral blood and mouse spleen [Munn *et al* 2002, Mellor *et al* 2003, Wakkach *et al* 2003]. It has been suggested that APCs express active IDO can inhibit T cell proliferation by depleting tryptophan. This enzymatic reaction, which results in an accumulation of toxic catabolites, such as L-kynurenine and picolinic acid [Frumento *et al* 2002, Swanson *et al* 2003]. APC expression of IDO has been shown to upregulate after exposure to IL-10 [Munn *et al* 2002] or by CTLA-4-B7 interactions [Grohmann *et al* 2002]. Inhibition of immune responses, initiated by "suppressor" APCs and T cell deletion, may operate in conjunction with APC induction of Tregs, to maintain homeostasis in the periphery and at mucosal surfaces.

1.2 Induction of Tregs by APCs

An alternative hypothesis to "suppressor" APCs is that of APCs induction of Tregs. These Tregs are responsible for the suppression of immune responses. APCs are known to influence T cell development, and the induction of Tregs by APCs has been reported [Hawiger *et al* 2001, Liu *et al* 2002]. For example, following respiratory exposure to OVA, lung DCs have been shown to produce IL-10. IL-10 has been appears essential, since blockade of IL-10 with mAbs inhibits Treg development. Adoptive transfer of these DCs to recipient allergic mice induced Ag-specific unresponsiveness [Akbari *et al* 2002]. Both mDCs and pDCs have been observed to induce Tregs [Martin *et al* 2002, Geissmann *et al* 2002]. For example, murine CD8α+B220+ pDCs isolated from the thymus, spleen or LNs, have the ability to induce Treg differentiation from OVA-specific transgenic T cells. When these pDCs were co-cultured together with OVA-specific transgenic T cells, and stimulated with IL-2 and OVA, no T cell proliferation was observed [Martin *et al* 2002]. Both mDCs and pDCs, tend to secrete low levels of IL-12 and high levels of IL-10 [Jonuleit *et al* 2000, Gasche *et al* 2000, de Heer *et al* 2004]. Along with IL-10, TGF-β has also been observed as essential for the development of Tregs. TGF-$\beta^{-/-}$ mice have significantly reduced numbers of CD4+CD25+ Tregs in their peripheral blood,

compared to TGF-β competent mice [Marie *et al* 2005]. In addition, blockade of TGF-β with a neutralising mAb inhibited the function of Tregs induced by *M. vaccae* [Zuany-Amorim *et al* 2002b]. These data highlight the possible importance of IL-10 and TGF-β in the induction of tolerance by Tregs.

1.3 Contact dependent mechanisms of Tregs

The exact mechanisms adopted by Tregs to suppress immune responses are unclear. *In vitro* studies suggest that naturally occurring CD4+CD25+ Tregs operate via a contact dependent manner [Thornton *et al* 1998, Shevach *et al* 2002]. Possible receptors involved in contact dependent tolerance could include CTLA-4 and GITR. CTLA-4 is a negative co-stimulatory receptor, which inhibits proliferation of activated T cells. Although CTLA-4 shares ligation of the B7 ligands with CD28, it binds them with 500 times more avidity [Munn *et al* 2002]. This suggests it may out compete CD28 ligation, resulting in an inhibitory T cell signal. GITR is a positive co-stimulatory receptor, which ligates with GITR-L. Ligation results in T cell proliferation [Tone *et al* 2003]. Although the activation of the GITR pathway results in proliferation of T cells, studies have still associated it with Tregs function. For example, blockade of GITR with a mAb inhibits CD4+CD25+ Treg function *in vitro* [McHugh *et al* 2002]. *In vivo* injection of GITR mAb leads to induction of autoimmune diseases, such as autoimmune gastritis [Shimizu *et al* 2002]. More recent literature however suggests that GITR expression is upregulated on non-regulatory CD4+CD25+ T cells, and ligation renders them resistant to suppression by Tregs [Stephens *et al* 2004]. Hence the role of GITR, along with contact dependent mechanisms, is still unclear.

1.4 Cytokine dependent mechanisms of Tregs

It has been suggested that naturally occurring Tregs operate by contact dependant mechanisms. In contrast, Tregs induced by foreign Ag have been observed to secrete IL-10 and TGF-β, and act in a cytokine mediated manner [Chen *et al* 1994, Groux *et al* 1997, Cottrez *et al* 2004]. These cytokines are responsible for initiating mechanisms that lead to inhibition of immune responses and maintaining peripheral tolerance at mucosal surfaces.

IL-10 has been shown to be essential in inhibiting local immune responses, since addition of IL-10 into the lungs by viral vector reversed OVA induced localised eosinophilic inflammation in the lungs [Stampfli *et al* 1999]. The actual mechanisms activated by IL-10 are still unclear. One such mechanism, the role of IL-10 in Ig class switching, has however long been understood. IL-4 influences B cells to secrete IgE, which leads to mast cell and basophil activation and histamine release, resulting in inflammation. The presence of IL-10 influences B cells to secrete IgG4, rather than IgE. IgG4 is an Ig found abundantly in the serum of healthy volunteers, and does not cause mast cells or basophils to release histamine [Banchereau *et al* 1994]. It has been shown that IL-10 secreted by inducible Tr1 cells plays a role in Ig class switching [Satoguina *et al* 2005]. Culturing IL-10 secreting Tr1 cells and B cells together with tetanus toxoid, anti-CD3 and anti-CD28 resulted in enhance levels of IgG4 production, which was dependent upon the presence of the Tr1 cells. This suggests that Ig class switching may be one mechanism by which IL-10 secreting Tregs inhibit airway inflammation.

TGF-β, although not exclusively secreted by Tregs, has been shown as essential in Treg development and activity [Maloy *et al* 2001, Shevach *et al* 2002]. TGF-β also has an inhibitory effect on T cells undergoing differentiation. CD4+ T cells isolated from naïve transgenic DO11.10 mice and treated with OVA and TGF-β proliferated poorly, when compared to CD4+ T cells treated OVA alone [Ludviksson 00]. TGF-β has also been observed to influence the development of Foxp3+ T cells. Foxp3 is a transcription factor, which has been described as "unique" to Tregs.

1.5 Foxp3

Foxp3 is a recently described marker, which has been shown to be expressed only by Tregs. The *Foxp3* gene codes for the transcription factor protein Scurfin, which is expressed in naturally occurring CD4+CD25+ Tregs and is essential for Treg development and activity [Hori *et al* 2003, Khattri *et al* 2003, Fontenot *et al* 2003, Ling *et al* 2004]. This is highlighted by mutations in the *Foxp3* gene. Transgenic mice, which have a disrupted *Foxp3* gene, are known

as *scurfy.* Mutations in the *Foxp3* gene lead to development of autoimmune diseases, such as type-1 diabetes [Brunkow *et al* 2001, Khattri *et al* 2003]. In addition, whereas normally Tregs proliferate poorly, but have the ability to suppress proliferation of other T cells. CD4+CD25+ cells isolated from Foxp3-/- mice are able to proliferate themselves, and do not suppress the proliferation of effector T cells. This suggests that they do not have regulatory properties [Fontenot *et al* 2003, Khattri *et al* 2003]. The lymphoproliferative disorder observed in *Foxp3*$^{-/-}$ mice is inhibited by transfer of CD4+CD25+ Tregs from healthy Foxp3$^{+/+}$ mice, suggesting that *Foxp3* is essential for functional Tregs. In contrast, *Foxp3*$^{-/-}$ mice injected with CD4+CD25- T cells from healthy animals went on to develop clinical and pathological symptoms of the lymphoproliferative autoimmune disease [Fontenot *et al* 2003]. This suggest that only CD4+CD25+Foxp3+ T cells have the ability to suppress the onset of the autoimmune diseases observed in mice lacking Foxp3 expression.

TGF-β has been shown to be essential for the development of *Foxp3*+ Tregs in a murine model of diabetes. Peng and colleagues showed that, in a NOD/SCID murine model of diabetes, pulsing the islets in the pancreas with TGF-β during the priming phase of the disease inhibits the onset of diabetes by promoting the expansion of CD4+CD25+*Foxp3*+ T cell populations. This protection can also be transferred to recipient NOD/SCID mice by cell transfer [Peng *et al* 2004]. The importance of TGF-β in the maintenance of Foxp3 expression and regulatory function of peripheral CD4+CD25+ Tregs has been shown using TGF-β$^{-/-}$ mice. Cells isolated from TGF-β$^{-/-}$ mice were used to investigate the effect of absence of TGF-β *in vitro.* Purified splenic CD4^{+} T cells from TGF-β$^{-/-}$ mice were activated with anti-CD3 and anti-CD28 in the presence or absence of TGF-β *in vitro. Foxp3* expression was elevated in the CD4+ T cells treated with TGF-β. TGF-β also lead to the differentiation of CD4+CD25+ Tregs from peripheral CD4+CD25- T cells *in vitro* [Chen *et al* 2003, Fantini *et al* 2004, Marie *et al* 2005]. These results suggest that, in addition to playing a role in Treg function, TGF-β also stimulates Treg differentiation. The presence of the cytokines IL-10 and TGF-β, along with elevated *Foxp3* expression would be suggestive of Treg development.

1.6 Hypothesis

M. vaccae-induced pulmonary CD11c+ APCs have been shown in this thesis to express immunoregulatory cytokine message and protein (Chapter 4). This thesis proposes that CD11c+ APCs may play a pivotal role in the induction of Treg development. *M. vaccae* has already been observed to induce Tregs. As well as producing IL-10, the function of these Tregs was reported to be IL-10 and TGF-β dependent [Zuany-Amorim *et al* 2002b]. The pulmonary CD11c+ APCs described herein may therefore be good candidate source of IL-10 and TGF-β, and may play a role in Treg induction.

This chapter attempts to further characterize the role played by *M. vaccae*-induced CD11c+ APCs. To establish whether or not the protective effect of *M. vaccae* could be transferred via the CD11c+ APCs, these cells were passively transferred to recipient allergic mice. In addition, to determine if these cells had Treg inducing abilities, an *in vitro* assay was set up by co-culturing CD11c+ cells with naïve CD4+ T cells. Immunoregulatory cytokines and Treg markers were investigated to establish whether these CD11c+ cells are capable of influencing Treg function.

2 Results

2.1 Cellular infiltration of the BAL fluid in donor mice

Two possible mechanisms for immunoregulation mediated by *M. vaccae*-induced pulmonary CD11c+ APCs were investigated. The first being that the cytokine production from the APCs directly causes downregulation of Th2 cell effector function. The second is that CD11c+ APCs induce Tregs, which are responsible for downregulating the immune response.

To determine whether the pulmonary CD11c+ cells had regulatory function per se, a passive transfer experiment was carried out. CD11c+ cells isolated from the lungs of *M. vaccae* or saline treated mice (donors), were transferred i.t. to untreated mice with allergic pulmonary inflammation (recipients). Donor mice were treated with *M. vaccae* or saline on day -21, and underwent a preventive allergy protocol (Figure 2-1). Animals were sacrificed on day 23 and the number of cells infiltrating the BAL fluid was determined to confirm the OVA challenge had induced airway inflammation, and to determine whether the *M. vaccae* treatment had suppressed inflammation.

In the BAL fluid of donor mice, a significant decrease was observed in the number of cells recovered from mice treated with *M. vaccae* when compared to animals treated with saline (Figure 5-1). In summary, treatment of donor mice with *M. vaccae* significantly alleviated airway inflammation, when compared to the cellular infiltrate in the BAL fluid of mice treated with saline. This finding suggests that the donor allergy protocol worked. The CD11c+ cells isolated from the lungs of these animals were subsequently transferred to mice with pulmonary inflammation.

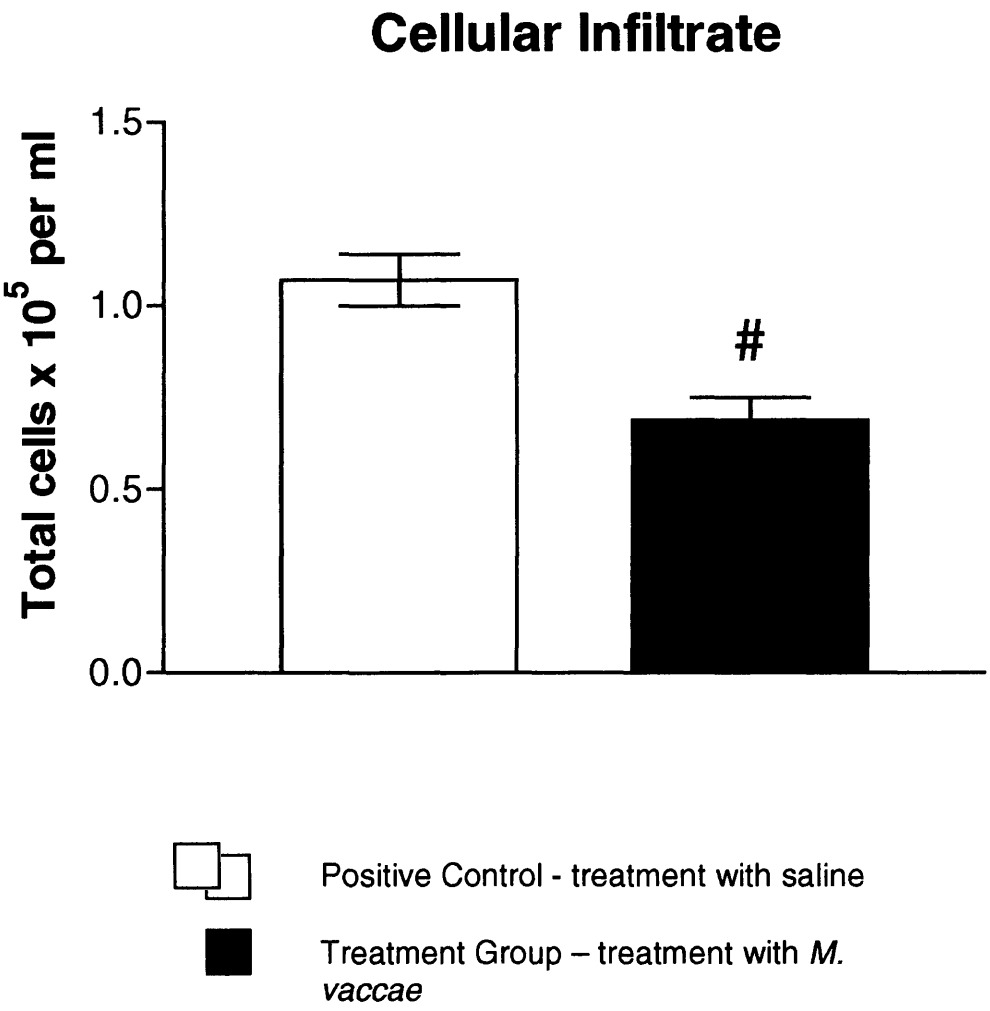


Figure 5-1: Pulmonary inflammation of donor mice. Donor animals were subjected to a preventive allergy protocol. Mice were treated with *M. vaccae* (black bar) or saline (white bar), sensitised with OVA/Alum, and challenged with OVA. Upon sacrifice, the BAL fluid was obtained to determine cellular infiltrate. Pulmonary CD11c+ cells from the lungs of these animals were then used in a passive transfer experiment. (n=6-8 per group, # $p<0.05$ compared to animals treated with saline, data expressed as mean ± SEM, one tailed student *t*-test used to test for significant differences).

2.2 Cellular infiltration in the BAL fluid of recipient mice upon CD11c+ cell transfer

To determine whether pulmonary CD11c+ cells from mice treated with *M. vaccae* had regulatory abilities per se, CD11c+ cells isolated from the lungs of the donor mice treated with saline or *M. vaccae* were transferred i.t. to recipient animals with allergic pulmonary inflammation (Figure 2-3). As an additional control, a group of mice received an i.t. injection of saline alone. After injection of CD11c+ cells directly into the lungs, mice were challenged i.t. with OVA and sacrificed on day 23. Cellular counts from the BAL fluid were then obtained to determine whether the affect seen after treatment with *M. vaccae* could be transferred by pulmonary CD11c+ APCs (Figure 5-2).

After CD11c+ cell transfer and OVA challenge, no significant difference in the number of cells obtained from the BAL fluid was observed in mice injected with CD11c+ cells from animals treated with *M. vaccae* (Figure 5-2), when compared the cell number in the BAL fluid of animals injected with either saline alone or with CD11c+ cells from animals treated with saline. This lack of effect was not due to a failure of *M. vaccae* to reduce inflammation in donor mice, since cellular infiltrate was significantly decreased in the BAL fluid of these mice (Figure 5-1).

