University of Southampton

REGULATION OF INFLAMMATORY RESPONSES IN ALLERGIC ASTHMA

LUIGI CAMPOROTA MD, MRCP

Thesis submitted for the Degree of
Doctor of Philosophy
Department of Medicine, Faculty of Medicine,
Health and Biological Sciences

May 2006

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
MEDICINE

Doctor of Philosophy

REGULATION OF INFLAMMATORY RESPONSES IN ALLERGIC ASTHMA

By Dr Luigi Camporota

Allergic asthma is the result of a Th2-mediated immune response against allergens, with Th2 cytokines, such as IL-4, IL-5 and IL-13 playing a crucial role. T helper cells are thought to be able to cross-regulate each other and a number of possible treatments for allergic disorders have been tested in order to either potentiate Th1 responses or block Th2 cytokines or their receptors. In my thesis I have sought to inhibit Th2 cytokine responses and, thereby, modulate allergic responses, using either cytokine inhibitors or microbial agents which are known to down-regulate cytokine responses.

I have tested a soluble form of IL-4 receptor (sIL-4R) with the aim of blocking IL-4 and preventing the signalling cascade leading to cell activation and gene transcription. Recombinant human IL-4 caused an increase in IL-5 production by PBMC which peaked at 20 nM. sIL-4R caused a significant concentration-dependent inhibition of IL-5 secretion. The extent of inhibition with sIL-4R was comparable to that achieved by using anti IL-4 and IL-4R antibodies. Th2 down-regulation was not secondary to reduced survival of PBMC as tested in proliferation assays. Soluble IL-4R exerted a differential effect on Th2 and Th1 cytokines at low concentrations. It caused a significant ($p<0.05$) inhibition of *Der p*-induced release of IL-5 at 1 μg/ml, without affecting IFN-γ production. A 2.6-fold increase in IFN-γ concentration was observed only when sIL-4R was used at 10 μg/ml, suggesting that inhibition of Th2 cytokines is not necessarily associated with promotion of Th1 responses.

Modifications of the pattern of microbial exposure associated with Westernisation represent a theoretically critical factor underlying the rising prevalence of atopic disorders as proposed by the so-called 'hygiene hypothesis'. Microbes most likely to prevent allergic diseases are environmental bacteria such as saprophytic mycobacteria. To test the hypothesis that the non-pathogenic *Mycobacterium vaccae* could reduce airway inflammation and the asthmatic reaction *in vivo* following allergen challenge and the Th2 response of *ex vivo* challenged PBMC, I conducted a clinical trial where an *M. vaccae* extract, SRL172, was administered intradermally. *M. vaccae* was able to cause a reduction in the late asthmatic response (LAR). During the LAR, the mean maximum fall in FEV_1 was 35.8% [16.5–66.7%] in subjects receiving SRL172 and 29.5% [16.9–39.4%] in subjects receiving placebo. In a subgroup of patients with mild asthma, *M. vaccae* caused a mean 47.2% relative reduction in the AUC of the LAR ($p=0.026$). However, the difference between treatment groups did not achieve statistical significance. Similarly, no difference was found in the early asthmatic response, (expressed as maximum % fall in either FEV_1 or AUC) and in $PC_{20}FEV_1$ between the two treatment groups ($p=0.98$). Sputum analysis showed no difference in the differential cell count or in the levels of ECP and tryptase. Studies on peripheral blood mononuclear cells (PBMC) of these patients showed showed a trend towards reduction in IL-5 synthesis *in vitro* and serum IgE levels three weeks post-treatment with *M. vaccae* ($p=0.07$) but not placebo. No difference in PBMC proliferation in response to allergen was found between the two groups. To understand the mechanisms responsible for the observed changes in the allergen challenge parameters as well as the down-regulation in IL-5 and IgE, I studied the *in vitro* effects of *M. vaccae* (SRP299) on cytokine production by PBMC from atopic asthmatics. SRP299 caused a potent inhibition of IL-5 and IL-13 synthesis, with a significant ($p<0.01$) mean 65.2% inhibition in *Dermatophagoides pteronyssinus*-induced IL-5 and a smaller but still highly significant ($p<0.01$) mean 36.7% inhibition in IL-13 synthesis. This effect did not seem to be entirely due to IL-12 induction but involved IL-10, as demonstrated by the fact that the levels of IL-5 in cultures of *M. vaccae/Der p*-stimulated PBMC were significantly increased in the presence of antibodies blocking IL-10 ($p<0.05$), suggesting a role for regulatory T cells. Furthermore, the inhibitory effect of *M. vaccae* appeared to involve the generation of prostaglandins, as indomethacin completely inhibited the ability of *M. vaccae* to down-regulate both IL-5 and IL-13 in response to allergen. On the contrary no significant effect was seen on IFN-γ production.

The hygiene hypothesis suggests that harmful infections *per se* may not be as critical as the exposure to microbial burden. Microbial components are found in varying concentrations in many indoor and outdoor environments. Among those components, lipopolysaccharide (LPS) is a potent inducer of IL-12 and IFN-γ, cytokines that stimulate Th1-mediated immunity and have the potential to down-regulate the Th2 response. In *in vitro* experiments I showed that LPS, when administered before allergen stimulation, down-regulated IL-5 responses and increased IL-12 production. The different timing of LPS exposure showed that IL-12 induction was greater when PBMC were exposed to LPS 24 hours prior to allergen stimulation ($p=0.02$, ANOVA) with a mean±SD increase above *Der p*-stimulated levels of 4293 ±1497 pg/ml. This increase was significant when compared to pre-exposure to allergen 2523± 38.4 pg/ml ($p<0.05$). Similarly, the degree of inhibition of IL-5 secretion was dependent on the timing of exposure to LPS in relation to allergen stimulation. The greatest inhibition of IL-5 (55±33%) was achieved when PBMC were exposed to LPS 24 hours prior to allergen stimulation ($p=0.04$, ANOVA) compared to pre-exposure to allergen 5.7±38.4%, ($p<0.05$) and co-culture condition 32.4 ±37.2%; ($p>0.05$). These effects seemed to be IL-12 mediated since anti-IL-12 antibodies restored both IL-5 ($p<0.05$) and IL-13 ($p<0.05$) IFN-γ synthesis was completely inhibited by anti-IL-12 antibodies, showing that in this system, IFN-γ was entirely IL-12 dependent ($p<0.01$).

In conclusion, I have shown that Th2 responses can be inhibted either by inhibiting the central Th2 cytokine, IL-4, by inducing IL-10 (probably reflecting regulatory T cell involvement) or by promoting Th1 responses. I have shown preliminary evidence that *M. vaccae* can reduce airway responses to allergen in atopic asthmatics which might involve down-regulation of Th2 responses.