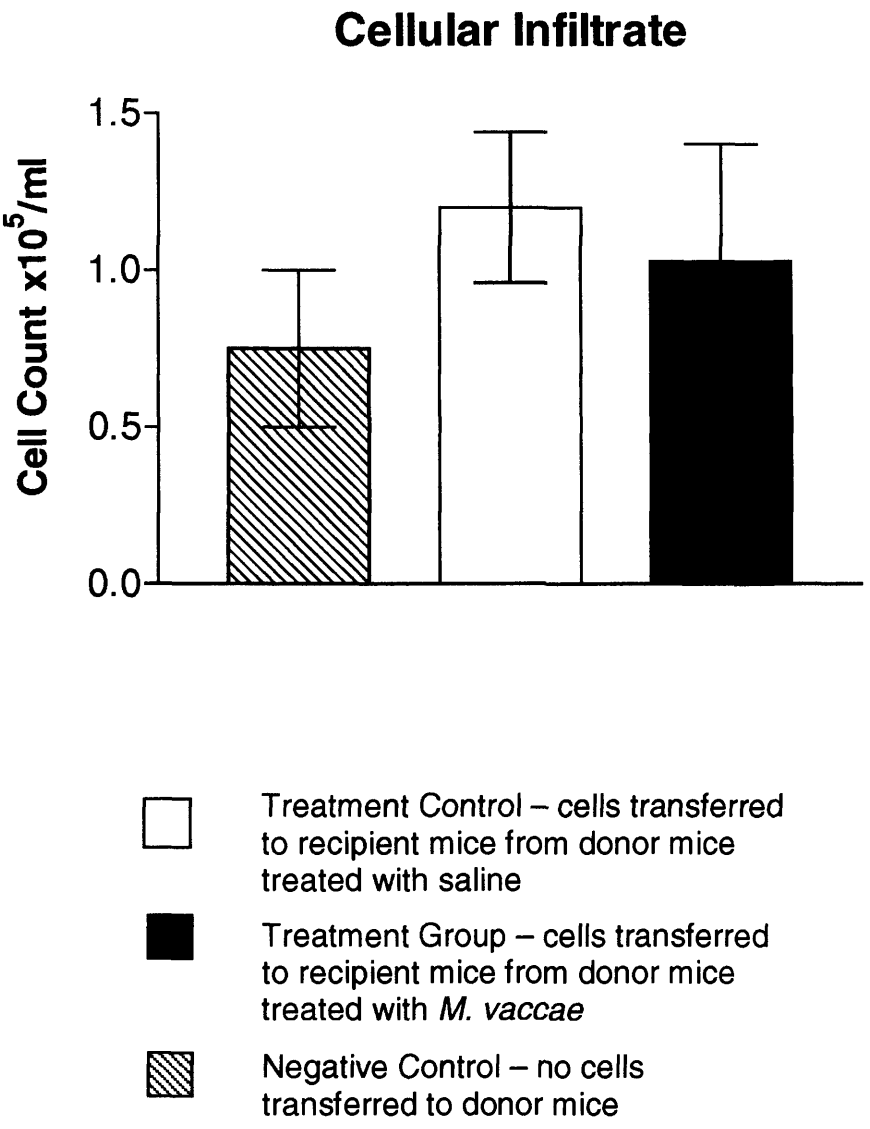


Figure 5-2: Passive Transfer Experiment. Pulmonary CD11c+ cells were isolated from *M. vaccae* (black bar) and saline-treated (white bar) donor allergic mice (cells pooled, n=6-8 per group). 2 x 10^5 CD11c+ were transferred to recipient mice with allergic pulmonary inflammation. A recipient group, which underwent the allergy protocol but had only saline transferred was also included (shaded bar). Cellular influx in the BAL fluid was determined (n=4 per group, data expressed as mean ± SEM, ANOVA used to determine significant differences).

2.3 Purity of CD4+ and CD11c+ cell isolations

Since injection of *M. vaccae*-induced CD11c+ APCs into the lungs of recipient mice with pulmonary allergic inflammation did not transfer the protective effect of *M. vaccae* (Figure 5-2), alternative mode of action was investigated. To determine whether the APCs have the ability to induce development of Tregs, an *in vitro* assay was set up, with pulmonary CD11c+ cells and naïve splenic CD4+ T cells, an aliquot of the CD4+ and CD11c+ separated cells were stained and analysed by FACS to establish purity. CD4+ cells had a purity of >90% (Figure 5-3 a & Figure 5-3 b), and the CD11c+ cells had a purity of >70% (Figure 5-4). This leads to the possibility that results obtained from the *in vitro* assay may be a consequence of contaminating APC or B cell populations in the T cell fraction. However, FACS staining of the CD4 separated fraction suggested that they were not contaminated with B220+ B cells. Although only of 70% purity, the CD11c+ cells stained negative for B220 and CD3, suggesting that lymphocyte contamination was limited.

2.4 *In vitro* assay optimisation

Optimisation of APC:T cell ratio *in vitro* was necessary to determine the most efficient Effector to Stimulator ratio, to give optimal cytokine levels. Preliminary experiments were carried out using three different CD11c+:CD4+ ratios, 1:100, 1:40, 1:20. Cultures set up with the ratio of 1 CD11c+ cell to every 100 CD4+ T cells produced minimal levels of cytokines, such as IL-4 and IL-10 (Figure 5-5 a &Figure 5-5 b). The 1:40 and 1:20 CD11c+:CD4+ T cell ratio appeared to give higher levels of cytokines than the 1:100 CD11c+:CD4+ T cell ratio. Availability of pulmonary CD11c+ APCs was a limiting factor of these experiments, so the 1:40 APC:T cell ratio was used in all further experiments, since this ratio gave higher levels of cytokines compared to the 1:100 but fewer cells are required to set up an *in vitro* assay than when using the 1:20 ratio. All wells received a set number of CD4+ T cells, 5 x 10^5, and the numbers of CD11c+ APCs were varied accordingly, in a final volume of 200µl.

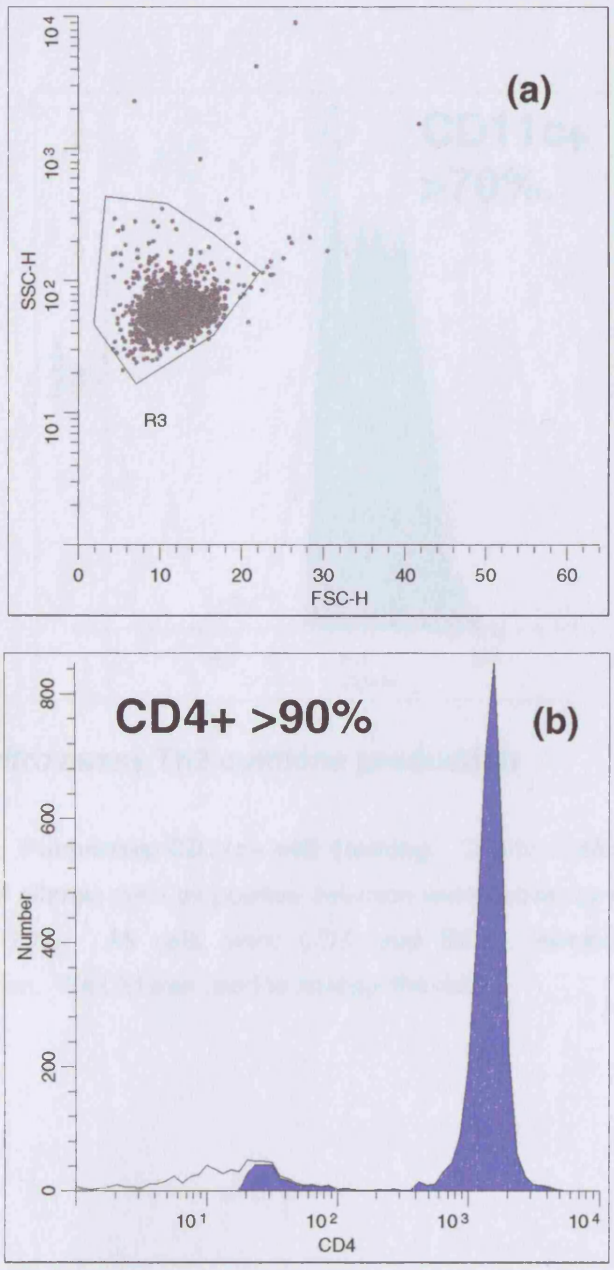
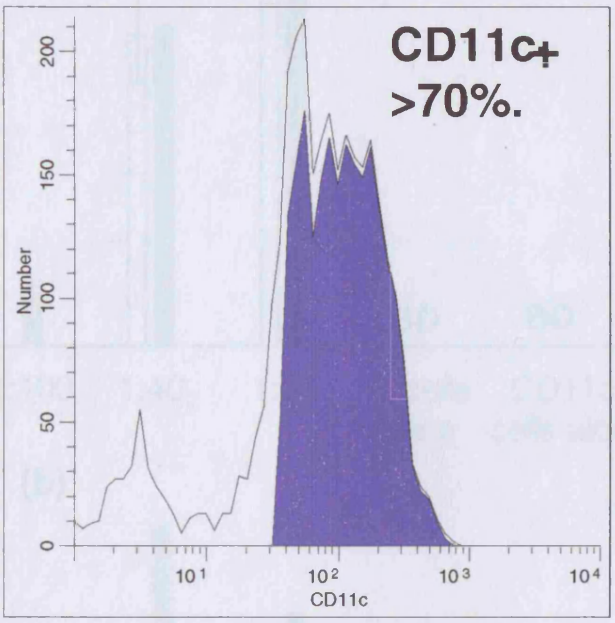


Figure 5-3: CD4+ splenocytes staining. CD4+ T cells isolated from the spleens of naïve mice by negative selection are shown by both the forward/side scatter (a) and by CD4+ staining (b) to be a heterogeneous population, above 90% positive. Contaminating cells consisted of a small number of CD8+ cells (<5%) but no B cells (B220-). Cells were used in an *in vitro* culture with CD11c+ cells. WinList was used to analyse the data.



2.5 *In vitro* assay Th2 cytokine production

Figure 5-4: Pulmonary CD11c+ cell staining. CD11c+ cells separated from the lungs of allergic mice by positive selection were shown by FACS staining to be above 70%. All cells were CD3- and B220-, eliminating lymphocyte contamination. WinList was used to analyse the data.

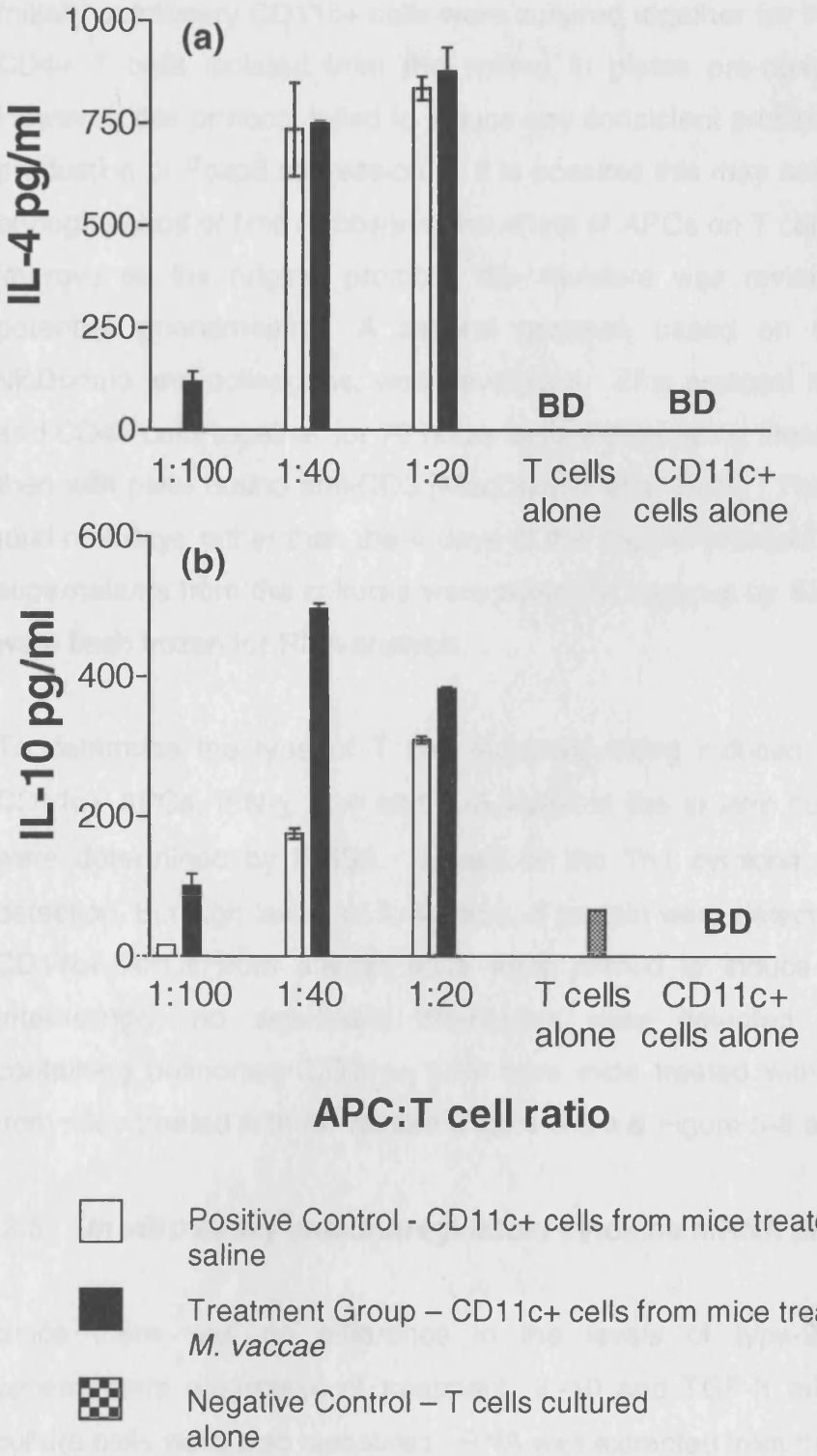


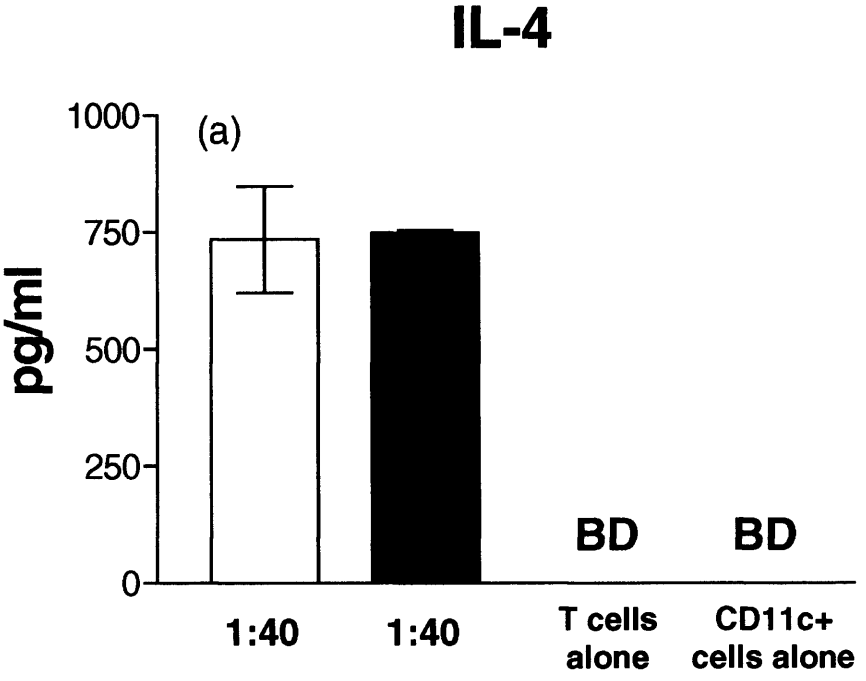
Figure 5-5: Optimisation of *in vitro* culture. Pulmonary CD11c+ cells were isolated from allergic mice treated with *M. vaccae* (black bar) or saline (white bar). These cells were co-cultured with naïve CD4+ T cells at a ratio of 1:100, 1:40 and 1:20 CD11c+:CD4+ cells. T cells were also cultured alone with anti-CD3 and IL-2 (checked bar). CD11c+ cells were culture alone in Growth Medium (without anti-CD3 and IL-2). Levels of IL-4 (a) and IL-10 (b) protein were measured in the culture supernatants. The 1:40 ratio gave a high cytokine release whilst using less cell numbers than the 1:20 ratio (CD11c+ cells from each treatment group were pooled, n=6-8 per group, data expressed mean ± SD).

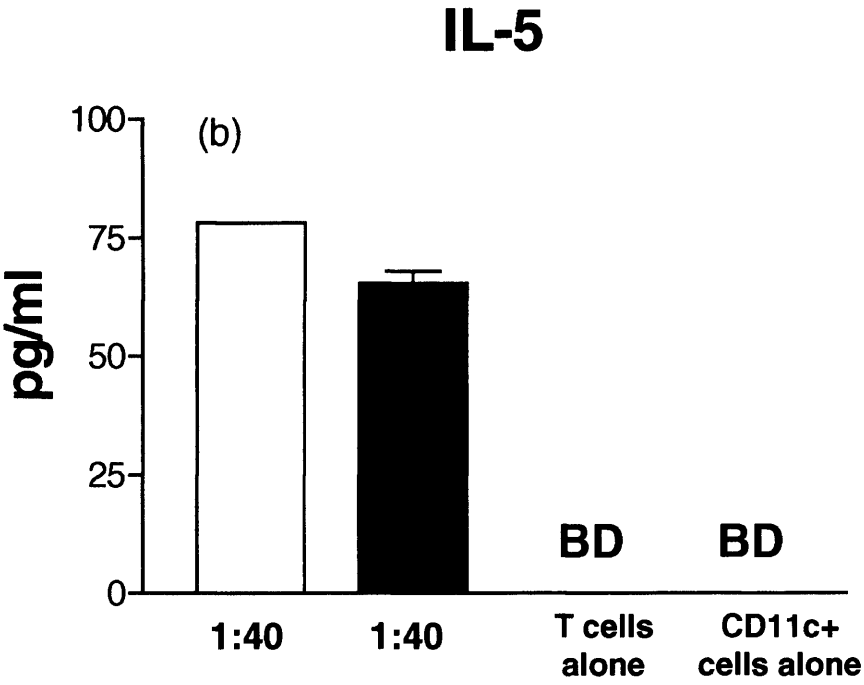
Initially pulmonary CD11c+ cells were cultured together for 96 hours with naïve CD4+ T cells isolated from the spleen in plates pre-coated with anti-CD3. However, this protocol failed to induce any consistent profiles in either cytokine production or Foxp3 expression. It is possible this may not have been a long enough period of time to observe the effect of APCs on T cell development. To improve on the original protocol, the literature was reviewed to investigate potential amendments. A second protocol, based on one described by McDonald and colleagues, was developed. This protocol rested the CD11c+ and CD4+ cells together for 72 hours before stimulating them first with IL-2 and then with plate bound anti-CD3 [MacDonald *et al* 2001]. This protocol lasted a total of 7 days rather than the 4 days of the original protocol. After 7 days, the supernatants from the cultures were taken for analysis by ELISA, and the cells were flash frozen for RNA analysis.