TABLE OF CONTENTS

Chapter one: Introduction

Chapter Two:Material and Methods

Chapter Three: IL-4 and allergic inflammation

Chapter Four: The effect of treatment with *Mycobacterium vaccae* in atopic asthma

Chapter Five: Effects of *Mycobacterium vaccae* on *in vitro* allergic inflammation

Chapter Six: Effects of LPS in an *in vitro* model of allergic inflammation

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AM	Alveolar Macrophage
APC	Antigen Presenting Cells
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar lavage fluid
BCG	Bacillus Calmette-Guerin
BHR	Bronchial Hyperresponsiveness
CI	Confidence interval
COPD	Chronic Obstructive Pulmonary Disease
COX-1	Cyclo-oxygenase-1
COX-2	Cyclo-oxygenase-2
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DC	Dendritic Cells
DC-SIGN	DC-specific intercellular adhesion molecule-3 grabbing nonintegrin
Der p	*Dermatophagoides pteronyssinus*
EAR	Early allergic responses
EGFR	Epidermal Growth Factor Receptors
ELISA	Enzyme-Linked Immunosorbent Assay
FcεRI	High-affinity IgE receptors
FcεRII	Low-affinity IgE receptors
FEV_1	Forced Expiratory Volume in 1 second
Foxp3	Forkhead/winged helix transcription factor-3
FVC	Forced Vital Capacity
GCP	Good clinical practice
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GRO-α	Growth-Regulated Oncogene-Alpha
HLA	Human leukocyte antigen
HPS	Heat shock protein
HuPBLSCID	Chimera, human peripheral blood lymphocytes into SCID mice
HVA	Hepatitis Virus A
ICAM-1	Intercellular adhesion molecule 1
IFNR	Interferon gamma receptor
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL-	Interleukin
IL-4R	Interleukin-4 receptor
IRAK	IL receptor-associated kinase
IRF-1	Interferon regulator factor 1
IRS	Insulin receptor substrate
ISS	Immunostimulatory sequences
JAK	Janus family kinase
LAR	Late allergic responses
LFA-1	Lymphocyte Function-Associated Antigen-1

LPS	Lipopolysaccharide
LT	Leukotriene
mAb	Monoclonal antibodies
MAP	Mitogen-Activated Protein
MAPK	Mitogen-activated Protein Kinase.
MHC	Major histocompatibility complez
MIP 1α	Macrophage Inflammatory Protein 1 alpha
mRNA	Messenger Ribonucleic Acid
NF-kB	Nuclear Transcription Factor Kappa B
NHS	National Health Service
NK	Natural Killer Cells
NRAMP-1	Natural resistance-associated macrophage protein-1
OR	Odds Ratio
OVA	Ovalbumin
P13K	Phosphoinositol 3' kinase
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cells
PC_{20}	Provocation Concentration of bronchconstrictor required to reduce the FEV_1 by 20%
PEF	Peak Expiratory Flow
PHA	Phytohaemagglutinin
PMA	Phorbol Myristate Acetate
RANTES	Regulated on Activation, Normal T-Cell Expressed and Secreted
RAST	Radioallergosorbent Test
RCT	Randomised controlled trial
rIL-4	Recombinant IL-4
SCID	Severe-Combined Immunodeficiency
SD	Standard Deviation
SEM	Standard Error of the Mean
sIL-4R	Soluble interleukin 4 receptor
STAT	Signal transducer and activator of transcription
TcR	T-cell Receptor
TGF-β	Transforming Growth Factor-beta
Th	T-helper
Th1	T Helper-1 Lymphocytes
Th2	T Helper-2 Lymphocytes
TICAM	Toll/IL-1 receptor-containing adaptor molecule
TIM 1	T cell Immunoglobulin Mucin 1
TLR	Toll-like receptor
TNF- α	Tumour Necrosis Factor-alpha
TRAF-6	Tumour necrosis factor receptor-associated factor-6
Treg	Regulatory T cells
VCAM-1	Vascular cell adhesion molecule-1
VLA-1	Very late antigen-1
WHO	World Health Organisation

ACKNOWLEDGEMENTS

The work contained in this thesis was carried out at the Department of Respiratory and Molecular Cell Biology at the University of Southampton.

My first acknowledgements go to my family who so unselfishly supported my decision to move to England, and to Prof. Stephen Holgate for allowing me to work in his Department and for being such an inexhaustible source of inspiration.

I would particularly like to acknowledge the continuous support, encouragement and advice that I have received from my supervisor Prof. Ratko Djukanović. I am grateful to Prof. Djukanović for offering me the opportunity to work in an internationally respected department and in the field of Respiratory Medicine and for letting me benefit from his wide academic experience. I am also extremely grateful to my co-supervisor, Dr Gordon Dent for the helpful advice in designing the experiments and in writing this thesis. The advice and expert supervision provided by both Prof. Ratko Djukanović and Dr Gordon Dent have been key factors in the completion of my work.

The studies detailed in this thesis would not have been possible without the successful collaboration with Prof. J. Stanford and Prof. G. Rook, UCL, London and SR Pharma.

A special thank you goes to the Dr Luminita Stanciu, for being my 'Cicero' in the laboratory, particularly at the beginning of my PhD, and to Dr Kevan Roberts and Dr Zeina Jaffar who were infinitely supportive and full of suggestions and good humour.

My special thanks goes to my Italian colleagues and friends Bianca Beghé and Carlo Capristo for their unfailing friendship, support and fun. I am also indebted to Prof. Leonardo Fabbri and Prof. Serafino Marsico for the trust and the support they offered me over the years.

I would also like to thank the administrative staff in the Department of Respiratory and Molecular Cell Biology including Mr Frank Anderson, Mrs Chris Vincent, Ms Leanne Darby and Mrs Lesley Morris.

Thanks must also be expressed towards the many volunteers who took part in the study and were uncomplaining in their bronchial challenges and blood tests.

Last but not least, I am deeply indebted to Jo who has been endlessly supportive, always able to offer suggestions that allowed me to overcome the inevitable 'writer's block' and for sacrificing so many free weekends and postponing numerous holidays so that I could finish this thesis.