To determine the type of T cell response being induced by the pulmonary CD11c+ APCs, IFN-γ, IL-4 and IL-5 levels in the *in vitro* culture supernatants were determined by ELISA. Levels of the Th1 cytokine IFN-γ were below detection, but high levels of IL-4 and IL-5 protein were detected, suggesting the CD11c+ APCs from allergic mice were primed to induce a Th2 response. Interestingly, no significant differences were detected between cultures containing pulmonary CD11c+ cells from mice treated with saline and those from mice treated with *M. vaccae* (Figure 5-6 a & Figure 5-6 b).

2.5 *In vitro* assay immunoregulatory cytokine mRNA production

Since there was no difference in the levels of type-2 cytokine in the supernatants regardless of treatment, IL-10 and TGF-β mRNA levels in the culture cells were also measured. RNA was extracted from the culture cells and RT-PCR analysis carried out. Significantly elevated levels of IL-10 and TGF-β mRNA were observed in the cell cultures containing the pulmonary CD11c+


IL-4
(a)
1000
750
500
250
0
pg/ml
BD
BD
1:40
1:40
T cells alone
CD11c+ cells alone


IL-5
(b)
100
75
50
25
0
pg/ml
BD
BD
1:40
1:40
T cells alone
CD11c+ cells alone

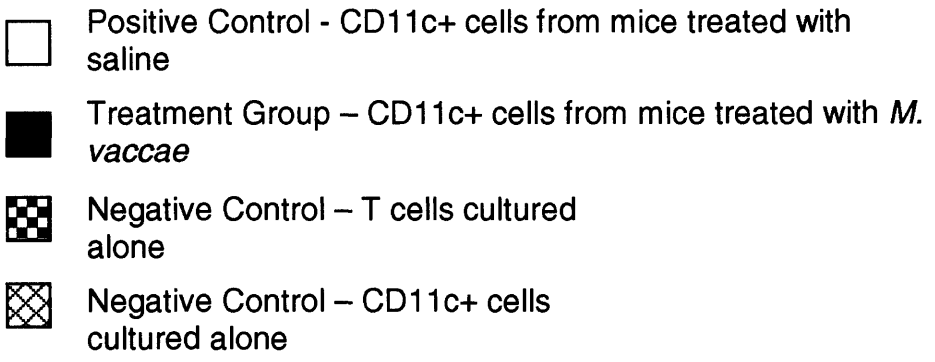
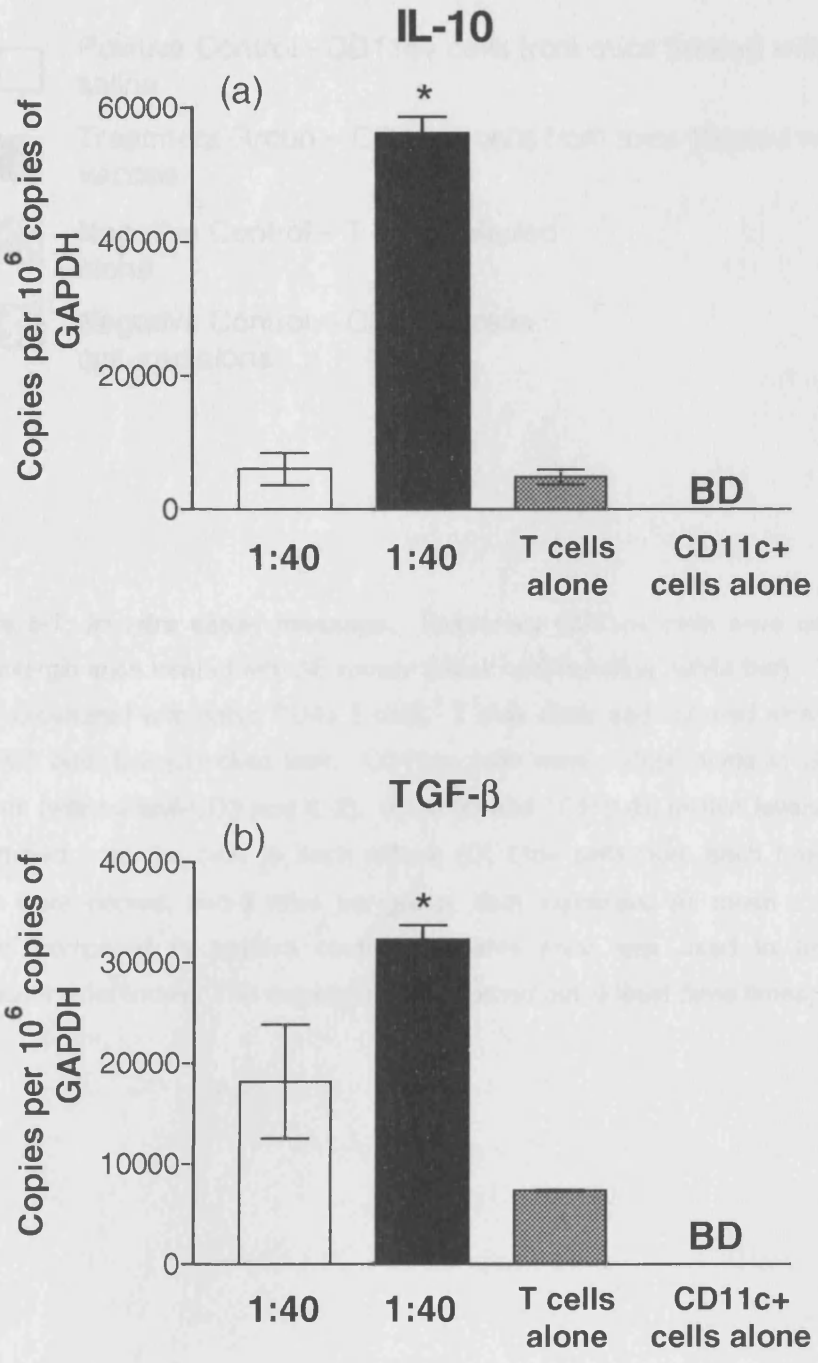


Figure 5-6: *In vitro* assay Th2 cytokines. Pulmonary CD11c+ cells were isolated from allergic mice treated with *M. vaccae* (black bar) or saline (white bar). These were co-cultured with naïve CD4+ T cells at a ratio of 1:40 CD11c+:CD4+ T cells. T cells were also cultured alone with anti-CD3 and IL-2 (checked bar). CD11c+ cells were culture alone in Growth Medium (without anti-CD3 and IL-2). IL-4 (a) and IL-5 (b) protein measurements were determined from the culture supernatants (CD11c+ cells from each treatment group were pooled, n=6-8 mice per group, data expressed mean ± SD, Students *t*-test used to test significant differences).


IL-10
(a)
Copies per 10^6 copies of GAPDH
60000
40000
20000
0
*
BD
1:40
1:40
T cells alone
CD11c+ cells alone
TGF-β
(b)
Copies per 10^6 copies of GAPDH
40000
30000
20000
10000
0
*
BD
1:40
1:40
T cells alone
CD11c+ cells alone

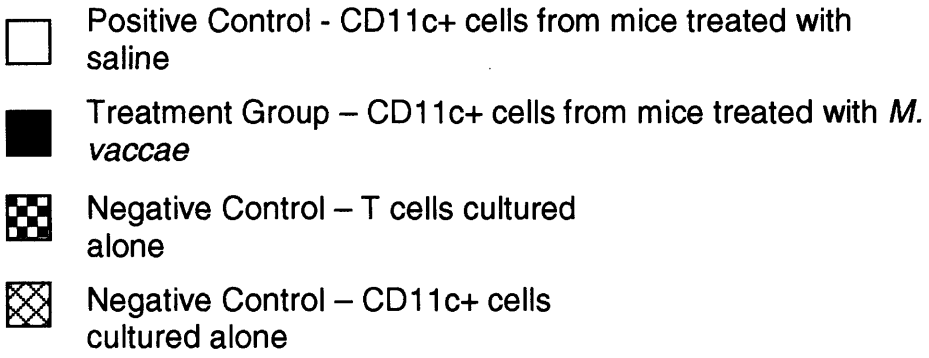


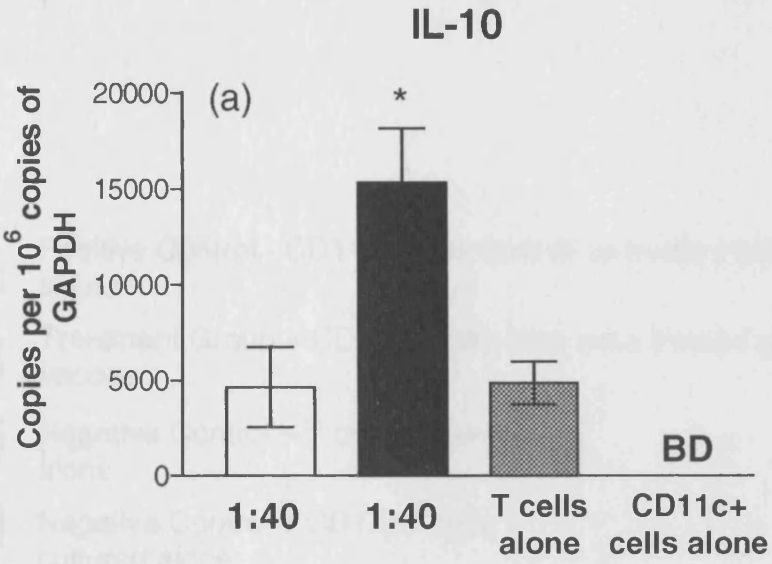
Figure 5-7: *In vitro* assay message. Pulmonary CD11c+ cells were isolated from allergic mice treated with *M. vaccae* (black bar) or saline (white bar). These were co-cultured with naïve CD4+ T cells. T cells were also cultured alone with anti-CD3 and IL-2 (checked bar). CD11c+ cells were culture alone in Growth Medium (without anti-CD3 and IL-2). IL-10 (a) and TGF-β (b) mRNA levels were determined from the cells in each culture (CD11c+ cells from each treatment group were pooled, n=6-8 mice per group, data expressed as mean ± SD, * $p<0.05$ compared to positive control, Students *t*-test was used to test for significant differences. This experiment was carried out at least three times).

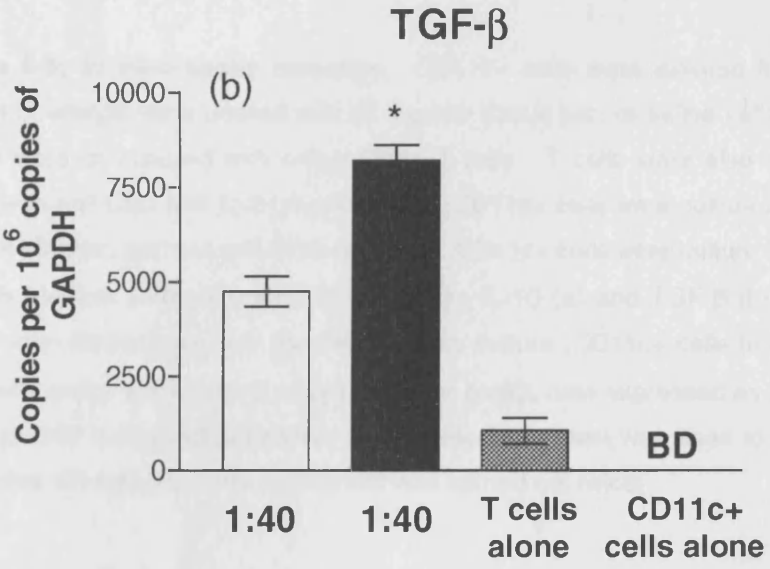
cells from allergic mice treated with *M. vaccae* (Figure 5-7 a and Figure 5-7 b).

When an APC internalises and processes Ag, it usually migrates to the LNs before it presents the Ag on its MHC receptor. The lung is not a lymphoid organ, and this may affect the ability of CD11c+ cells isolated from this organ to induce Tregs. For this reason, the same experiment was also carried out using CD11c+ cells isolated from the spleens of mice with allergic pulmonary inflammation, treated with *M. vaccae* or saline. Significantly elevated levels of IL-10 and TGF-β mRNA were also observed when CD11c+ cells from the spleen of allergic mice treated with *M. vaccae* were co-cultured with naïve CD4+ T cells (Figure 5-8 a and 5-8 b).

2.6 *In vitro* assay immunoregulatory cytokine production

To establish whether the increase in IL-10 and TGF-β mRNA observed is also observed at the protein level, the supernatants from the *in vitro* cultures were analysed by ELISA. Despite no difference being seen in the levels of Th1 or Th2 cytokines, an elevated amount of IL-10 protein was found in the supernatants from the wells, which contained cells from animals treated with *M. vaccae* (Figure 5-9). TGF-β levels were below detection. This increase in IL-10 was also observed in the supernatants from the wells that contained splenic CD11c+ cells from mice treated with *M.* vaccae (Figure 5-10). Interestingly, less IL-10 was secreted from the cultures containing CD11c+ cells isolated from the spleen, compared to those containing pulmonary CD11c+ APCs. In summary, co-culture of CD11c+ APCs from allergic mice treated with *M. vaccae* with naïve CD4+ T cells showed an increase in IL-10 protein production. The IL-10 was not secreted from the T cells cultured alone, nor was detected in CD11c+ cells cultured alone in medium.


IL-10
(a)
*
20000
15000
10000
5000
0
Copies per 10^6 copies of GAPDH
BD
1:40
1:40
T cells alone
CD11c+ cells alone


TGF-β
(b)
*
10000
7500
5000
2500
0
Copies per 10^6 copies of GAPDH
BD
1:40
1:40
T cells alone
CD11c+ cells alone

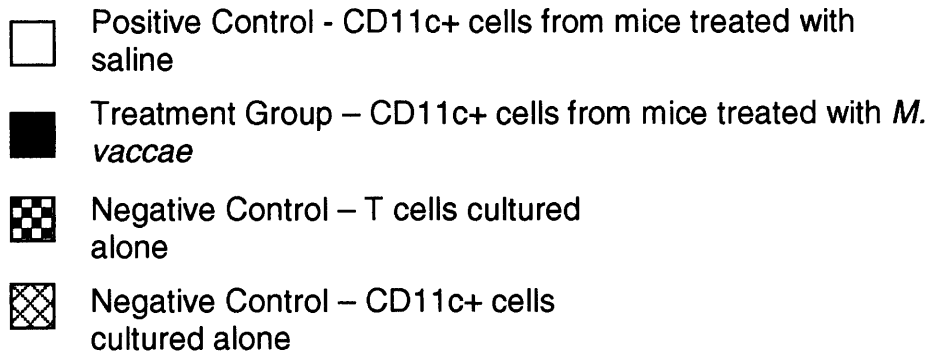


Figure 5-8: *In vitro* assay message. CD11c+ cells were isolated from the spleen of allergic mice treated with *M. vaccae* (black bar) or saline (white bar). These were co-cultured with naïve CD4+ T cells. T cells were also cultured alone with anti CD3 and IL-2 (checked bar). CD11c+ cells were culture alone in Growth Medium (without anti-CD3 and IL-2). CD11c+ cells were culture alone in Growth Medium (without anti-CD3 and IL-2). IL-10 (a) and TGF-β (b) mRNA levels were determined from the cells in each culture (CD11c+ cells from each treatment group were pooled, n=6-8 mice per group, data expressed as mean ± SD, * $p<0.05$ compared to positive control, Students *t*-test was used to test for significant differences. This experiment was carried out twice).

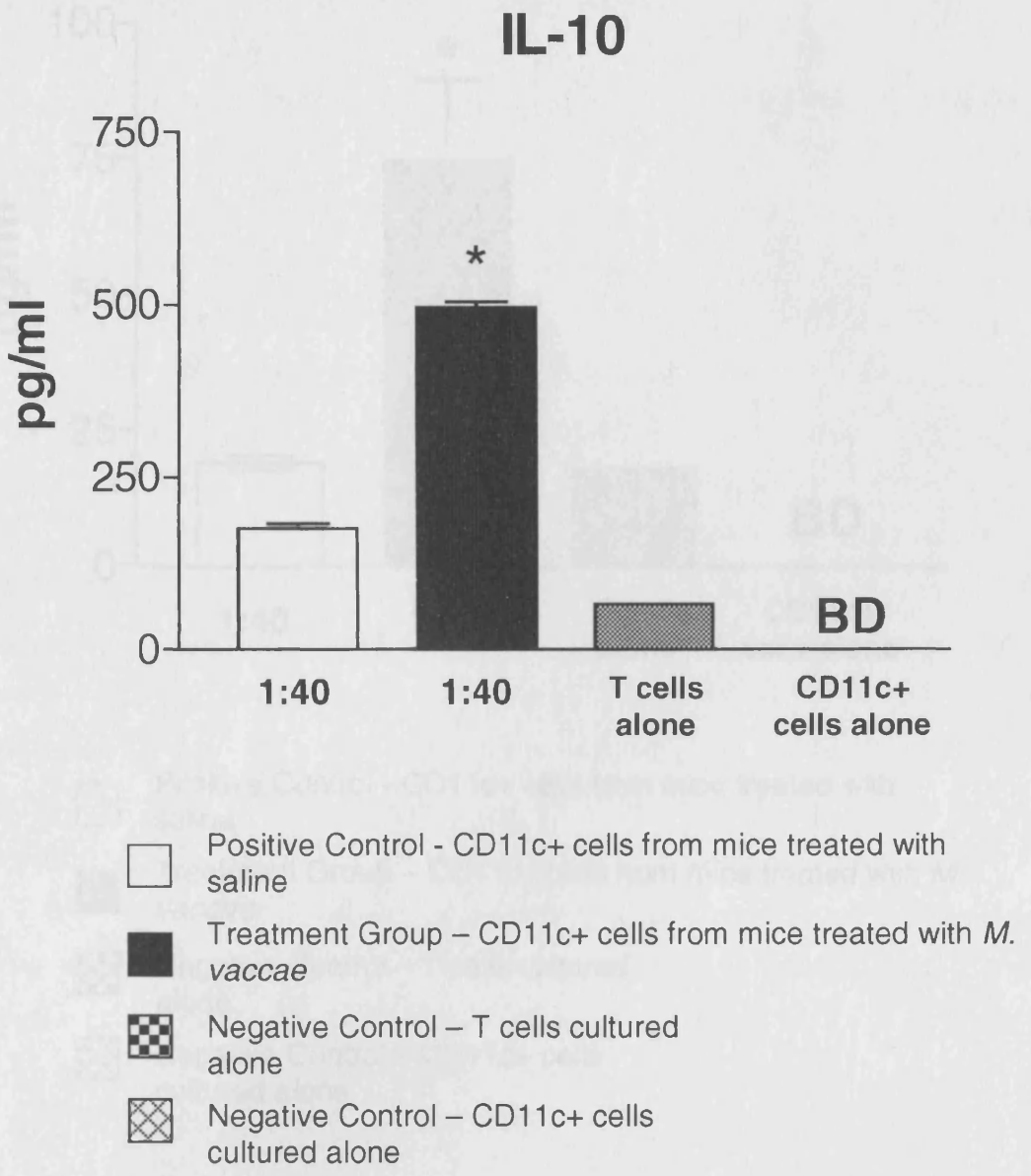


Figure 5-9: *In vitro* assay IL-10 protein. Pulmonary CD11c+ cells were isolated from allergic mice treated with *M. vaccae* (black bar) or saline (white bar). These cells were co-cultured with naïve CD4+ T cells. CD4+ T cells were also cultured alone with anti-CD3 and IL-2 (checked bar). CD11c+ cells were culture alone in Growth Medium (without anti-CD3 and IL-2). IL-10 protein measurements were determined from the culture supernatants (CD11c+ cells from each group were pooled, n=6-8 mice per group, data expressed mean ± SD, * $p<0.05$ compared to positive control, Students *t*-test was used to test for significant differences).

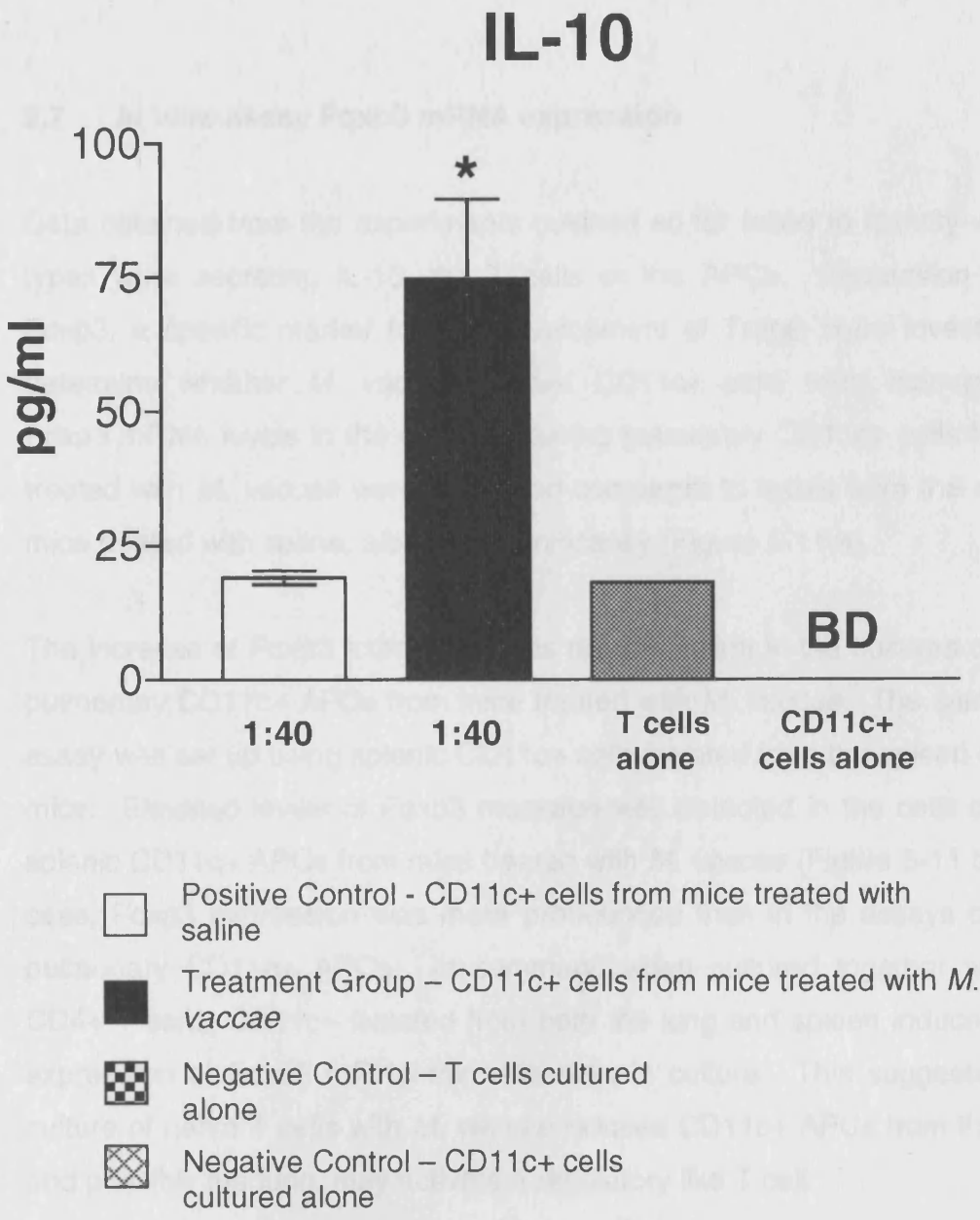
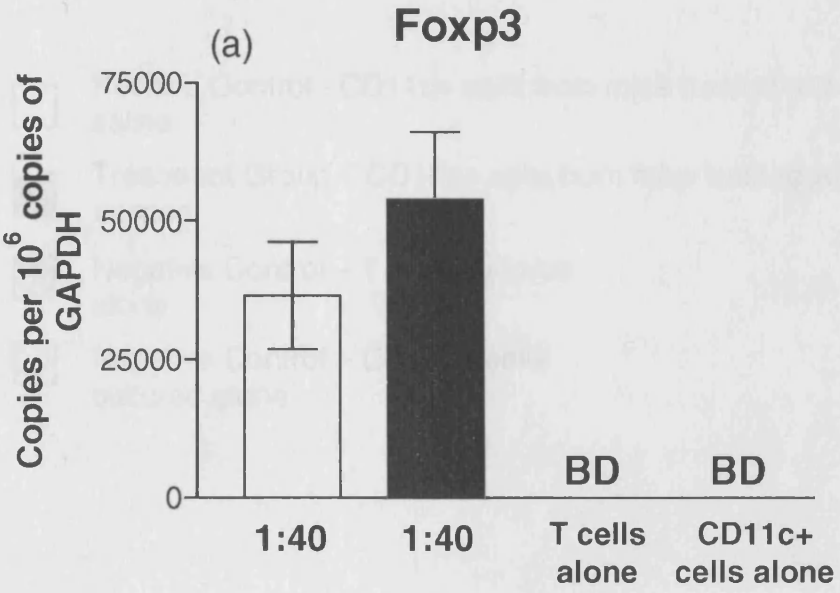


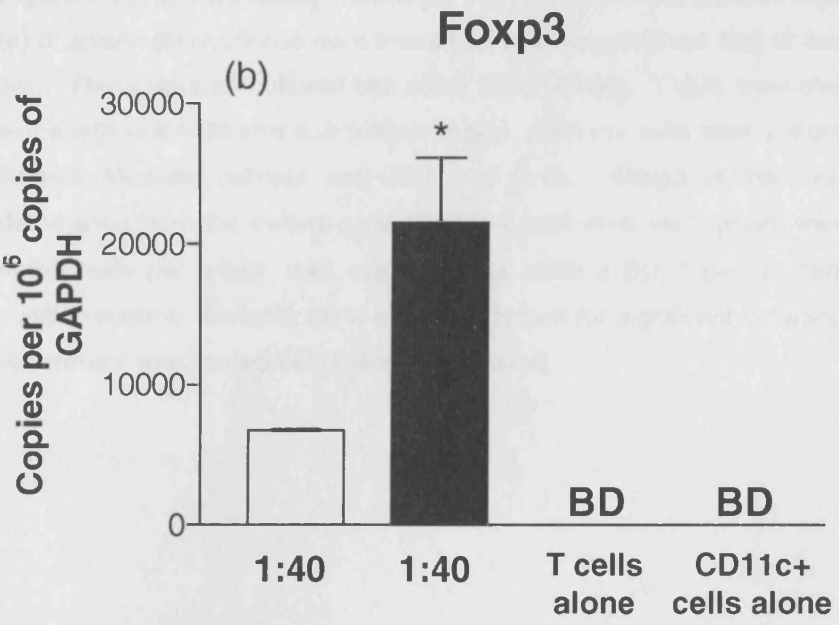
Figure 5-10: *In vitro* assay IL-10 protein. Splenic CD11c+ cells were isolated from allergic mice treated with *M. vaccae* (black bar) or saline (white bar). These cells were co-cultured with naïve CD4+ T cells at a ratio of 1:40 CD11c+:CD4+ cells. T cells were also cultured alone with anti-CD3 and IL-2 (checked bar). CD11c+ cells were culture alone in Growth Medium (without anti-CD3 and IL-2). IL-10 protein measurements were determined from the culture supernatants (CD11c+ cells from each treatment group were pooled, n=6-8 mice per group, data expressed mean ± SD, * $p<0.05$ compared to positive control, Students *t*-test was used to test for significant differences).

2.7 *In vitro* assay Foxp3 mRNA expression

Data obtained from the experiments outlined so far failed to identify which cell types were secreting IL-10, the T cells or the APCs. Expression levels of Foxp3, a specific marker for the development of Tregs, were investigated to determine whether *M. vaccae*-induced CD11c+ cells were inducing Tregs. Foxp3 mRNA levels in the cells containing pulmonary CD11c+ cells from mice treated with *M. vaccae* were increased compared to levels from the cells from mice treated with saline, albeit not significantly (Figure 5-11 a).

The increase of Foxp3 expression was not significant in the cultures containing pulmonary CD11c+ APCs from mice treated with *M. vaccae.* The same *in vitro* assay was set up using splenic CD11c+ cells isolated from the spleen of allergic mice. Elevated levels of Foxp3 message was detected in the cells containing splenic CD11c+ APCs from mice treated with *M. vaccae* (Figure 5-11 b). In this case, Foxp3 expression was more pronounced than in the assays containing pulmonary CD11c+ APCs. In summary, when cultured together with naïve CD4+ T cells, CD11c+ isolated from both the lung and spleen induce elevated expression of Foxp3 mRNA from the cells in culture. This suggests that co-culture of naïve T cells with *M. vaccae*-induced CD11c+ APCs from the spleen, and possibly the lung, may activate a regulatory like T cell.


Foxp3
(a)
Copies per 10^6 copies of GAPDH
75000
50000
25000
0
BD
BD
1:40
1:40
T cells alone
CD11c+ cells alone


Foxp3
(b)
Copies per 10^6 copies of GAPDH
30000
20000
10000
0
*
BD
BD
1:40
1:40
T cells alone
CD11c+ cells alone

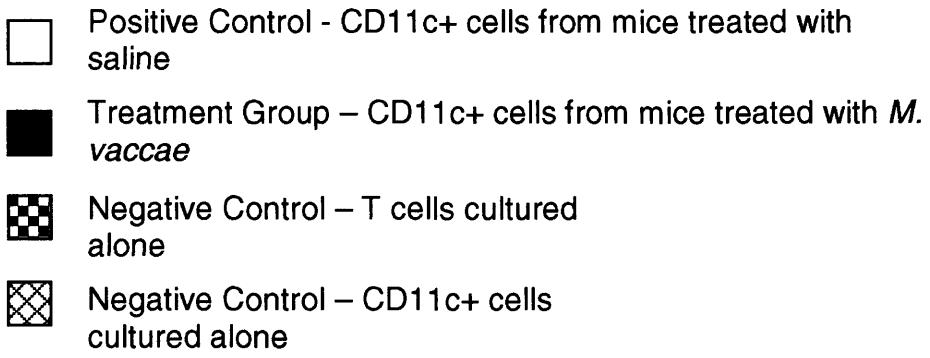


Figure 5-11: *In vitro* assay message. CD11c+ cells were isolated from the lung (a) or spleen (b) of allergic mice treated with *M. vaccae* (black bar) or saline (white bar). These were co-cultured with naïve CD4+ T cells. T cells were also cultured alone with anti-CD3 and IL-2 (checked bar). CD11c+ cells were culture alone in Growth Medium (without anti-CD3 and IL-2). Foxp3 mRNA levels were determined from the culture cells (CD11c+ cells from each group were pooled, n=6-8 mice per group, data expressed as mean ± SD, * $p<0.05$ compared to positive control, Students *t*-test was used to test for significant differences. This experiment was carried out at least three times).

3 Discussion

This chapter attempts to address the immunoregulatory role played by *M. vaccae*-induced CD11c+ APCs. The two possible hypotheses explored are; a) that the APCs act as regulatory cells themselves, and are "Suppressor APCs" [Rook *et al* 2004], or b) that they are involved in inducing Tregs, which then act as suppressor cells [Hawiger *et al* 2001, Lui *et al* 2002]. A passive transfer experiment was carried out, isolating pulmonary CD11c+ APCs from of mice treated with *M. vaccae* or saline, and injected to the lungs of mice with allergic pulmonary inflammation. The extent of inflammatory symptoms was determined to establish if these CD11c+ APCs were regulatory themselves. In addition, pulmonary and APCs were also cultured with naïve T cells and the cytokine release was analysing by ELISA and RT-PCR to determine if these APCs may be capable of inducing Tregs.

3.1 Transfer of CD11c+ APCs from mice treated with *M. vaccae* does not transfer protection

Results from this chapter suggest that the pulmonary CD11c+ APCs isolated from mice with pulmonary inflammation treated with *M. vaccae* may not be "regulatory" APCs per se. A passive transfer experiment was carried out by injecting the pulmonary CD11c+ cells from allergic mice treated with either *M. vaccae* or saline, directly into the lungs of allergic mice. It has been previously shown that IL-10 secreting cells, both T cells and APCs, can inhibit OVA-induced airway inflammation in a murine model [Akbari *et al* 2001, Zuany-Amorim *et al* 2002b, Akbari *et al* 2002]. These IL-10 secreting APCs may have the potential to inhibit inflammatory symptoms. In this thesis, no difference was observed in the number of cells in the BAL fluid between the recipient mice that received pulmonary CD11c+ APCs derived from mice treated with *M. vaccae,* the control groups (no cells injected) and the group injected with cells derived from allergic mice treated with saline. The animals that received an i.t. injection of pulmonary CD11c+ cells actually appeared to have an elevated number of cells in the BAL fluid. This may be due to the CD11c+ cells transferred into the lungs. These CD11c+ cells may have exacerbated inflammation and attracted other cells to the lungs.

From analysing the BAL fluid of donor mice, it was shown that treatment of the donor group with *M. vaccae* was successful in reducing pulmonary inflammation. This suggests that, the failure of the transfer experiment, where CD11c+ APCs were transferred from the lungs of mice treated with *M. vaccae* into the lungs recipient animals, was not because the *M. vaccae* treatment did not improve inflammation in the donor animals.