PUBLICATIONS

The following publications have arisen from work presented in this thesis:

Original Articles

L. Camporota, G. Dent, J. Tocker, C. Maliszewski, R.Djukanovic. Effects of soluble interleukin-4 receptor (sIL-4R) on in vitro Th1 and Th2 cytokine responses in mild atopic asthma. *To be submitted*

L. Camporota, G. Dent, and R. Djukanovic. Effects of bacterial lipopolisaccharide on *in vitro* cytokine responses to allergen. *To be submitted*

L. Camporota, G. Dent, J. G.A.W. Rook, and R. Djukanovic. Effects of *Mycobacterium vaccae* on *in vitro* cytokine responses to allergen. *To be submitted*

L. Camporota, A. Corkhill, H. Long, J. Lordan, L. Stanciu, N. Tuckwell, A. Cross, J.L. Stanford, G.A.W. Rook, S.T. Holgate, R. Djukanovic: A randomised, placebo-controlled study of the effects of *Mycobacterium vaccae* (SRL172) on allergen-induced airway responses in atopic asthma *Eur. Resp. J.* 2003; 21: 287-293.

Reviews

L. Camporota, J.W. Holloway: Interleukin-12 and allergic tissue response. *Clinical and Experimental Allergy*, 1999; 29:1298-1300.

L.Camporota. Interleukin-12 and development of atopy. *Clinical and Experimental Allergy*, 2001; 31:1481-4.

Book Chapters

C. Calabrese, L. Camporota, S.A. Marsico, G. Mazzarella. [Etiology and pathogenesis of Asthma: airways inflammation in asthma]. Eziopatogenesi dell'asma: la flogosi delle vie aeree nell'asma. In: Rapporto sull'asma, Edited by M. De Palma and C. Grassi. 1997 Syntagma srl.

L. Camporota, R. Djukanovic : [Costimulatory Molecules and asthma] Molecole di costimolazione ed asma. Edited by L.M. Fabbri. Pacini Ed.

Abstracts

L. Camporota, G. Dent, J. Gereda, R. Djukanovic. Effects of bacterial lipopolysaccharide on allergen-induced responses *in vitro*. ATS 100th International Conference. San Diego, May 2005. *Proceedings of the American Thoracic Society.* 2005; 2 A382.

L. Camporota, G. Dent, J. Gereda, G.A.W. Rook, R. Djukanovic. Effects of *Mycobacterium vaccae* on *in vitro* cytokine response to allergen. ATS 100th International Conference. San Diego, May 2005. *Proceedings of the American Thoracic Society.* 2005; 2 A382.

L. Camporota, G. Dent, J. Gereda, J. Tocker, C. Maliszewski, R. Djukanovic: Effects of soluble interleukin-4 receptor (sIL-4R) on *in vitro* Th1 and Th2 cytokine responses in mild atopic asthma. American Thoracic Society. 96th International Conference. San Francisco, May 2001. *Am J Resp Crit Care Med.* 2001; 163 (5): A520.

L. Camporota, A. Corkhill, H. Long, L. Lau, J. Lordan, J. Stanford, G. Rook, S. Holgate, R. Djukanovic. Effects of *Mycobacterium vaccae* vaccine on allergen-induced airway responses and IL-5 generation by PBMC in mild to moderate asthma. American Thoracic Society. 95th International Conference. Toronto May 2000. *Am J Resp Crit Care Med.* 2000; 161 (3): A477.

CHAPTER ONE

Introduction

1.1 EPIDEMIOLOGY OF ASTHMA

Allergic disorders are common throughout the United Kingdom (UK) and currently represent a substantial burden of morbidity and cost for the National Health Service (NHS). Thirty-nine percent of children and 30% of adults are affected by one or more atopic condition, accounting for 36% of general practice consultations and 0.8% of hospital admissions (Gupta *et al.* 2004). Treatments for asthma and other allergic disorders currently account for 10% of primary care prescribing costs and over one billion UK pounds per annum of NHS costs (Gupta *et al.* 2004).

Asthma is a common cause of morbidity and mortality worldwide. Between 100,000,000 and 150,000,000 people around the globe suffer from asthma and this number is rising. Worldwide, deaths from asthma have reached over 180,000 per year. The multicentre International Study of Asthma and Allergies in Childhood (ISAAC) has shown that the prevalence of symptoms of asthma varies between populations, with differences of 20-fold (range 1.6-36.8%) between different nations (ISAAC 1998),(Figure 1.2-1). Similarly, marked variation has been reported from the European Community Respiratory Health Survey (ECRHS) (ECRHS 1996). A longitudinal survey conducted in about 30,000 primary school children in England between 1973 and 1986 has shown an increasing prevalence of asthma for each annual birth cohort (boys, 6.9%; girls, 12.8%) and wheeze (boys, 4.3% per cohort; girls, 6.1% per cohort). These results suggest that the increase in morbidity is largely due to a true increase in prevalence rather than reflecting a change in diagnostic fashion. A better understanding of the aetiology and pathogenesis of asthma is therefore needed to devise appropriate prophylactic measures and therapeutic interventions (Burney *et al.* 1990).