There are several possible explanations as to why CD11c+ cells failed to transfer the therapeutic effect of *M. vaccae.* The experimental design may have been at fault. CD11c+ cell numbers obtained in each experiment were a limiting factor. For this reason, 2×10^5 CD11c+ cells were injected per recipient mouse. This may not have been enough cells, since when carrying out passive transfer experiments other groups have transferred between 1×10^6 and 5×10^6 APCs [Lambrecht *et al* 2000, Akbari *et al* 2001]. However, very few cells were actually obtained from the lungs of 8 mice. If there were so few pulmonary CD11c+ APCs *in vivo,* it would be expected that few cells would be needed to have a suppressive effect, if indeed these APCs are capable of suppressing immune responses.

The route of delivery of these cells may also have an effect on suppressive ability of the APCs. These CD11c+ cells were injected i.t. into recipient mice. One other group has injected IL-10-secreting APCs i.v., which transferred suppression of inflammation to recipient mice [Akbari *et al* 2001]. However, another group has induced sensitisation to OVA by transferring OVA-pulsed DCs i.t. [Lambrecht *et al* 2000]. This was why this route was selected.

The timing of the experiment may also have played a role in the transfer experiment. Mice were sacrificed 3 days after passive transfer of CD11c+ cells. This may not have been enough time for the pulmonary CD11c+ cells to exert any "bystander" effects on the local cell population. However, the CD11c+ APCs were already secreting IL-10, TGF-β and IFN-α when transferred into the allergic recipient animals. It would be assumed that the "bystander" effect of the secreted cytokines would be rapid, since inflammatory responses can cause irreversible tissue damage quickly if not kept under control. It is therefore unlikely that 3 days was too short a time.

Another possible reason for the failure of the experiment may be that the source of the CD11c+ cells. When other groups have carried out similar transfer experiments, the APCs were isolated from lymphoid organs, such as the spleen, PPs or MLN [Akbari *et al* 2001, Akbari *et al* 2002, Chambers *et al* 2004]. APCs may need to migrate to these lymphoid organs to receive instructions before developing into "regulatory" APCs.

A final possible explanation as to why passive transfer of pulmonary CD11c+ cells did not result in reduced pulmonary inflammation is that these cells are simply not regulating immune responses themselves, and instead act by inducing regulatory cells. Injection of the CD11c- fraction from animals treated with *M. vaccae* into allergic recipients resulted in a small reduction in cellular influx, albeit not a significant one (data not shown). This may be due to T cells contained in the CD11c- cell fraction. An improvement in pulmonary inflammation has been observed when IL-10 secreting Tregs isolated from the LNs of allergic mice treated with *M. vaccae* are transferred into recipient allergic mice [Zuany-Amorim *et al* 2002b]. Any Tregs already in the lungs of mice treated with *M. vaccae* could be expected to be in the CD11c- cell fraction. Transfer of these cells, even in small numbers, could possibly reduce cellular infiltration. Therefore, it may be a possible hypothesis that these pulmonary CD11c+ cells are inducing Tregs.

3.2 CD11c+ APCs induce a Th2 response

Pulmonary CD11c+ cells were co-cultured with splenic CD4+ T cells from naïve mice, to determine if the CD11c+ APCs were capable of inducing T cells with regulatory characteristics. The CD11c+ cells induced a Th2 response, initiating IL-4 and IL-5 secretion. A decrease in IL-4 and IL-5 production was expected from the cultures containing CD11c+ cells from mice treated with *M. vaccae*, since *M. vaccae* treatment has been shown to decrease type-2 responses [Zuany-Amorim *et al* 2002a, Adams *et al* 2004]. It may be possible that more time is needed for any Tregs to inhibit type-2 cytokine production by activated T cells in the culture.

3.3 *M. vaccae*-induced CD11c+ APCs may be inducing T cells with regulatory like properties

IL-10 protein was increased in the supernatants of the cultures containing APCs from allergic mice treated with *M. vaccae*. TGF-β in the culture supernatants was below detection, although this is not surprising since elevated TGF-β was not observed in the BAL fluid of mice treated with *M. vaccae* (Chapter 3). Incidentally, these results were accompanied by an increase in both IL-10 and TGF-β mRNA from the wells containing CD11c+ cells from mice treated with *M. vaccae*. The inconsistencies in the TGF-β results may be due to the fact that Tregs are not producing TGF-β, and simply need it for development. Any TGF-β being secreted by the CD11c+ cells may have been used up by Tregs, hence none would be detected in the supernatants. Alternatively, it may be due to the fact that TGF-β is post-transcriptionally regulated [Chang *et al* 2002], so that TGF-β mRNA may have been transcribed but the TGF-β protein not translated.

From these experiments, the source of, IL-10 and TGF-β message remains unclear. Neither naïve CD4+ T cells nor CD11c+ APCs cultured alone in just Growth Medium, produced any IL-10 protein. Unfortunately, the CD11c+ cells alone were not cultured with IL-2 and anti-CD3. This should have been done, so that conditions were kept consistent and the effect of anti-CD3 and IL-2 on these APCs was known. IL-2 and anti-CD3 should have a minimal effect on CD11c+ APCs alone, since CD3 is part of the TCR signalling complex involved in activating T cells, and IL-2 is responsible for T cell activation and proliferation. However, without these controls the effect of IL-2 and anti-CD3 on CD11c+ APCs is not known. The only way to determine the exact source of the cytokines is to separate the T cells and the CD11c+ cells at the end of the *in vitro* assay, possibly using CD11c and CD4 magnetic microbeads. To determine the source of IL-10 and TGF-β, either intracellular staining or RT-PCR could be carried out on the individual cell populations. Technically, this would be a difficult experiment to do, since cell numbers were very limited, and amounts of RNA extracted from the individual cell populations would be minimal.

3.4 Foxp3 expression is induced in naïve T cells by *M. vaccae*-induced CD11c+ APCs

Since the source of IL-10 and TGF-β was unclear, the levels of Foxp3 mRNA in the culture cells was determined, since Foxp3 should only be expressed by T cells. Foxp3 is a transcription factor protein, which has been observed as defective in autoimmune diseases [Bennett *et al* 2001, Khattri *et al* 2003, Gambineri *et al* 2003]. Recently it has been described as a marker for Tregs, since it is highly expressed by naturally occurring CD4+CD25+ Tregs [Hori *et al* 2003, Read *et al* 2003, Ling *et al* 2004]. Since CD4+CD25- do not express Foxp3 once they have been activated, it was suggested that Foxp3 was not simply a marker of T cell activation, but a marker unique to Tregs [Fontenot *et al* 2003]. However this is now an area of conflict, since stimulation of human CD4+CD25- effector T cells with PHA enhances Foxp3 expression [Morgan *et al* 2005]. However, to date this has not been observed in mice.

In this thesis, Foxp3 mRNA levels were observed to be increased in the wells containing pulmonary CD11c+ APCs from mice treated with *M. vaccae,* albeit not significantly. Foxp3 message is not be expressed by the APCs, and is unique to Tregs. An increase of Foxp3 would suggest possible Treg development. However, not all Tregs have been observed to express Foxp3 and several different Treg subtypes have been described, each with a distinctive phenotype [Vieira *et al* 2004].

Naturally occurring CD4+CD25+ Tregs have been reported to express both Foxp3 and secrete IL-10. They constitutively express the IL-2 receptor α chain, CD25, and play a critical role in the prevention of autoimmunity and allograft rejection [Sakaguchi *et al* 1995, Salomon *et al* 2000]. They are naturally produced in the thymus and make up approximately 10% of all CD4+ T cells [Sakaguchi *et al* 1995, Salomon *et al* 2000, Stephens *et al* 2001]. However, it has been suggested that naturally occurring CD4+CD25+ Tregs are specific only for autoantigens expressed by the thymus. Naturally occurring CD4+CD25+ Tregs are important for inducing tolerance against self-Ag [Anderson *et al* 2000, Salomon *et al* 2000]. In contrast, Tr1 is a type of Tregs shown to be specific for Ags derived from, for example, bacteria and pathogens

[Cong *et al* 2002, Satoguina *et al* 2002]. Tr1 cells are induced by repetitive stimulation in the presence of IL-10, in both mice and humans. These cells produced high levels of IL-10, low levels of IL-2 and no IL-4 and have been shown to inhibit the onset of colitis in SCID mice [Groux *et al* 1997]. However, these cells have been reported to lack Foxp3 expression [Vieira *et al* JI 2004]. This may be a reason as to why the increase in Foxp3 mRNA is not significant in the CD4+ cells from the cultures containing CD11c+ cells derived from mice treated with *M. vaccae*. Another possible explanation for the unconvincing increase observed in Foxp3 expression may be because these cells were isolated from the lungs. When presenting Ag to T cells, APCs first migrate to the lymphoid organs. It may be possible that "regulatory" APCs need to migrate to lymphoid tissue before being able to affect T cell development and function. To test this theory, CD11c+ APCs were isolated from the spleens of allergic mice and the same assay carried out.

3.5 IL-10, TGF-β and Foxp3 expression induced in naïve T cells by CD11c+ APCs from the spleen of mice treated with *M. vaccae*

To establish whether APCs from a lymphoid organ had a similar effect on inducing Tregs, CD11c+ cells were isolated from the spleen of allergic mice and co-cultured with naïve CD4+ T cells. Increases in IL-10 protein and message, and TGF-β message were observed. This result was similar to that reported when pulmonary CD11c+ APCs were co-cultured with naïve T cells. The expression of Foxp3 was however significantly elevated in the cells containing CD11c+ spleen cells isolated from mice treated with *M. vaccae*, compared to those containing CD11c+ APCs from animals treated with saline. These data may suggest that CD11c+ APCs isolated from mice treated with *M. vaccae* have the capability to induce Tregs, but they may first need to have migrated to a lymphoid organ.

3.6 CD11c+ cell purity

The purity of the CD11c+ cell separations, when stained with CD11c+ Ab and analysed by FACS, was not always very high (>70%). This means that the cells could have contained additional cell types, such as lymphocytes.

Contamination could have had an affect on the results obtained from the *in vitro* assay, since cytokine secretion could have come from an unknown contaminating cell source. The CD11c+ fractions did however stain negative for CD3 and B220, suggesting minimal lymphocyte presence. In addition, the CD11c+ cells were incubated overnight in plastic flasks, before being used in the *in vitro* assay. This would have removed the majority of MΦs, which would have adhered to the plastic. The reason for such a low purity is therefore unclear.

There are several possible explanations. As discussed in Chapter 4, the natural autofluorescence of APCs is very high, covering a wide spectrum. It is therefore possible that the CD11c+ cells are purer than the FACS analysis is detecting. Some of the staining, particularly staining of cells with weaker CD11c signals, may be masked by the populations' own autofluorescence. Another possibility is that the CD11c Ab to which the magnetic MACS microbead is attached is the same clone as used by BDPharmigen for FACS stains. If this were the case, treatment of the pulmonary cells with the microbeads would sequester all the CD11c+ surface Ag specific for that clone. Further treatment with a FITC or PE conjugated Ab would be unsuccessful, since all cell the surface Ag would be blocked. However, microbeads are meant to drop off after approximately 6 hours, and these CD11c separated APCs were cultured overnight. It is therefore unlikely that the FACS Ab did not bind to the cell surface CD11c.

As discussed previously, CD11c alone is not an ideal marker for DC isolation. It is expressed not only by DCs, but also by MΦs and NK cells [Ancuta *et al* 2000, Hogg *et al* 2003, Lima *et al* 2002]. A more appropriate method for isolating DCs from organs such as pulmonary tissue would be to sort them using the FACSCalibur. Different DC subsets could be separated this way using 3 DC specific markers. For example, CD11c+CD11b+CD8α+ pDCs could be isolated by staining with each Ab on a different fluorochrome. This would involve staining the cells with flurochrome conjugated Abs, and setting the gates on the CellQuest program to sort cells which appeared positive at all 3 wavelengths. However, the FACSCalibur is not designed for such complicated, high quality sorting. A more sophisticated cell sorter exists, known as the MoFlo (made by

BD). The MoFlo is capable of four way sorting, using four different flurochrome conjugated Abs, giving purities of >95%. However, this piece of equipment was not available during the performance of this thesis, so other methods of DC separation had to be used.

4 Conclusion

This chapter analyses the possible role of *M. vaccae*-induced CD11c+ APCs in the lung. When they are isolated from the lungs, they do not transfer the protective effect of *M. vaccae* to other animals with allergic pulmonary inflammation. When cultured *in vitro,* elevated IL-10 detected in the supernatants and in the cells from the cultures suggests that pulmonary CD11c+ APCs derived from allergic mice treated with *M. vaccae* may have the ability to influence T cell development when cultured together with naïve T cells. More work is required to determine the exact source of IL-10 and TGF-β in these cultures, since both CD11c+ APCs and T cells from allergic animals treated with *M. vaccae* have been shown to produce these cytokines. Although more work is needed, these data provide some tantalising preliminary data to suggest CD11c+ APCs isolated from allergic mice treated with *M. vaccae* may have the ability to induce Tregs.

Chapter 6

Chapter 6: General Discussion

1.1 Introduction

The last three chapters have described the preventive and therapeutic effect of treatment with *M. vaccae* on the symptoms of allergic pulmonary inflammation in a murine model. Treatment with *M. vaccae* alleviated symptoms by reducing eosinophilia and IL-4 production. Treatment with *M. vaccae* was also shown to induce secretion of IL-10 and TGF-β, and increased expression of IFN-α, by pulmonary CD11c+ APCs. Culture of these CD11c+ APCs with naïve T cells resulted in increased IL-10 levels in the culture supernatants, and elevated expression of Foxp3. This suggests that CD11c+ APCs derived from mice treated with *M. vaccae* may have the ability to induce T cells with a regulatory phenotype. The main focus of this discussion will be on the role of *M. vaccae* in immunoregulation, and how it may interact with the immune system to induce tolerance to allergens.

1.2 *M. vaccae*

Although *M. vaccae* was first described in 1965 [Bonicke *et al* 1965], the strain used as treatment in this thesis, *M. vaccae* R887R, was described later in 1973 and was isolated from mud samples obtained from the Lango district of central Uganda [Stanford *et al* 1973]. *M. vaccae* has been used as an immunotherapy for several different diseases, including cancer [Baran *et al* 2004], leprosy [Katoch *et al* 1996] and tuberculosis [Stanford *et al* 2004]. This thesis has focussed on the use of *M. vaccae* as an immunotherapy for allergy, in particular asthma.

1.2.1 Effect on type-2 responses

Several groups have investigated the effect treatment with *M. vaccae* has on the symptoms of asthma, both in murine models and in human clinical trials. Treating allergic mice with *M. vaccae* results in a decrease in eosinophils numbers in the

BAL fluid [Hopfenspirger *et al* 2002, Zuany-Amorim *et al* 2002a, Smit *et al* 2003], which suggests a reduction in inflammation. This decrease in eosinophilia was therefore an important outcome measurement in this thesis, and was expected and observed every time mice with pulmonary inflammation were treated with *M. vaccae* (Chapter 3). Eosinophilic inflammation is a result of an excessive type-2 response. If eosinophilia decreased, it would be expected that a decrease in other type-2 cytokines would be observed.

Research on the treatment with *M. vaccae* has shown conflicting results in regards to its effects on Th2 cytokine levels such as IL-4 and IL-5 in the BAL fluid. One group observed no difference between IL-4 and IL-5 levels from mice treated with saline and mice treated with *M. vaccae* [Zuany-Amorim *et al* 2002a]. Other groups have however observed reductions in either IL-4 levels [Smit *et al* 2003] or IL-5 [Hopfenspirger *et al* 2002, Smit *et al* 2003] in the BAL fluid of mice treated with *M. vaccae*. Until now alterations to the cytokine milieu in the lung tissue itself, which is the actual site of inflammation, has never been investigated. This thesis is the first to report a decrease in IL-4 expression in the lung, which was not associated with an increase in type-1 cytokines IFN-γ and IL-12. This suggests that the observed decrease in type-2 responses in not due to the development of a type-1 response.

1.2.2 Effect on immunoregulation

Treatment with *M. vaccae* has been observed to induce elevated levels of IL-10 in the BAL fluid, both in this thesis (Chapter 3) and by collaborators [Zuany-Amorim *et al* 2002a]. This suggests the development of immunoregulatory mechanisms, since the decrease in local type-2 responses is not due to an antagonistic increase in a type-1 response. Both IL-10 and TGF-β are involved in regulating the immune response, although the exact mode of action induced by these cytokines remains unclear. IL-10, for example, has been observed to play a major role in Ig class switching, influencing B cells to produce IgG4 rather than IgE [Banchereau *et al* 1994]. A reduction of IgE would reduce inflammation, since less mast cells and

basophils would be activated to degranulate. On the other hand, TGF-β has been shown to inhibit IL-2 production, which would affect the proliferation capability of naïve T cells [Seder *et al* 1999]. *M. vaccae* is likely to trigger a combination of different mechanisms concerning both IL-10 and TGF-β, and possibly other cytokines, which contribute to the suppression of type-2 inflammatory responses.

One additional mechanism by which IL-10 and TGF-β influence immunoregulation is though the differentiation and function of Tregs. Indeed, treatment with *M. vaccae* has been shown to induce CD4+CD45RB^{Low} Tregs. Transfer of these sorted Tregs isolated from the spleens of mice treated with *M. vaccae* into sensitized recipient mice significantly reduced the OVA-induced eosinophilic inflammation. In contrast, transfer of CD4+CD45RB^{High} T cells from animals treated with saline had no suppressive effect [Zuany-Amorim *et al* 2002b]. This suggests that the Tregs induced by treatment with *M. vaccae* may be responsible for alleviation of allergic symptoms [Zuany-Amorim *et al* 2002b, Adams *et al* 2004]. In this thesis, the increase observed in IL-10 protein levels in the BAL fluid of mice treated with *M. vaccae* (Chapter 3), may be consistent with Treg activity.

The source of IL-10 detected in the BAL fluid of mice treated with *M. vaccae* is still unclear. Although *M. vaccae*-induced Tregs were observed to secrete IL-10, the suppressive effect on pulmonary inflammation is inhibited by anti-IL-10 and anti-TGF-β neutralising Abs [Zuany-Amorim *et al* 2002b]. The function of these Tregs must be dependent on these cytokines. In addition to the function, the development of Tregs has also been shown to require IL-10 and TGF-β [ref]. If the presence of IL-10 and TGF-β is necessary for Treg development and function, then there must be an initial source of these cytokines. Tregs cannot be the only major source of IL-10 and TGF-β, as these cytokines are required for their initial development. This primary source of IL-10 and TGF-β remains elusive, but examples of "regulatory" APCs which are capable of inducing Tregs have been observed. For example, tolerogenic DCs isolated from the LNs of mice exposed to OVA secrete IL-10. In addition, when cultured with naïve T cells, these DCs induced OVA-specific T cells *in vitro*, which also secreted IL-10. These IL-10-

secreting T cells blocked AHR development and eosinophilia when transferred to allergic recipient animals [Akbari *et al* 2001].

In this thesis, CD11c+ APCs isolated from the lungs of mice with pulmonary inflammation treated with *M. vaccae* were observed to produce increased levels of IL-10 and TGF-β protein, compared to CD11c+ APCs isolated from the lungs of animals treated with saline. In addition, increased levels of IFN-α mRNA were observed in the CD11c+ APCs from the lungs of mice treated with *M. vaccae*. IFN-α has been observed to upregulate IL-10 production by DCs [Bilsborough *et al* 2003]. This suggests that secretion of IFN-α by APCs may contribute to the inhibition of inflammatory responses, through Treg development. From these data, it would appear that *M. vaccae* has the ability to regulate immune responses. There are a number of other microbial organisms with similar characteristics that will be discussed in the next sections.

1.3 Microorganisms and immunoregulation

All microorganisms, whether pathogenic and harmless, influence the immune system. Typically, bacterial infection induces a type-1 response, whereas infection with helminths induces a type-2 response. Mycobacteria in particular are known for their strong induction of Th1 responses upon infection [Rook *et al* 1998, Manca *et al* 2001]. Under certain conditions, however, microorganisms can induce immunoregulation in the host. Examples will be discussed in the next sections.

1.3.1 Induction of immunoregulation by Lactobacilli

The "Old Friends" hypothesis suggests that harmless microorganisms which have been encountered by humans throughout evolution have influenced the immune system, so that excessive immune responses are not mounted against non-pathogenic Ags. The constant presence of lactobacilli in the natural intestinal flora leads to their inclusion as an "Old Friend". As the increased use of antibiotics coincides with an increase in the prevalence of allergy [Taylor *et al* 1984, Downs *et*

al 2001] it may be inferred that the destruction of lactobacilli also correlates with a rise in prevalence of allergy, since the increased use of antibiotics in modern medicine has led to lowered bacteria populations, including lactobacilli, in the gut. Evidence that the correlation exists comes from the association of lower incidence of allergy with the presence of large amounts of lactobacilli in the intestinal flora [Sepp *et al* 1997, Bjorksten *et al* 1999]. These indirect evidences have led to the hypothesis that the presence of lactobacilli may induce tolerance against allergy [Reviewed in Rook *et al* 2004].

Direct evidence of a correlation between lactobacilli and tolerance against to is available. The ingestion of *Lactobacillus rhamnosus* by children with atopic dermatitis does result in a rise in IL-10 levels both in the patients' serum, and by their stimulated PMBCs [Pessi *et al* 2000]. The source of this IL-10 may be inducible Tregs, since *Lactobacillus reuteri* and *Lactobacillus casei* primed DCs have been shown to induce an IL-10-secreting Treg, when they are cultured with naïve T cells. These Tregs were also capable of inhibiting proliferation of stimulated T cells, an effect that was suppressed by addition of anti-IL-10 neutralising Ab [Smitts *et al* 2005].

There is an evolutionary advantage to developing immunoregulation to harmless Ags, such as lactobacilli and *M. vaccae*. An excessive reaction of the immune system can cause tissue damage and may under certain conditions be fatal. It is therefore likely that the host immune system has developed mechanisms by which tolerance is induced upon recognition of, for example, lactobacilli-specific Ag. One such mechanisms include the induction of Tregs and the secretion of immunosuppressive cytokines.

1.3.2 Induction of immunoregulation by helminths

Infection with parasitic helminths typically induces a type-2 response from the host, with the production of cytokines IL-4, IL-5 and IL-13, increased serum IgE levels and the activation and degranulation of mast cells, eosinophils and basophils [Maizels *et al* 2004]. Since allergy is also a result of excessive Th2 responses, the

expectation is for infection with helminths to exacerbate allergic symptoms. However, in tropical countries where helminth infection is commonplace, allergies are rare [Wilson *et al* 2004]. One study compared the rate of infection with *Schistosoma haematobium* with positive skin tests to house dust mite in school children from The Gambia. It was observed that fewer *Schistosoma haematobium* infected children gave a positive skin test result compared to those children who were parasite free [van der Biggelaar *et al* 2004]. In a murine model, infection of OVA or Derp1-sensitized mice with the nematode *Heligmosomoides polygyrus* or *Litomosoides sigmodontis* resulted in a significant decrease in eosinophilia in the BAL fluid and epithelial goblet cell proliferation in the lungs [Maizels *et al* 2004, Wilson *et al* 2005].

Following murine infection with helminths, the host develops type-2 responses, with high levels of IL-4, IL-5 and IL-13. This response reaches its peak approximately 8 weeks after infection, and then starts to gradually decline. By week 16, the type-2 response has significantly downmodulated, but does not completely cease. Significantly, the decrease of a type-2 response is accompanied by an increase in IL-10 production [King *et al* 1993, Yazdanbakhsh *et al* 2002, Cooper *et al* 2001]. Indications of the involvement of Tregs in helminth infection have been highlighted by studies with *Schistosoma mansoni.* CD4+CD25+ T cells which secrete IL-10 and express Foxp3, have been isolated from the LNs of *Schistosoma mansoni* infected mice. When cultured *in vitro* with CD4+CD25- cells, these cells inhibit proliferation of naïve T cells [McKee *et al* 2004]. Similarly, IL-10 secreting CD4+CD25+ T cells have been isolated from egg-induced granulomas in the liver. Again, these T cells have the ability to inhibit *in vitro* proliferation of naïve T cells [Hesse *et al* 2004]. Induction of Tregs has also been observed during experimental infection with the filarial nematode, *Litomosoides sigmodontis.* CD4+CD25+ T cells isolated from the thoracic cavity, the site of infection, expressed high levels of CTLA-4, GITR and Foxp3, all markers associated with Treg activity [Taylor *et al* 2005]. These studies suggest that Tregs are induced during helminth infection.

1.3.3 Induction of immunoregulation by *Bordetella pertussis*

Respiratory infection with the Gram-negative bacterium *Bordetella pertussis,* which causes whooping cough, induces a Th1 response [Mills *et al* 1993, Ryan *et al* 1997, Ivanoff *et al* 1997]. *Bordetella pertussis* produces a number of virulence factors which aid the survival of the bacterium in the respiratory tract by mediating adherence to the epithelium [Bromberg *et al* 1991, Steed *et al* 1992]. One such virulence factor is filamentous haemagglutinin (FHA). FHA has been observed to inhibit IL-12 production by MΦs and the development of Th1 responses by the host [McGuirk *et al* 2000a, McGuirk *et al* 2000b]. Incubation of DCs with FHA induced a significant production of IL-10. These DCs were shown to direct the generation of Tregs in the lungs of mice infected with *Bordetella pertussis.* The DC-induced Tregs secreted high amounts of IL-10, and perhaps surprisingly IL-5. These Tregs were able to suppress a Th1 response to an unrelated Ag, influenza virus hemagglutinin [McGuirk *et al* 2005]. *Bordetella pertussis* is a highly pathogenic microbe, which induces this mechanism of tolerance to prolong its survival in the host.

1.3.4 Evolution

Data suggest that both pathogens and harmless microorganisms have evolved regulatory mechanisms to avoid detection by the host immune system, and to control immune-mediated pathology. The need of regulating immune responses is critical for host survival. For example, IL-$10^{-/-}$ mice infected with the malaria parasite, *Plasmodium chabaudi chabaudi* mount a vigorous immune response which cannot be suppressed even once the pathogen is cleared. Mice die from irreversible tissue damage [Li *et al* 1999]. Hence morbidity is a direct consequence of the host immune response, rather than of the pathogen. In the case of *M. vaccae,* a non-pathogenic organism which only survives briefly in the host, the exposed host would waste energy and potentially incur immune mediated pathology if it were to mount an immune response. Hence, by taking specific cues from *M. vaccae,* the host may regulate the immune response and prevent immune-mediated pathology by developing IL-10-secreting Tregs. Once induced these

immunoregulatory mechanisms can be useful under other conditions where developing an immune response would be harmful. It is possible that pathogens may have evolved to mimic this mechanism adopted by harmless, non-pathogenic microorganisms, such as *M. vaccae.* This is an example of the evolutionary arms race between the immune system and pathogenic organisms. Pathogens are constantly evolving new ways of preventing elimination from the host. In turn, this is a major selective force for the immune system to evolve new and improved ways of detecting and clearing potentially dangerous infections.

1.4 *M. vaccae* and the hygiene hypothesis

M. vaccae is a harmless pathogen, which has been described as an "Old Friend" [Rook *et al* 2004], and incorporated into the revised version of the hygiene hypothesis [Rook *et al* 1998]. *M. vaccae* has several unique characteristics which make it an interesting immunomodulator. It is a ubiquitous, saprophytic organism, found abundantly in the environment, in the soil and untreated water. The fact that exposure to *M. vaccae* was once a common occurrence warrants its inclusion as an "Old Friend". The existence of a subset of CD1-restricted T cells lends further supporting evidence to the exposure of saprophytic mycobacteria throughout human evolution. These appear to recognize only mycobacterial lipids and glycolipids [Dutronc *et al* 2002]. Mycobacterial lipids have also been detected in healthy human lymphoid tissue, such as the tonsils [Hanngren *et al* 1987]. Whereas in the past exposure to *M. vaccae* would have been a common occurrence, in modern living conditions limited exposure may have resulted in immunodysregulation. The lack of immunoregulatory signals may have lead to the observed increased in the development of inappropriate immune responses to common allergens, to self and to gut content.

One of the most unique characteristics of *M. vaccae* is that it retains its immunotherapeutic properties even after it has been heat-killed. The paper by Hopfenspirger and Agrawal is the only study to look at the effect of live *M. vaccae* on mice with allergic pulmonary inflammation, and showed similar results to those presented in this thesis, which addressed treatment with heat-killed *M. vaccae.* A

significant reduction in total cell numbers in the BAL fluid was observed, along with reduced eosinophil numbers [Hopfenspirger *et al* 2002].

Similar studies using BCG as a treatment for allergic pulmonary inflammation have been attempted. BCG is an attenuated strain of *M. bovis,* which is used as a vaccine to prevent TB, with a protective efficacy that ranges from 0-80% in different locations [Colditz *et al* 1994]. Similarly to treatment with *M. vaccae*, in a murine model of OVA-induced pulmonary inflammation, treatment BCG i.n. was shown to be affective in inhibiting airway eosinophilia and IL-4 and IL-5 in the BAL fluid and MLN respectively [Major *et al* 2002]. In humans, according to a single study, vaccination of asthmatic children with BCG results in improvement of symptoms. In addition, it was claimed that revaccination of the same children resulted in further improvement of lung function [Choi *et al* 2002]. This observation needs to be confirmed, because epidemiological studies do not find decrease in atopic symptoms in BCG-vaccinated compared to non-vaccinated children [Alm *et al* 1998].

Unlike *M. vaccae,* data suggest that BCG reduces symptoms of allergic disease by inducing a type-1 response, which may antagonise the type-2 response associated with asthma. In a murine model of allergic pulmonary inflammation, treatment with BCG significantly inhibits eosinophil numbers, IL-4 production in the BAL fluid and IgE levels in the peripheral blood [Major *et al* 2002]. In addition, a shift towards a type-1 response was observed in the peripheral blood of asthmatic children treated with BCG. The ratio of IFN-γ to IL-4 protein in the supernatants of PBMC cultures increased [Choi *et al* 2002], suggesting that BCG vaccination was inducing an increase in the Th1 cytokine, IFN-γ. In spite of this, evidence of a Th1 switch following treatment with BCG remains controversial, as other research groups have not successfully replicated these data.

1.4.1 Th1 responses

The use of *M. vaccae* as a Th1 stimulator has been investigated. In the case of

cancer, Th1 responses promote cytotoxic immunity, and hence cell destruction. Indeed, Th1 mediated immunity has been shown to be impaired in patients suffering from many types of cancer, including prostate, colon and renal cancer [Elsasser-Beile *et al* 1998, Heriot *et al* 2000]. The treatment of several types of cancer with *M. vaccae* has been attempted, including prostate cancer, melanoma and lung cancer [Hrouda *et al* 1998, Maraveyas *et al* 1999, O'Brien *et al* 2000]. It was hypothesized that *M. vaccae* was rich in heat shock proteins largely homologous with those of cancer cells [Ferrarini *et al* 1992], hence would initiate a Th1 response that may directly attack the tumour cells. It was also suggested that that treatment would downregulate a Th2 response, possibly via the induction of Tregs. In the case of prostate cancer, 10 patients were treated with heat-killed *M. vaccae* intradermally at regular intervals, using the prostate specific Ag (PSA) as a marker of cancer advancement. The levels of PSA in the serum rise as the cancer progresses. In half the patients, the rate at which PSA increased slowed down significantly after treatment with *M. vaccae*. Although treatment with *M. vaccae* was successful in slowing the rate of PSA in half the patients, this study showed no induction of IFN-γ, and only in three responding patients was a reduction in PBMC secretion of IL-4 observed [Hrouda *et al* 1998]. The improvements of PSA levels, however, lead to more trials using *M. vaccae* as an adjuvant in combination with chemotherapy.

It is hypothesized that chemotherapy treatment induces release of tumour-specific Ags or tumour-associated Ags from the treated tumour, and that a non-specific immunostimulator, such as *M. vaccae,* could enhance recognition of tumour-specific or tumour-associated Ag in patients, who may be suffering from immunosuppression. The encouraging results from the prostate cancer Phase I/II trial [Hrouda *et al* 1998] suggested that *M. vaccae* was a potential immunostimulator. Trials were carried out on patients with inoperable small-cell lung cancer (SCLC) non-small-cell lung cancer (NSCLC) and mesothelioma (a less common cancer arising from the serosa of the lung and closely associated with asbestos exposure), using *M. vaccae* as an adjuvant in combination with chemotherapy [O'Brien *et al* 2000, Assersohn *et al* 2002, Mendes *et al* 2002]. Promising results were observed in all these trials. In the case of SCLC, mean

survival time increased from 8.6 months to 12.9 months, for patients treated with chemotherapy alone versus patients treated with a combination of chemotherapy and *M. vaccae* respectively [Assersohn *et al* 2002]. A trend towards improved mean survival and improved 1-year survival was observed when *M. vaccae* was given in combination with chemotherapy in with NSCLC and mesothelioma. Sleep and appetite also were noted to improve, after treatment with *M. vaccae*. In addition, an increase in the activation of NK cells, and a decrease in the percentage of IL-4 producing T cells were observed in the peripheral blood after treatment with *M. vaccae* [O'Brien *et al* 2000].

Unfortunately, results from a Phase III clinical trial into NSCLC failed to confirm significant beneficial results (www.srpharma.com/documents/SRL172_ updated_050617.pdf). Little work has hence been carried out using *M. vaccae* as a treatment for cancer. Interestingly, it has been suggested that naturally occurring Tregs play a major role in the development of cancer. Indeed, increased numbers of CD4+CD25+ T cells have been observed in the peripheral blood of patients suffering from epithelial malignancies [Wolf *et al* 2003]. It would have been interesting to observe whether *M. vaccae* treatment had any effect on the numbers of CD4+CD25+ Tregs circulating in the peripheral blood or tumour tissue of cancer patients.

1.4.2 Immunoregulation

The treatment of allergic mice with *M. vaccae* induces immunoregulation via IL-10 production. The actual source of the IL-10 remains, however, unclear. Treatment with *M. vaccae* has shown to induce the development of Tregs, which secrete IL-10. Transfer of these *M. vaccae*-induced CD4+CD45RB^{Low} Tregs i.v. to recipient allergic mice significantly reduced the number if eosinophils in the BAL fluid [Zuany-Amorim *et al* 2002b]. The importance of these Tregs in reducing pulmonary inflammation, suggests that these cells may be the source of IL-10. However, in this thesis pulmonary CD11c+ APCs were isolated from mice treated with *M. vaccae,* which also secrete enhanced levels of IL-10.