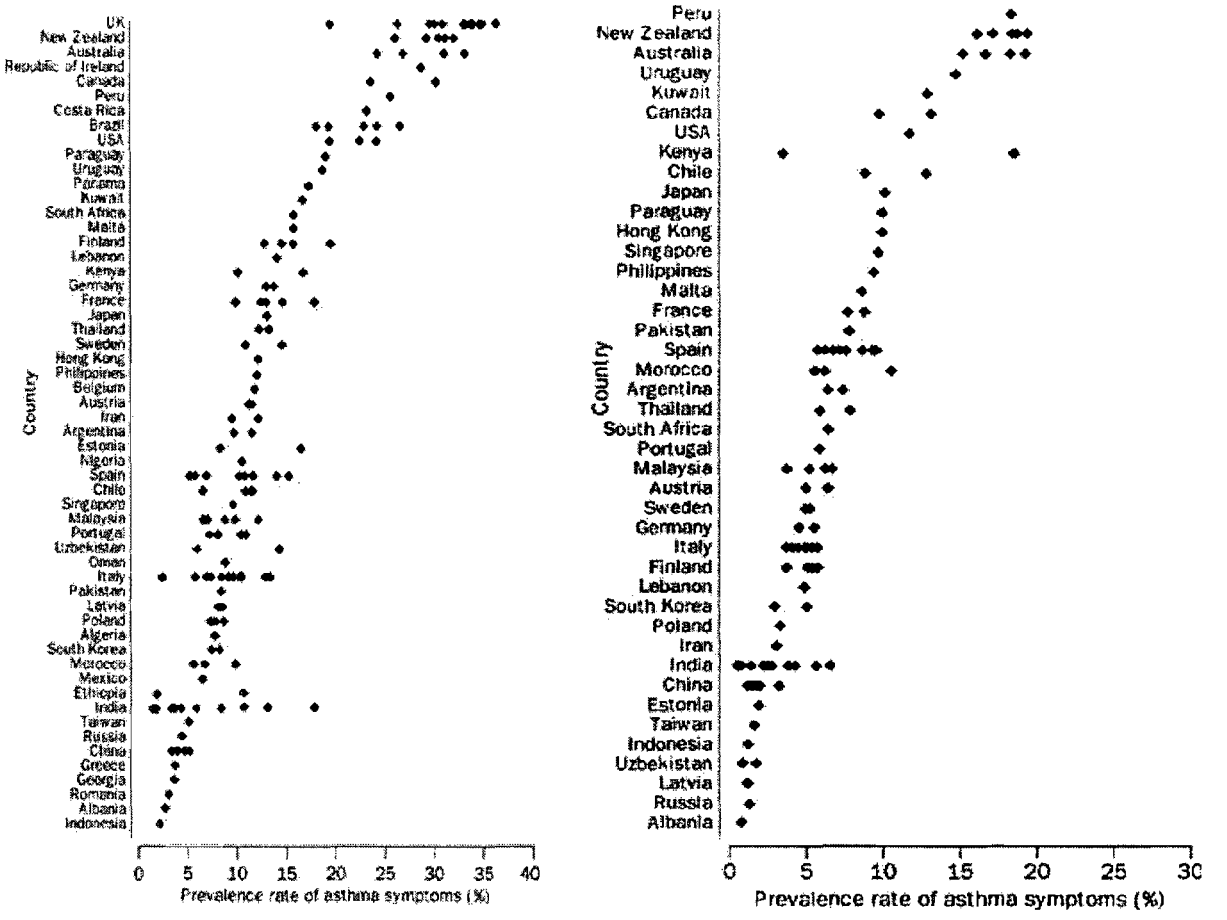


FIGURE 1.2-1: *Prevalence of asthma symptoms worldwide.*
Prevalence obtained using written questionnaire (left) or video questionnaire (right). Reproduced from ISAAC, 1998.

1.1.1 'Westernised' lifestyle and atopy

Epidemiological studies show that along with the difference among nations, there seems to be a difference in the prevalence of allergic disorders between industrialised and rural regions. Allergic diseases are less common in rural regions where people have a traditional lifestyle as compared to industrialised regions where the lifestyle is 'westernised' (Van Niekerk *et al.* 1979; Woolcock *et al.* 1997; Yemaneberhan *et al.* 1997; Bach 2002). Several studies have now demonstrated that concomitant to the rise in allergic disorders there has been a decline in the prevalence of early childhood infections and this has been thought to be the major factor influencing the development of allergic diseases (Smit *et al.* 2004) (Figure 1.2-2).

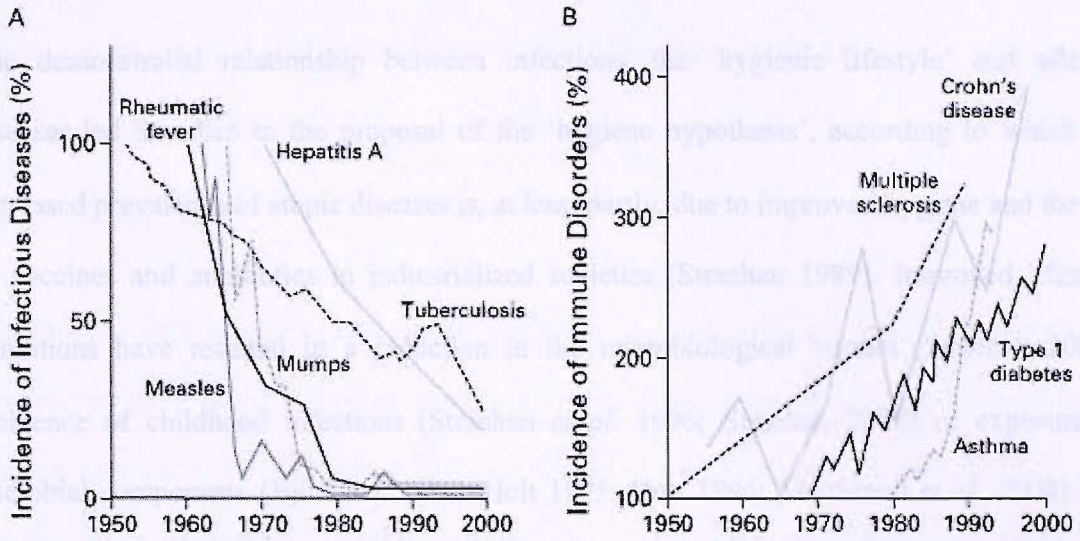


FIGURE 1.2-2: *Inverse relationship between the incidence of infectious diseases (A) and the incidence of immune disorders (B).*

Almost twenty years ago Gerrard *et al* observed that the prevalence of atopic diseases such as asthma, eczema and urticaria in individuals from white families living in central Saskatchewan was higher than in the rural living Metis Indians in Canada (Gerrard *et al.* 1976). This difference was attributed to the increased prevalence of helminth infestation as well as of other untreated viral and bacterial diseases in the Metis community. The authors suggested that "atopic disease is the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria and helminths" (reviewed by (Smit *et al.* 2004)). More recently, other studies have confirmed a lower prevalence of atopic disorders in people living in rural areas as compared with people living in urban environment (reviewed in (Bach 2002)).

Further data on the role of environmental conditions on atopic diseases comes from studies showing an increased trend of allergic sensitisation in former East Germany only six to eight

years after German reunification, which has led to lifestyle which are more similar to western societies (Matricardi 2001).

The demonstrated relationship between infections, the 'hygienic lifestyle' and allergic diseases led Strachan to the proposal of the 'hygiene hypothesis', according to which the increased prevalence of atopic diseases is, at least partly, due to improved hygiene and the use of vaccines and antibiotics in industrialized societies (Strachan 1989). Improved lifestyle conditions have resulted in a reduction in the microbiological burden (Strachan 2000), incidence of childhood infections (Strachan *et al.* 1996; Strachan 2000) or exposure to microbial components (Bjorksten 1994; Holt 1995; Holt 1996; Matricardi *et al.* 2000) that would normally stimulate the maturation of the immune system in such a way as to have a preventive effect on the subsequent development of allergies (Martinez *et al.* 1999) (Figure 1.2-3). Other epidemiological studies that provide support for this hypothesis include observations that exposure to farm animals and raw milk early in life reduces the likelihood of developing allergic disorders (Umetsu *et al.* 2002).

Indirect evidence linking the exposure to microorganisms and allergy in schoolchildren aged nine to eleven years shows that the prevalence of atopic sensitisation decreases linearly with increasing number of siblings (von Mutius *et al.* 1994). Furthermore, a large prospective trial. involving 1,035 children followed since birth as part of the Tucson Children's Respiratory Study, reported a reduced risk of developing asthma in relation to both the total number of siblings and enrolment in day-care (Ball *et al.* 2000). Other cross-sectional surveys that support this hypothesis include observations demonstrating that long-term exposure to a farming environment - which provide exposure to microbial compounds, farm animals and raw milk - early in life, reduces the likelihood of developing asthma, hay fever and atopic sensitisation (Riedler *et al.* 2001). These observations suggest that environmental exposure to

microbial components in stables or farmhouses stimulates the development of immune tolerance towards allergens in children. Exposure to bacterial endotoxin may be important in producing this protective effect (Gereda *et al.* 2000; Gereda *et al.* 2001).

Childhood infections

Childhood infections such as measles, hepatitis A, and infection with *Mycobacterium tuberculosis* have all been associated with a decrease in the incidence of atopy (Shaheen *et al.* 1996; Matricardi *et al.* 1997; Matricardi *et al.* 2000). A study looking at a historical cohort in Bandim, a semi-rural district of Bissau, the capital of Guinea-Bissau, has shown that measles infection protects against the development of atopy in children aged zero to six years (Shaheen *et al.* 1996). In a retrospective case-control study on 240 atopic cases and 240 non-atopic controls from a population of Italian male cadets aged 17-24, Matricardi *et al* investigated the correlation between markers of exposure to foodborne and orofaecal microbes and prevalence of atopy and respiratory allergies.When compared with controls, there was a lower prevalence of positive serology for *Toxplasma gondii*, hepatitis A and *Helicobacter pylori* in atopic participants. Allergic asthma was rare (0.4%) and allergic rhinitis infrequent (7%) among the participants exposed to at least two orofaecal and foodborne infections (Matricardi *et al.* 2000). Furthermore, hepatitis A seropositivity remained inversely associated with atopy after adjusting for family education, number of older siblings, and the area of residence (based on the number of inhabitants). In contrast, it increased with a decreasing number of older siblings among seronegative subjects (Matricardi *et al.* 1997). Further epidemiological data show that children with more exposure to other children at home (older siblings) or at day care are more likely to have frequent wheezing at the age of two years than children with little or no exposure but are less likely to have

frequent wheezing from the age of six through the age of 13, again suggesting that the transmission of early childhood infections have a protective role against the development of allergic disorders (Ball *et al.* 2000). These cross-sectional studies provide indirect evidence that common infections acquired early in life, because of the presence of older siblings or because of living conditions, may reduce the risk of developing atopy.

The inverse association between family size and allergic symptoms, positive skin-prick test and specific IgE levels is well recognised (Strachan 1997; Strachan 2000). In particular, studies have found that children with no siblings or only one sibling were at increased risk of asthma when compared with children with more than one sibling (Wickens *et al.* 1999). Other studies have found that subjects from large families with shared bedrooms and pets were less often atopic as adults. These findings led to the hypothesis that the living conditions that favoured the transmission of bacterial or viral agents during childhood would prevent the development of atopy (Svanes *et al.* 1999).

A newly identified asthma susceptibility gene, *Tim-1* (T cell Immunoglobulin Mucin 1), provides evidence for a direct role of hepatitis A virus (HAV) in the prevention of atopic disease. The human homologue of *Tim-1*, which lies at human chromosome 5q33.2, encodes the cellular receptor for HAV (hHAVcr-1) (McIntire *et al.* 2001). In addition, in mice, the *Tim-1* gene product is expressed on T cells and appears to regulate the production of IL-4 in T cells by affecting $CD4^+$ T cell differentiation, the development of Th2 cells, with production of IL-4 and IL-13, and the development of BHR (McIntire *et al.* 2001). By interacting with HAV, human *Tim-1* may directly alter the T helper cell balance of the infected individual and may have significant effects on Th2 differentiation and survival. HAV may selectively eliminate allergen-specific Th2 cells by clonal deletion and, thus, specifically protect against the development of atopy or by altering T cell development conferring protection against

asthma (Umetsu *et al.* 2002). The homology between human hepatitis A virus receptor and *Tim-1* may explain the inverse relationship between hepatitis A and atopy (McIntire *et al.* 2001).

The possibility of influence by environmental factors on the prevalence of allergic disease in populations with similar genetic background has been raised by studies carried out in Germany soon after its reunification (von Mutius *et al.* 1998). These studies have shown that the prevalence of asthma, allergic sensitisation and bronchial hyperresponsiveness (BHR) was considerably higher in West German adults and children than in their East German peers (von Mutius *et al.* 1994; Nicolai *et al.* 1997).

Many bacteria and their components can exert a powerful modulating effect on immune cells. These include cell wall components of Gram-negative bacteria (lipopolysaccharide, LPS) (Raetz *et al.* 2002), mycobacteria (Rook *et al.* 1998) and DNA containing immune-stimulating sequences (ISS, CpG motifs) (Roman *et al.* 1997). Amongst the agents that have the potential for protecting against the development of Th2 driven allergic diseases are mycobacteria (Rook *et al.* 1998), the immunity to which has been shown to be associated with lower risk of atopy (Shirakawa *et al.* 1997).

Because of changes in lifestyle, and changes in vaccination programmes with Bacillus Calmette-Guerin (BCG) in the industrialised world, exposure to mycobacteria may have decreased whilst that to allergens has increased. This may have influenced the postnatal development of immune function so as to increase the pre-disposition to chronic allergic diseases in later years (Martinez *et al.* 1999; Prescott *et al.* 1999)

The inverse relationship between mycobacteria and allergy, and the theoretical possibility that non-pathogenic mycobacteria could alter the immune response to allergen both *in vivo* and *in*

vitro, has represented the leading hypothesis for the studies presented in Chapters four and five of this thesis.

1.2 Definition and Pathogenesis of Allergic Asthma

Asthma is a chronic inflammatory disorder of the airways which is characterised by reversible airflow obstruction and chronic airway inflammation leading to persistent bronchial hyperresponsiveness (BHR) and airway remodelling (Busse *et al.* 2001). The pathologic alterations of asthma were historically derived from post-mortem studies of patients who died of asthma (Busse *et al.* 2001). The advent of fibre-optic bronchoscopy has provided the opportunity to obtain bronchoalveolar lavage fluid (BALF) and endobronchial mucosal biopsy specimens from patients with milder forms of asthma, allowing the demonstration that the histopathological features present in fatal asthma are also found in mild forms of the disease (Djukanovic *et al.* 1990). Structurally, the bronchial mucosa of asthmatics is characterised by chronic inflammation, featuring lymphocytes, eosinophils and mast cells, along with epithelial desquamation, goblet cell hyperplasia (Cohn *et al.* 1997; Cohn *et al.* 1999) and thickening of the submucosa (Dunnill *et al.* 1969; Dunnill 1971; Beasley *et al.* 1989; Saetta *et al.* 1991). These pathological findings, consistent with chronic inflammation, appear independent of the aetiology of the disease and have consistently been associated with the main physiological abnormalities of the disease, such as variable airflow obstruction and BHR (Cockcroft *et al.* 1977; Casale *et al.* 1987; Wills-Karp 1999).