When these IL-10-producing CD11c+ APCs were incubated with naïve CD4+ T cells, an increased amount of IL-10 was produced. From the experiments carried out, it is unclear whether the IL-10 is produced by the CD4+ T cells, or by CD11c+ APCs. Colleagues from the same laboratory have observed similar data. Murine pDCs were generated by incubating bone-marrow derived DCs with flt3-ligand, as previously described [Bjorck *et al* 2001]. These pDCs were the incubated overnight with either *M. vaccae* or *Propionibacterium acnes*. When co-cutured with naïve CD4+ T cells, significantly elevated levels of IL-10 were observed in the supernatants from the cultures containing pDCs treated with *M. vaccae*. The cultures containing pDCs treated with *P. acnes* did not have elevated IL-10 production [Martinelli *et al* unpublished observations]. It is still unclear whether the pDCs or the naïve T cells are secreting the IL-10. To determine this, the pDCs could be isolated from the bone marrow of IL-$10^{-/-}$ mice. If, once treated with *M. vaccae*, and cultured with wild type naïve T cells they still produce IL-10, then it can be concluded the T cells are producing the IL-10. If no IL-10 is produced, then it is the *M. vaccae* treated pDCs are the source of IL-10.

These data correlate with the data observed in this thesis. Furthermore, these data suggest that the IL-10-producing CD11c+ APCs described in this thesis may be pDCs. The fact that these CD11c+ APCs also expressed elevated levels of IFN-α supports this hypothesis. However, the limited FACS staining that was carried out suggests that they are not pDCs, since they are CD8α- and B220-. However, not all groups have observed pDCs as being B220+, so analysis of more pDC associated markers, such as Gr-1 and CD11b, may be necessary. In addition, problems were encounter when analysising the pulmonary CD11c+ APCs FACS staining, which was discussed in detail in Chapter 4. Briefly, excessive autofluorescence of cells stained with the isotype control made staining difficult to detect. B220 staining may have been present, but not detected using FACS analysis.

1.4.3 Role of APCs in immunoregulation

Although it is known that *M. vaccae* promotes the development of Tregs, the mechanism by which they are induced is unclear. APCs are likely to play a major role, as they not only are involved in the initiation of inflammation but also in the initiation of mucosal tolerance [Holt *et al* 2000]. Microorganisms associated with the hygiene hypothesis have been observed to induce Tregs via APC stimulation. For example, human DCs incubated with *Schistosoma*-derived lysophosphatidylserine are characterised by decreased IL-12 production and increased IL-10 production. They are also observed to drive the generation of IL-10 secreting Tregs *in vitro* [van der Kleij *et al* 2002].

In this thesis, pulmonary CD11c+ APCs from mice treated with *M. vaccae* expressed elevated levels of IL-10, TGF-β and IFN-α mRNA (Chapter 4). *M. vaccae* may possibly influence "regulatory" APCs to induce Tregs. The way in which *M. vaccae* actually interacts with the APCs remains unclear, and should be further investigated. For example, additional FACS analysis is required to establish which cell surface receptors upregulate after treatment with *M. vaccae.* Although it appeared that *M. vaccae* did not upregulate expression of MHC II, CD80 or CD86, many other co-stimulatory pathways exist through which *M. vaccae* may signal (examples discussed in detail below). In addition to identifying through which pathway *M. vaccae* signals, the relevant *M. vaccae*-derived Ag responsible for inducing tolerance could also be identified. If the actual component that is responsible for inducing Tregs can be isolated, this could have positive implications for treatment. Once the active component has been identified, being that a protein, carbohydrate or a lipid, a synthetic mimic can be synthesised. Already, one component of *M. vaccae* has been synthesised (termed SRP312™), and patent applications for SRP312™ have been submitted. Pre-clinical data suggest that, like heat-killed *M. vaccae,* treatment with SRP312™ significantly reduces allergic pulmonary inflammation. It is however likely, that a number of individual components from *M. vaccae* may act in concert and induce immunoregulation, or that a number of them may have similar ability and act in synergy.

1.5 "Regulatory" DCs

DCs are believed to play an important role in the initiation on any immune response. The three main types of DC, "lymphoid" DCs, "myeloid" DCs (mDCs) and "plasmocytoid" DCs (pDCs), were described and discussed in Chapter 4. Most of the work in regard to regulation has been done on mDCs and pDCs, but all have been observed to have some regulatory properties [Dhadopkar *et al* 2001, Gasche *et al* 2000, Kobayashi *et al* 2004]. "Regulatory" DCs can influence T cell activation and development through two main mechanisms; expression of co-stimulatory markers and cytokine production. For example, immature APCs with low levels of CD80 and CD86 are less effective at activating T cells, since these ligands will preferentially bind CTLA-4 on T cells, which has a negative regulatory effect, rather than CD28 which activates T cell proliferation [Jonuleit *et al* 2000]. In addition, through the secretion of cytokines such as IL-10, APCs can regulate the induction of Tregs [Jump *et al* 2002, de Heer *et al* 2004]. It is probable that *M. vaccae* influences "regulatory" APCs through more than one receptor or pathway.

1.5.1 APC receptors

An important feature of the innate immune system is that it can recognize and target microbial components. The innate immune system has evolved receptors that can recognize these pathogen-associated molecular patterns (PAMPs). PAMPs on the surface of *M. vaccae* and other "Old Friends" may interact with pattern recognition receptors on host APCs. This interaction may influence APCs maturation, and induce specific cytokine secretion [Rook *et al* 2004, Christensen *et al* 2002, Akbari *et al* 2003]. It is likely that the unusual fatty acid with its double bond at position 10-11 (SRP312) that has been isolated from *M. vaccae* and synthesised, is able to interact with pattern recognition receptors (PRR) of APCs, though the nature of this interaction has not yet been defined and is an important target of future research.

1.5.2 Toll-like receptors

The Toll-like receptors (TLRs) are a family of pattern recognition receptors that recognize a broad range of microbial products. TLRs are expressed by APCs and are involved in the recognition of foreign and sometimes self-Ag [Takeda *et al* 2003]. Stimulation of the TLRs activates nuclear factor kappa B (NF-κB), a transcription factor that plays a major role in initiating the production of pro-inflammatory cytokines. Eleven TLRs are known to date, and some form heterodimers with other TLRs. A wide variety of microbial components are known to stimulate the TLRs, including LPS (TLR4), bacterial flagellin (TLR5), double stranded RNA (TLR3), bacterial DNA (TLR9) and single stranded viral RNA (TLR7) [Underhill 2005].

Studies using mouse models deficient in TLRs have demonstrated the importance of TLR signaling in host defense against microbes. For example, $TLR4^{-/-}$ mice are more prone to infection with Gram-negative bacteria [Hagberg *et al* 1984]. Interestingly, $TLR2^{-/-}$ mice are more susceptible to mycobacteria [Heldwein *et al* 2002, Seya *et al* 2002] and Gram-positive bacteria [Takeuchi *et al* 2000, Echchannaoui *et al* 2002]. A lack in TLRs means that the host cannot recognize PAMPS from the particular microorganism, and cannot mount an effective immune response.

TLR4 appears to be essential in the detection of LPS and in the defense against Gram-negative bacteria. LPS is used as a marker for bacterial contamination. Its has been observed that children in German farming communities who have high levels of LPS in their bedding are less likely to develop allergies, than those children whose bedding contains little LPS [Braun-Fahrlander *et al* 2002]. This study supports the hygiene hypothesis, which suggests that exposure to a variety of microbes should educate the immune system, so it will not respond inappropriately to harmless Ag. If LPS is a marker of bacterial presence, and high levels of LPS correlates with low incidence of allergy, it might be expected that

TLR4 expression is also associated with allergy. One study of children in Sweden has established a link between a polymorphism in the TLR4 gene and asthma. Children with this polymorphism (Asp299Gly) had decreased levels of LPS-induced IL-12 secreted by PBMCs. This suggests that in children with an altered TLR4 receptor, LPS may not be signaling through TLR4 adequately. These same children also had a four-fold higher probability of developing asthma [Fageras Bottcher *et al* 2004]. This suggests signaling through TLR4 may prevent the development of asthma. However, other studies have shown no evidence that a variety of polymorphisms, and hence genetic variation, in the TLR4 gene contributed to asthma susceptibility [Raby *et al* 2002].

Infection with mycobacteria tends to trigger APC through TLR2. For example, the cell wall of BCG and liporabinomannan from saprophytic mycobacteria species is recognized by TLR2 [Heldwein *et al* 2002]. Indeed, deficiencies in the TLR2 gene result in increased susceptibility to *Mycobacterium leprae* and *Mycobacterium tuberculosis* [Kang *et al* 2002, Bochud *et al* 2003, Reiling *et al* 2002]. In addition, TLR2 has been associated with allergy and the hygiene hypothesis. A study conducted in Germany and Austria compared TLR2 expression in children living in rural farming communities. Children carrying a particular polymorphism in the TLR2 gene were significantly more likely to be diagnosed with allergic asthma and hay fever compared to those children without this particular TLR2 allele [Eder *et al* 2004]. This suggests that genetic variation in TLR2 is a major determinant of the susceptibility to asthma and allergies in the children of farmers. A negative association between children from farming communities and the incidence of allergy has already been discussed in Chapter 1, so taken together with the study of TLR2 on children from farming communities it would be interesting to see if treatment with *M. vaccae* has any effect on TLR2 signaling.

A simple experiment to determine whether treatment of *M. vaccae* alleviates inflammation by activating TLR2 signaling would be to set up a pulmonary inflammation model using $TLR2^{-/-}$ transgenic mice. If treatment no longer decreases pulmonary inflammation, it can be concluded that TLR2 is essential for *M. vaccae* recognition and induction of regulatory mechanisms. Alternatively, an *in*

vitro experiment could be set up to determine the effect of blocking TLR2. For example, the *in vitro* assay described in Chapter 5 (and Chapter 2 section 7.3) could be modified, with the addition of an anti-TLR2 neutralising Ab. The effect of TLR2 blockade on naïve T cell proliferation could be determined, along with the effects it has on cytokine secretion and Foxp3 expression. Experiments such as these could determine whether *M. vaccae* binds the TLR2 receptor, and whether this has an effect on the induction of Tregs. It would also be interesting to know if SRP312 interacts with TLR2. TLR2 forms heterodimers with TLR1 or TLR6, and these alternative dimers recognize subtly different lipid structures.

1.5.3 CD14

Another receptor that has been linked to asthma susceptibility is CD14. CD14 is expressed and secreted by myeloid cells. It plays a crucial role in binding many microbial components, in particular LPS, and facilitating their interaction with cell membrane associated signaling molecules, such as TLRs. In some studies, a specific polymorphism in the 5' promoter region of CD14 is associated with high levels of IgE in the peripheral blood. Children who were homozygous for the base T at −159 had less serum IgE and more serum sCD14 compared to children who were homozygous C or heterozygous, suggesting that the increase in sCD14 and decrease in serum IgE may be the result of a recessive allele [Baldini *et al* 1999]. This suggests that CD14 may play a role in asthma susceptibility. Indeed, studies in farming communities have shown that the PBMCs cells from children of farmers, who have a lower risk of developing allergies, expressed higher levels of CD14 mRNA [Lauener *et al* 2002].

Mycobacteria do not contain LPS in their thick outer wall, but do have other lipids and glycolipids. CD14 expression on APCs may be involved in binding such lipids, and activating pattern recognition receptors, such as TLRs. For example, infection of mycobacteria through TLR2 signaling has been shown to be enhanced by CD14 activation [Bochud *et al* 2003]. As with TLR2, the effect of *M. vaccae* treatment on CD14 expression would be an interesting observation. To determine if *M. vaccae* influences CD14 expression, an *in vitro* experiment can be set up using an APC

cell line, such as Thp1. Thp1 is a human monocyte/macrophage cell line. Thp1 cells could be treated with either *M. vaccae* or saline, and the expression of CD14 analysed either by FACS or RT-PCR. If *M. vaccae* does activate APCs via CD14, it would be expected that CD14 expression in Thp1 cells would be enhanced by treatment with *M. vaccae.*

1.5.4 Co-stimulatory pathways

In this thesis, FACS analysis of *M. vaccae*-induced pulmonary CD11c+ cells showed no difference in staining for co-stimulatory markers MHC II, CD80 and CD86 (Chapter 4). This is an unexpected finding as others have shown that an elevation of IL-10 decreases MHC Class II and CD80 expression [Koppelman *et al* 1997]. Surprisingly, expression of CD40 was not detected on the CD11c+ cells. This was unexpected, since CD40 has been shown to be upregulated after allergen challenge [Vermaelen *et al* 2003]. Interestingly, CD40 has also been shown to be upregulated upon infection with *Mycobacterium tuberculosis* [Gonzalez-Juarrero *et al* 2001]. One explanation for this puzzling result is that a technical fault has occurred. The CD11c+ APCs autofluorescence was very high, so that fluorescence from fluorochrome-conjugated Abs was masked. This possibility was discussed in detail in Chapter 4. An alternative explanation is that other co-stimulatory pathways are involved in the Ag presentation of *M. vaccae.* CD40 signaling leads to increased signaling through the B7 pathway. This in turn leads to increased cytokine release, especially IL-4 from naïve T cells, via the OX40-OX40-L pathway [Flynn *et al* 1998]. However, since IL-4 is decreased in the lungs of mice treated with *M. vaccae* (Chapter 3), it may be that the CD40 pathway is not involved, and that another co-stimulatory pathway is activated.

One possible co-stimulatory pathway, the ICOS-ICOS-L pathway, has been shown to be essential for Treg induction by DCs [Akbari *et al* 2002, Akbari *et al* 2003]. In addition, it also to plays an essential role in the development of AHR and airway inflammation [Gonzalo *et al* 2001, Tesciuba *et al* 2001, Akbari *et al* 2002]. Since ICOS-ICOS-L engagement superinduces IL-10, the involvement of the ICOS pathway in *M. vaccae* induction of Tregs warrants further investigation.

Unfortunately, in this thesis, no difference in ICOS-L expression by CD11c+ cells could be detected between OVA-sensitized and challenged mice treated with either saline or *M. vaccae* (Chapter 4). This may be due to limitations of the experimental design. CD11c+ cells were isolated from murine lungs 24 hours after the OVA-challenge. A longer period between challenge and sacrifice may be needed, since ICOS doesn't reach maximum upregulation till 48 hours after activation [Hutloff *et al* 1999]. Although this does not rule out the possibility that ICOS is affected by *M. vaccae,* no evidence was found during the course of this thesis to suggest so.

Another possible co-stimulatory pathway through which *M. vaccae* may signal is the 4-1BB (CD137) pathway. Like ICOS and OX-40, the 4-1BB receptor [Kwon *et al* 1989] is not expressed on naïve T cells, but is upregulated about 24 hours after T cell activation on both CD4+ and CD8+ T cells [Pollok *et al* 1993, Schwarz *et al* 1996, Croft *et al* 2003]. However, 4-1BB expression is not exclusive to T cells, and is present on B cells, MΦs and DCs. Its ligand, 4-1BB-L, is found on B cells, DCs and MΦs, and can be regulated by LPS [Futagawa *et al* 2002, Goodwin *et al* 1993, Pollock *et al* 1994]. 4-1BB has the ability to promote CD8+ cell survival, and most work regarding 4-1BB has been done using CD8+ T cells [Cooper *et al* 2002]. More recently, 4-1BB has been shown to induce proliferation of CD4+CD25+ Tregs, both *in vitro* and *in vivo,* without IL-2 production. Murine CD4+CD25+ cells isolated from the spleen were stimulated with anti-CD3 and soluble 4-1BB-Fc. No proliferation was detected and no IL-2 protein was detected in the supernatants from the culture [Zheng *et al* 2004, Lee *et al* 2005]. 4-1BB may therefore be important for the expansion of Tregs. *M. vaccae* may have the ability to signal through the 4-1BB pathway, enabling Treg expansion. Analysis of 4-1BB-L expression on the *M. vaccae*-induced CD11c+ cells, both of RT-PCR and FACS, may be an interesting observation and could be included in any future work.

1.6 Real-time RT-PCR

In this thesis, the real-time RT-PCR method was adopted and optimised, giving very consistent results. Real-time RT-PCR has advantages over end point PCR,

mainly in terms of sensitivity and accuracy. It does however, have some disadvantages associated with its use when quantifying RNA levels. These problems, which extend to basic end point PCR also, include the variability of the RNA extracted, along with the efficiencies of the reverse transcription and PCR reactions. Consequently, for accurate quantification of RNA levels, a precise method of normalisation is necessary to take into account all these possible variables. Several strategies have been put forward for normalising real time RT PCR data, but unfortunately all these have their limitations.

1.6.1 Normalization of real-time RT-PCR

In this thesis, all RT-PCR data from genes of interest are normalised to the housekeeping gene, GAPDH. This method of normalisation estimates the amount of mRNA extracted from each individual sample. A housekeeping gene is a gene that encodes an enzyme, which is essential for the upkeep and survival of the cell (hence known as a housekeeping gene). By using the mRNA transcribed from a housekeeping gene, the efficiencies of the RNA extraction, the cDNA conversion and the PCR reaction are all taken into account for each sample. A housekeeping gene is used to make an estimate of the amount of mRNA present.

In the laboratory, the most commonly used housekeeping genes are GAPDH, β-actin, HPRT and 18S rRNA. All these genes messages are expressed at relatively high levels in all cells, so detection of a housekeeping gene can also act as a positive control. If a specific gene, for example IL-10, is not expressed in a tissue, successful detection of the housekeeping gene by PCR serves to confirm the presence of cDNA. If the gene of interest in not detected by PCR, but the housekeeping gene is, then the gene of interest must be either very weakly expressed, or not expressed at all, in that particular tissue or sample. Expression levels of some of these housekeeping genes do, however, vary between tissues or may be altered under certain conditions. For example, HPRT is more highly expressed in parts of the CNS compared to the rest of the human body [Stout *et al* 1985]. Hence, if comparing gene expression between different tissues, an increased expression of the housekeeping gene in one tissue could alter the

results significantly. If the housekeeping gene is expressed at a higher level in the CNS compared to spleen tissue, but expression of IL-10 remains unchanged, it would appear that IL-10 expression was downregulated in the CNS. HPRT would not be an appropriate housekeeping gene to use if comparing expression of genes in different tissue types. The expression of a housekeeping gene can also vary between individual patients. For example, β-actin has been observed to be expressed at different levels in different leukaemia patient tumour samples [Blomberg *et al* 1987], probably due to the varying numbers of infiltrating cells into the tumour. Depending on the stage and size of the tumour, the number of cells infiltrating it will vary. A similar sized biopsy may contain different numbers of cells, depending on the individuals' stage of cancer. It is therefore important to select the most appropriate housekeeping gene, and to realise that any results obtained using this method are only an estimation and not absolute values.

In this thesis, the housekeeping gene used was GAPDH. In humans, variations in GAPDH expression probably exist between individuals, because of genetic diversity. However, all the experiments were carried out using BALB/c mice. These mice are highly inbred, and hence are genetically similar. This should minimise the differences in GAPDH expression between individual mice. However, GAPDH was not validated against any other housekeeping genes, and no attempt was made to ensure that expression of GAPDH relative to total RNA is not altered by the conditions of the experiments, for instance, by Th2 cytokines. Future work could use a housekeeping gene that has been validated in the experiments performed, and shown not to change in expression level. An alternative approach is the use of several different housekeeping genes to establish whether the use of one has advantages or disadvantages over another [Dheda *et al* 2004]. RNA should be extracted from all different tissues that are being analysed in a particular study, such as lung and spleen, from all treatment and control groups. A selection of common housekeeping genes, such as GAPDH, HPRT, β-Actin, and 18S rRNA, should be amplified by RT-PCR, and normalised to total RNA. Each copy number obtained from each sample should be averaged, and the SEM calculated to establish how variable a particular housekeeping gene is between tissue types,

and between treatment groups. The housekeeping gene with the least variability should then be chosen and used in each subsequent experiment [Dheda *et al* 2004]. This experiment should ideally be carried out for every study where a housekeeping gene is being used to normalise PCR data.

1.6.2 Other normalisation methods

Alternative methods of normalisation have been suggested, but all appear to present different limitations. The simplest method of normalisation is to ensure the sample volume or sample weight is always the same, for example the same volume of blood or weight of tissue. The problem with using this method alone is the fact that often, similar weights or volumes do not represent the same number of cells. Blood or tumour tissue, for example, will have varying numbers of cells in a given volume or weight, depending upon the hosts state of immunocompetence or immunosuppression [Alexander *et al* 2003]. For example, if comparing blood obtained from a healthy volunteer with blood from an HIV patient, the number of cells present in the same volume of blood is likely to vary significantly between the individuals. With *in vitro* cultures, cell may have proliferated, differentiated or clumped, leading to inaccurate estimates. This method of normalisation is hence rarely used.

A commonly used method for mRNA normalisation is normalising to total RNA. There are several methods for measuring RNA quantity, one of which is LabChip (Agilent 2100) [Dheda *et al* 2004]. Labchip is a kit that estimates the total RNA concentration. Normalisation to total RNA is advantageous, since it takes into account the efficiency of the RNA extraction process. Each RNA sample has been through the identical processes and has been exposed to the same conditions. Unfortunately, normalising to total RNA does not control the variation that can occur during the reverse transcription process or the PCR reaction. For example, individual RNA extractions may be converted into cDNA at different rates, resulting in some samples having more cDNA in than others. Normalising to total RNA measured before the cDNA transcription would not detect these differences. Another disadvantage is that measuring total RNA extraction will include

measuring rRNA, which makes up approximately 80% of all RNA. RT-PCR is the measurement of gene expression, meaning quantifying the amount of mRNA. mRNA only comprises approximately 5% of total RNA, with tRNA making up the last 15%. To use total RNA as a normalisation method, it must be assumed that the mRNA:rRNA ratio is unchanged between control and treatment groups, which is not always the case [Spanakis *et al* 1993].

Since normalising to total RNA does not take into account the efficiencies of the reverse transcription, genes can be normalised to the amount of cDNA obtained [Whelan *et al* 2003]. This has the advantage of quantifying the actual amount of mRNA present in the sample. A double stranded fluorescent dye, such as picogreen, can be used as long as the cDNA remains double stranded after reverse transcription. This can be achieved by using a reverse transcriptase with a limited RNase H activity. RNase H is an enzyme that degrades the RNA template strand of the double stranded cDNA. Most reverse transcriptase enzymes have a natural RNase H activity, so cDNA is usually single stranded. Some reverse transcriptases have been developed without RNase H activity, so the cDNA remains double stranded. As long as the second strand remains intact, cDNA quantification can be carried out, eliminating any RNA left over [Whelan *et al* 2003]. The disadvantage with this is that it does not control the differences in the efficiencies of the PCR reactions. Also, picogreen will bind non-specifically to double stranded DNA or RNA, meaning any hairpin loops in the total RNA will be erroneously included in the calculations.

Normalisation to genomic DNA has also been suggested [Talaat *et al* 2002]. As cells proliferate, their genomic DNA doubles at an exponential rate so that it will contain exponentially more copies of genomic genes than non-proliferating cells. A major problem with this normalization technique is that RNA extraction procedures are not usually designed to purify DNA and RNA from the same sample. This would mean that a sample would have to be split, and RNA extracted from one and DNA from the other. This would mean the extraction rate of genomic DNA might vary from that of RNA extraction, leading to variation. This method is therefore rarely used.

1.7 Clinical implications of work so far

1.7.1 Clinical trials involving *M. vaccae*

Heat-killed *M. vaccae* has been tested in clinical trials with patients suffering with atopic dermatitis and atopic asthma [Arkwright *et al* 2001, Camporota *et al* 2003]. In an atopic dermatitis, double-blinded placebo controlled clinical trial, 41 children aged 5 to 18 years old with moderate to severe eczema were enrolled. Split randomly into two groups, one group was given an intradermal injection of heat-killed *M. vaccae* and the other groups a buffer solution as a placebo. Children treated with *M. vaccae* showed a mean 48% reduction in surface area of skin affected by dermatitis, compared to a mean of 4% in the group treated with the placebo. The group treated with *M. vaccae* also saw a significant reduction in the severity of their dermatitis [Arkwright *et al* 2001]. This double-blinded placebo study suggested that treatment with heat-killed *M. vaccae* did have a positive effect on the symptoms of moderate to severe atopic dermatitis in children. Further work failed to show a difference in the levels of IFN-γ, IL-2 or IL-5 protein obtained from the supernatants of cultured PBMCs from children with atopic dermatitis, either treated with *M. vaccae* or with placebo. No change was observed in IL-4, IL-2 or TGF-β mRNA from the same cells either [Hadley *et al* 2005]. Although these trials resulted in improvement of atopic symptoms, no changes in immune responses were observed between the group treated with *M. vaccae* and those treated with the placebo.

A clinical study was carried out on patients suffering from atopic asthma. 24 males aged 12-53 years of age were enrolled for a placebo controlled clinical trial. All patients demonstrated both an early and a late airway response, and either suffered from mild or moderate asthmatic symptoms. Patients were injected intradermally with either 0.1mg of heat-killed *M. vaccae* or the placebo buffer. Serum IgE levels were measured 18 days later, and 21 days later patients were challenged with histamine, and their PBMCs cultured. Patients were challenged again with histamine on day 22 and PBMCs cultured later, at day 42. The group

treated with *M. vaccae* showed a significant improvement in the forced expiratory volume per second (FEV_1 – a frequently used index to measure lung capacity) before and after treatment. This improvement was not observed in the group treated with the placebo. When the PBMC cultures were investigated, although 7 out of 10 patients treated with *M. vaccae* showed a gradual decrease in IL-5 protein secreted by the cultured PBMCs, no significant difference was observed between the PBMC cultures from the group treated with *M. vaccae* and those obtained from the group treated with the placebo. No difference was observed in the serum IgE from the group treated with *M. vaccae* compared to the group treated with the placebo [Camporota *et al* 2003]. This clinical trial showed a trend towards an improvement in asthmatic symptoms following treatment with *M. vaccae,* albeit not significant. It may be that optimizing the dose and timing of administration of *M. vaccae* will give significant results.

Results from these published human clinical trials were encouraging, and additional phase II trials have now been completed. One trial has been performed on children suffering from atopic dermatitis. Children with moderate to severe atopic dermatitis were treated with either heat-killed *M. vaccae* or a placebo, and symptoms monitored using the six-area six-sign atopic dermatitis score (SASSAD). This score is obtained by grading six signs (erythema, exudation, excoriation, dryness, cracking and lichenification), each on a scale of 0 (absent), 1 (mild), 2 (moderate), or 3 (severe), at each of six sites; arms, hands, legs, feet, head and neck, trunk [Berth-Jones *et al* 1996]. 12 weeks after administration, no clinical difference was observed between the group treated with *M. vaccae* and the placebo group. It is not clear whether this is a meaningful result or not because there was a 30% improvement in the placebo group.

A second phase II trial was randomized, multicentered placebo controlled study conducted in Europe. Adults with mild to moderate persistent asthma were recruited and treated intradermally with either *M. vaccae* or placebo. Treatment with *M. vaccae* failed to significantly improve asthmatic symptoms. Upon reanalysis, however, it was observed that patients who received two high dose of heat-killed *M. vaccae* did show significant benefits. Patients had a reduction in

asthmatic symptoms score and asthma exacerbations, compared to patients treated with the placebo (http://www.srpharma.com/documents/SRP299_Updated_050617.pdf).

It still remains to be determined whether the effects of *M. vaccae* in human patients also depend on APCs cytokine secretion and Treg induction, and this needs to be addressed in any future work. Proving Treg and APC function in humans would be difficult, since BAL fluid and tissue samples are not readily available. Work would have to be done on the peripheral blood. PBMCs could be isolated from the blood, and FACS staining done for CD4+CD25+ T cells. These cells could then be sorted and analysed using RT-PCR for Treg markers. However, the number of Tregs circulating in the periphery is very small in healthy individuals (approximately 10% of all CD4+ T cells). This number would theoretically be much less in patients with atopic allergy. Obtaining enough Tregs to carry out any functional assays may be difficult. However, one study has isolated CD4+CD25+ from the peripheral blood of allergic and non-allergic donors. Allergen peptide immunotherapy was used to treat allergic patients with cat allergies in a double-blinded placebo study. CD4+CD25+ cells were isolated from peripheral blood and cultured with cat allergen *in vitro*. There was a significant reduction in both proliferation and IL-13 production by allergen-stimulated CD4+ T cells after therapy, compared to pre-therapy, whereas no change was seen after treatment with a placebo. CD4+CD25+ T cells suppressed proliferation by CD4+CD25- T cells before and after therapy. These data suggests that isolation of CD4+CD25+ Tregs from the peripheral blood of allergic patients is possible, and that this method should be considered in future *M. vaccae* related clinical trials.

1.7.2 Alternative delivery routes

Additional work is required on the immunotherapeutic use of *M. vaccae.* So far, all treatment with *M. vaccae* in human trials has been administered intradermally. However, the presence of *M. vaccae* in mud and untreated water suggests that the most likely route of *M. vaccae* exposure would have been orally. This would be a more practical route of delivery for treatment with *M. vaccae,* since injection causes

inflammation and scarring in some patient.

Oral administration of BCG to mice has been shown to protect against infection with *Mycobacterium tuberculosis* [Lagranderie *et al* 2000]. The size, charge and hydrophobicity of mycobacteria means that they are rapidly taken up by the PPs [Fujimura *et al* 1986, Lagranderie *et al* 2000]. This would suggest that oral administration of *M. vaccae* may be an efficient route of delivery, and it is possible that exposure to the gut mucosa may facilitate the induction of Tregs, since this site is considered an ideal environment for Treg development and maintenance [Chen *et al* 1994, Maloy *et al* 2001].

Preclinical investigations into the efficiency of oral delivery have already been undertaken. Stimulation of cells isolated from the MLNs of naïve mice treated by gavage with *M. vaccae* resulted in enhanced IL-10 production. The MLNs drain the small intestine, so cells would have been exposed to Ags present in the gut. In addition, oral treatment of *M. vaccae* is as effective at ameliorating inflammatory symptoms in a murine model as treatment of *M. vaccae* s.c. A single administration of heat-killed *M. vaccae* by gavage before sensitisation to OVA prevented the development of severe pulmonary inflammation upon OVA challenge [Hunt *et al* 2005]. The oral effectiveness of *M. vaccae* has not yet been investigated in human clinical trials.

1.7.3 *M. vaccae* in conjunction with probiotics

Delivering *M. vaccae* by the oral route would mean that it would be absorbed through the gut mucosa into the Peyer's Patches. The gut, along with its intestinal flora, has been deemed the most important site and source of microbial stimulation of the immune system [Salminen *et al* 1998]. Indeed, studies linking the composition of the intestinal flora and atopy in children have been carried out [Bjorksten *et al* 2004]. Disruption of the natural bacteria in the gut by antibiotic use in infant mice leads to impaired Th1 immune responses and Th2 dominant immunity with elevated serum IgE levels. Subsequent administration of *Enterococcus faecalis* or *Lactobacillius acidophilus* after antibiotic treatment

reversed this rise in IgE [Oyama *et al* 2001, Sudo *et al* 2002].

Several epidemiological studies have shown that antibiotic use in young children can affect susceptibility to allergy in later life [Droste *et al* 2000, Johnson *et al* 2005]. For example, a cohort study in the USA found that children who had received antibiotics within the first six months of life had an increased chance of developing allergy by the age of 7 years. Children who were treated with antibiotics were more likely to have a positive skin-prick test, and raised serum IgE levels, than children who did not receive antibiotic treatment. Interestingly, this incidence of allergy was higher in the group of children who had less than two pets at home [Johnson *et al* 2005].

There is evidence that probiotic use can reduce the risk of developing allergies. In a randomized-placebo study of infants from families with a history of atopic disease, children were treated with either *Lactobacillus rhamnosus* or a placebo. This was begun prenatally, by giving the mother an oral capsule and was continued after birth for 6 months. After 4 years, children who had been treated with *Lactobacillus rhamnosus* had a reduced risk of atopic eczema compared to children treated with the placebo [Kalliomaki *et al* 2003]. A more recent study treated children with atopic dermatitis with *Lactobacillus rhamnosus* for 4 weeks (twice daily mixed with their food). A significant decrease was observed in the groups SCORAD index by the end of 4 weeks [Viljanen *et al* 2005].

Although *M vaccae* is not naturally part of the commensal intestinal flora, it is likely that the humans have been exposed to *M. vaccae* via the oral route throughout human evolution. Abundantly found in mud and untreated water, the presence of mycobacteria during human evolution can be seen by the existence of a subset of CD1-restricted T cells which appear to only recognize mycobacterial glycolipids [Dutronc 2002]. The hypothesis that exposure to *M. vaccae* is effective when administered orally is supported by the decrease in type-2 responses observed when allergic mice were treated orally with *M. vaccae* [Hunt *et al* 2005].

1.8 Conclusions

Published data suggest the possibility treatment with *M. vaccae* might alleviate symptoms of allergic asthma and the data presented in this thesis concurs. The mechanism by which this alleviation occurs is still unclear. This thesis implies that treatment with *M. vaccae* influences APCs at the site of inflammation. This thesis explicitly demonstrates that *M. vaccae* induces CD11c+ APCs in the lungs to secrete IL-10, TGF-β and IFN-α. In addition this thesis demonstrates that co-culture of these CD11c+ APCs with naïve CD4+ T cells results in increased release of IL-10 and TGF-β in the supernatant. It also demonstrates an increased expression of Foxp3 in the cells, compared to those cells in the cultures containing CD11c+ APCs from saline treated mice. Taken together these increases suggest that these "regulatory" APCs may have the ability to induce Tregs.

The attitude to hygiene in the developed world means that exposure to *M. vaccae*, and other "Old Friends" is extremely limited. Evidence suggests that exposure to "Old Friends" at an early age induces mechanisms of immunoregulation and prevents the development of inappropriate immune responses to allergen. Consequently reintroduction of non-pathogenic microorganisms orally may help reduce the incidence of allergic disease. Although not naturally part of the intestinal flora, *M. vaccae* is effectively absorbed through the gut mucosa. *M. vaccae* is clearly an effective immunotherapy preclinically, and this thesis provides further evidence that supports this statement. Furthermore this thesis suggests one mode of action triggered by *M vaccae*. Given the potential, it is therefore of the highest importance that further work be undertaken to prove the mechanisms suggested by this thesis.

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