The aetiology of asthma is multifactorial and involves the interaction between genetic factors and environmental stimuli (Holgate 1999). Although many different mechanisms can determine the clinical syndrome of asthma, most asthma occurs in association with atopy – the propensity to synthesise and secrete immunoglobulin E (IgE) in response to various,

otherwise harmless, environmental antigens – and the consequent imbalance between the T lymphocyte (Th1 and Th2) phenotypes (Holgate 1993; Holgate 1999). Bronchial inflammation and BHR begin with the inhalation of environmental antigens. Inhaled antigens that escape muco-cilliary clearance and penetrate the underlying epithelial layer are intercepted by dendritic cells that then migrate to the regional lymph nodes, where they act as antigen-presenting cells to the B and T cells. In the presence of interleukin (IL)-4 and IL-13, the interaction between B and T cell results in immunoglobulin class-switching to produce IgE, the hallmark of the atopic status (Szabo *et al.* 2003).

1.2.1 Allergic sensitisation

The defining feature of atopy is the production of IgE in response to exposure to a variety of antigens. Such IgE production is a tightly regulated process and part of a complex network of cellular and molecular events necessary for the development of the allergic response. The initiation of this response occurs with the presentation of allergen by antigen-presenting cells (APCs) to $CD4^+$ T cells residing in the airway mucosa (a process referred to as sensitisation). In atopic individuals, allergic inflammation and IgE synthesis are known to be promoted by a distinctive allergen-specific T helper 2 cell subset (Th2) defined by the fact that they secrete a cytokine profile represented by IL-4, IL-5, IL-9 and IL-13. In contrast, Th1 cells synthesise mainly IL-2 and interferon-gamma (IFN-γ) (Abbas *et al.* 1996; Mosmann *et al.* 1996; Romagnani 1997). The presence of cytokines like IL-4 or IL-12 (along with IL-18 and IFN-γ) at the time of T cell priming and activation represents the single most important factor influencing the differentiation of $CD4^+$ T cells respectively into Th2 or Th1 (Abehsira-Amar *et al.* 1992; Seder *et al.* 1992; Seder *et al.* 1993; Romagnani 1995; Magram *et al.* 1996) (Figure 1.2-1).

After priming, the presence of IL-4 and CD40/CD40L interaction promote immunoglobulin class-switching to IgE (Busse *et al.* 2001). IgE released into the circulation binds to high-affinity IgE receptors (FcεRI) on the surface of mast cells and basophils and to the low-affinity IgE receptors (FCεRII or CD23) on the surface of lymphocytes, eosinophils, platelets and macrophages (Bachert *et al.* 1990; Tunon-De-Lara *et al.* 1996; Busse *et al.* 2001). FcεRI cross-linking on the mast cell surface following antigen binding causes cell activation resulting in the induction of signalling cascades and the release of preformed granule contents (histamine, tryptase, chymase, vasoactive and chemotactic mediators) and Th2 cytokines, as well as *de novo* synthesis of lipid mediators and reactive oxygen species (Busse *et al.* 2001). The release of pre-formed mast-cell mediators upon allergen stimulation, constitutes the *'early phase response'*. Histamine induces the contraction of airway smooth muscle and mucus secretion; it also causes vasodilatation and increased microvascular permeability, leading to exudation of plasma proteins into the airway walls, ultimately causing narrowing of the airway lumen (Bousquet *et al.* 2000). Tryptase potentiates the histamine-induced smooth muscle contraction, whereas chymase has a procollagen proteinase activity and is probably directly toxic to the airway cells (Bousquet *et al.* 2000).

The release of the cysteinyl leukotrienes, inflammatory cytokines and chemotactic factors results in the recruitment and activation of eosinophils, Th2 $CD4^+$ cells, macrophages and neutrophils, which ultimately leads to the inflammatory reaction known as the *'late phase response'* (Rothenberg 1998). Once the inflammatory reaction or *late phase* is initiated, eosinophils become one of the major mediators of chronic inflammation in allergic asthma, leading to the repetitive cycle of tissue damage and chronic inflammatory changes, which persist even in the absence of sustained allergen exposure (Holgate 1997) (Figure 1.2-1).

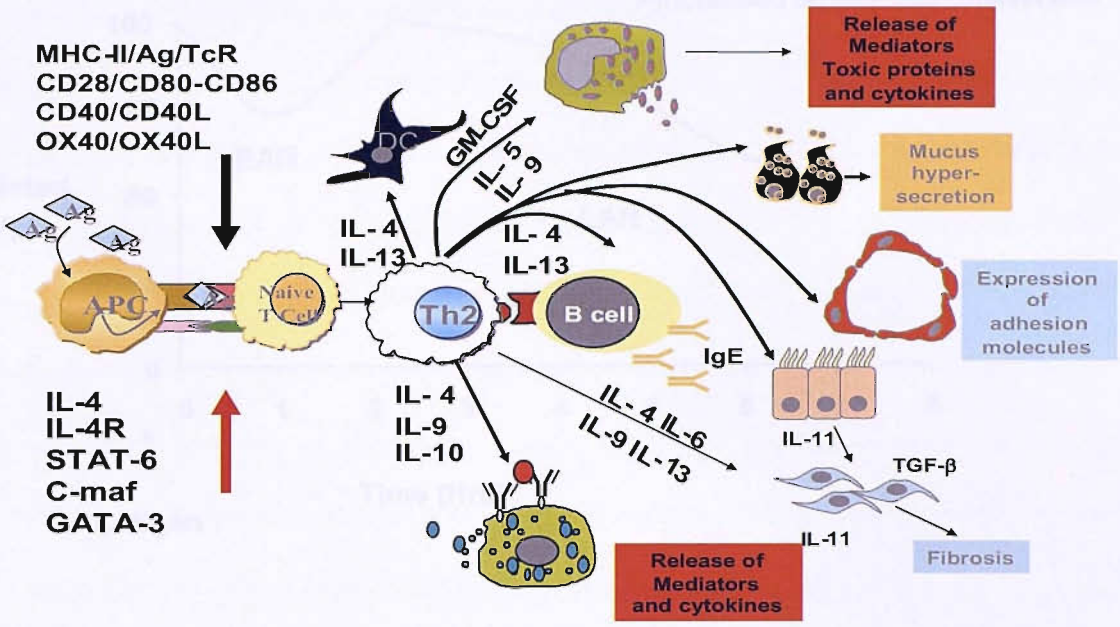


FIGURE 1.2-1: *Cellular and molecular mechanisms of allergic inflammation.*

1.2.2 Airway responses to allergen (EAR and LAR)

Inhalation of allergen by sensitised subjects, following natural exposure or allergen challenge test, can result in bronchoconstriction associated with symptoms experienced during asthma exacerbations. Bronchoconstriction can present as different patterns: isolated immediate, isolated late, or dual reactions. Measurements of the changes in airway function following allergen challenge have largely involved spirometry. The immediate or *'early asthmatic response' (EAR)* develops within 10 to 15 minutes of the inhalation challenge, reaches a maximum by 30 minutes and usually resolves between one and three hours (Figure 1.2-2). *'Late asthmatic responses (LAR)'* occur 3 to 4 hours post-allergen exposure and reach maximal intensity after four to eight hours, with a resolution that takes place between 12 and 24 hours (Figure 1.2-2).

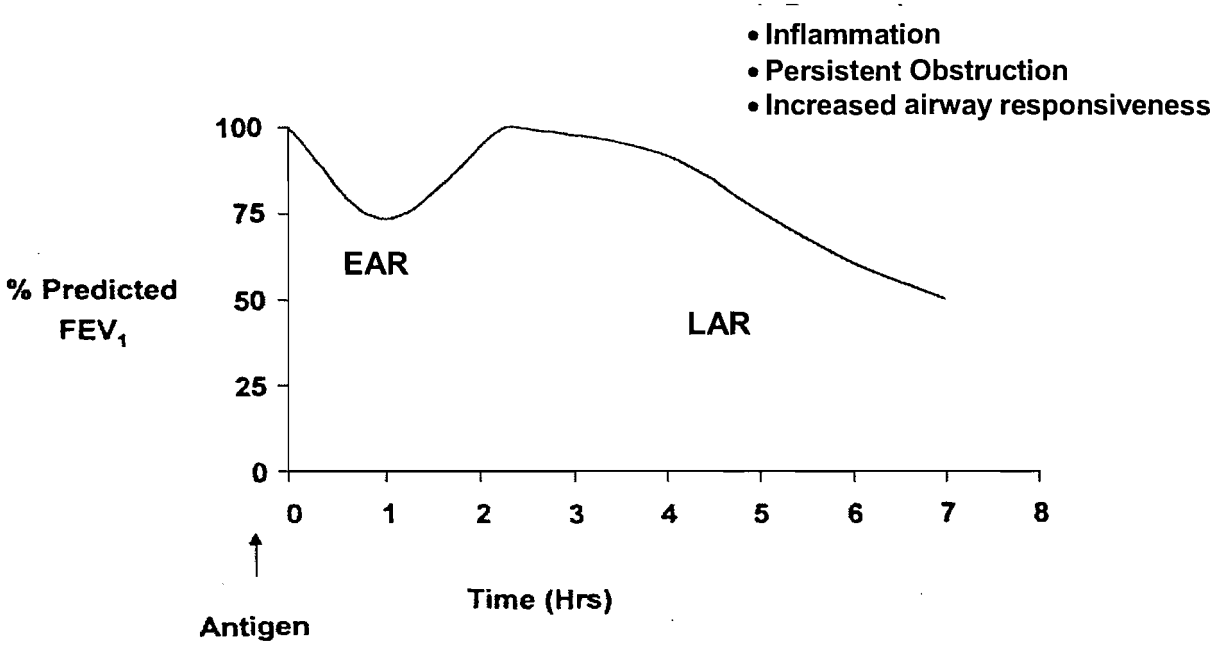


FIGURE 1.2-2: *The early and late-phase reactions to inhaled antigen. Modified from Nadel and Busse, 1998.*

The early (immediate) response is characterised by rapid onset of mucosal oedema, increase in airway smooth muscle tone and airway narrowing, associated with mast cell degranulation (Pauwels 1989).

In a proportion of subjects – 50% of adults (Robertson *et al.* 1974) and 70% or more of children (Van Lookeren *et al.* 1969) – airway narrowing persists or recurs after two to three hours, with a maximal peak at six to twelve hours post-allergen challenge, followed by a recovery phase which occurs within 24 hours. The *LAR* involves the recruitment and activation of eosinophils (De Monchy *et al.* 1985), $CD4^+$ T cells (Robinson *et al.* 1993), basophils (Guo *et al.* 1994), neutrophils (Koh *et al.* 1993; MacAry *et al.* 1997) and macrophages (Calhoun *et al.* 1993). Twenty-four hours after allergen challenge, an increase of activated $IL\text{-}2^+$ T cells and an increase in the expression of IL-5 or granulocyte-macrophage colony-stimulating factor (GM-CSF) messenger RNA (mRNA) is observed in bronchial biopsies, suggesting involvement of T cells (Bentley *et al.* 1993). The release of preformed

cytokines by mast cells is the likely initial trigger of the early selective recruitment and activation of inflammatory cells (De Monchy *et al.* 1985; Metzger *et al.* 1986; Zangrilli *et al.* 1995; Jarjour *et al.* 1997). The increased level of IL-5 during the EAR and LAR stimulates bone marrow production of eosinophil progenitors. This has recently been confirmed in a study in which bone marrow progenitors were directly measured in bone marrow aspirates taken 24 hours after allergen inhalation (Sehmi *et al.* 1996). Because of the good reproducibility and the resemblance to the asthmatic reaction that occurs after natural allergen exposure, the allergen-induced late-phase response to allergen challenge is considered to be a reliable experimental model to study the inflammatory mechanisms in asthma and test the efficacy of new anti-asthma drugs (Holgate 1993; Bochner *et al.* 1994).

1.2.3 Mechanisms of Airway Hyperresponsiveness

Bronchial hyperresponsiveness (BHR) is the term used to describe the tendency of the airways to constrict to both specific (allergens) and non-specific stimuli (e.g. histamine, methacholine, cold air, exercise). The more responsive the airways, the lower the amount of stimulus needed to cause a set decrease in lung function. Hyperresponsiveness of the airways to exogenous stimuli is considered to be one of the hallmarks of asthma. Alterations in the structure of the airways can account for the differences in airway responsiveness between asthmatic subjects and healthy individuals. This baseline hyperresponsiveness can be altered experimentally (Cockcroft *et al.* 1987) or naturally following exposure of mild asthmatic subjects to airborne allergens, and these events can reverse spontaneously following removal of antigen from the environment or following treatment. While structural remodelling is an important determinant of baseline airway responsiveness, the wide variability in BHR

indicates that mechanisms additional to structural remodelling *per se* contribute toward baseline hyperresponsiveness (Riffo-Vasquez *et al.* 2002).

Epidemiological studies demonstrate that atopy is associated with BHR (Peat *et al.* 1987), as well as asthma incidence (Stempel *et al.* 1980; Zimmerman *et al.* 1988), persistence and severity (Peat *et al.* 1987). In a large population-based study, atopy was the most important risk factor for developing BHR (Peat *et al.* 1992). Allergen sensitisation – predominantly to indoor allergens – confers an odds ratio of 1:6–1:20 for symptomatic BHR (Platts-Mills *et al.* 1997). Furthermore, the LAR is more likely to develop during seasons of the year when natural exposure is increased and prolonged allergen avoidance can attenuate LAR.

The relationship between the LAR and BHR has received considerable attention. It has been shown that airway responsiveness to histamine is significantly increased post allergen challenge in patients who develop a LAR but not in patients who develop an isolated EAR (Cockcroft *et al.* 1977), showing that the development of the LAR may be associated with more significant and persistent changes in airway responsiveness.

In Chapter five, I used the reduction in the EAR and LAR as outcome measures to assess the effects of *Mycobacterium vaccae* as possible asthma treatment.

1.2.4 Th2 cells in asthma

As primary orchestrators of the specific immune response, T lymphocytes have been implicated in the pathogenesis of allergic airway disease (Azzawi *et al.* 1990; Corrigan *et al.* 1990). Consistently elevated numbers of $CD4^+$ T lymphocytes have been found in the BALF and bronchial biopsies from asthmatics (Azzawi *et al.* 1990; Corrigan *et al.* 1990). $CD4^+$ T cells exhibit increased expression of activation markers such as interleukin-2 receptor (IL-2R), class II histocompatibility antigens (HLA-DR), and very late activation antigen-1 (VLA-

1) (Corrigan *et al.* 1990; Walker *et al.* 1992). Increased numbers of $CD4^+$ T lymphocytes that express mRNA for IL-4 and IL-5, but not IFN-γ, have been observed in the airway submucosa and sputum of patients with asthma at baseline and during allergen-induced late-phase asthmatic reactions (Till *et al.* 1995; Humbert *et al.* 1996; Ying *et al.* 1997; Olivenstein *et al.* 1999). Furthermore, the increase in airway responsiveness to methacholine following allergen challenge of immunised mice is abrogated by depletion of $CD4^+$ T lymphocytes or in mice with severe combined immuno deficiency (SCID), which lack T and B lymphocytes. The adoptive transfer of Th2 clones into SCID or BALB/c mice induces both pulmonary eosinophilia and BHR following antigen challenge. In contrast, the adoptive transfer of ovalbumin-specific Th1 cell clones into these mice results in a neutrophilic and monocytic infiltrate into the lungs, without the attendant BHR. Similarly, the adoptive transfer of T lymphocytes from genetically hyperresponsive mice can increase airway responsiveness in hyporesponsive-recipient mice (De Sanctis *et al.* 1997). T cells from both the BAL fluids and bronchial biopsies of allergic asthmatics express elevated levels of mRNA for IL-4, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5, consistent with a Th2 pattern of cytokine production (Robinson *et al.* 1992; Walker *et al.* 1992; Del Prete *et al.* 1993; Robinson *et al.* 1993), as well as the Th2 chemokine receptors CCR4 and CCR8 and the Th2 transcription factor GATA-3. (Nakamura *et al.* 1999; Ray *et al.* 1999; Caramori *et al.* 2001; Christodoulopoulos *et al.* 2001; Das *et al.* 2001; Panina-Bordignon *et al.* 2001). After allergen challenge there is an increase in activated T cells and Th2 cytokines in bronchial biopsies of asthmatic subjects (Bentley *et al.* 1993; Robinson *et al.* 1993). There is abundant evidence showing that IL-4 is excessively produced in asthma. Studies on asthmatic subjects demonstrate increased expression of IL-4 mRNA in the bronchial mucosa (Robinson *et al.* 1992), raised levels of IL-4 protein in serum and bronchoalveolar lavage (BAL) fluid (Walker

et al. 1994; Daher *et al.* 1995), increased frequency of T cells that express either IL-4 mRNA or protein in the airways mucosa and circulation (Chan *et al.* 1996; Humbert *et al.* 1996), and increased capacity of T cells to produce IL-4 in response to house dust mite antigen (Leonard *et al.* 1997).

There appears to be an association between the severity of asthma and the number of $CD4^+$ cells in BALF (Walker *et al.* 1991; Robinson *et al.* 1993). The presence of IL-4- and IL-5-producing cells was shown to correlate with BHR, FEV_1 and airway eosinophilia (Bradley *et al.* 1991; Walker *et al.* 1991; Robinson *et al.* 1993). Furthermore, successful treatment of asthma with corticosteroids is associated with a decrease in cells expressing mRNA for IL-4 and IL-5 (Robinson *et al.* 1993) as opposed to subjects who did not improve after steroid treatment or were steroid-resistant (Leung *et al.* 1995). IL-4 functions also in an autocrine fashion for Th2 cell growth and development; as indicated by the fact that, despite intensive exposure to allergen, in the absence of IL-4, Th2 cells either do not develop or cannot be sustained *in vivo* (Brusselle *et al.* 1995). In keeping with its potential to contribute to the asthmatic process, administration of inhaled recombinant human IL-4 to patients with allergic asthma causes a significant reduction in methacholine PC_{20} at 48 hours (increased BHR) and a significant increase in sputum eosinophilia, both hallmarks of asthma (Shi *et al.* 1998).

IL-4 also induces a variety of cellular and molecular responses that are important in the development of allergic airway diseases. One of the first recognised effects of IL-4 has been the induction of isotype switching to IgE production by initiating ε-germline transcription (Finkelman *et al.* 1988). Furthermore, IgE-mediated immune responses are enhanced by IL-4 through its ability to up-regulate the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils as well as the low-affinity IgE receptor (FcεRII; CD23) on B lymphocytes and monocytes cells (Gould *et al.* 2003). Although human and murine B-cells also synthesize

IgE in response to IL-13, robust IgE responses can proceed in the complete absence of IL-13 during allergen challenge *in vivo* (McKenzie *et al.* 1998). IgE-mediated activation of mast cells leads to the synthesis and release of a number of inflammatory mediators that may contribute to the bronchoconstriction, vascular changes and mucus secretion (Temann *et al.* 1997; Dabbagh *et al.* 1999) observed in the early-phase response to allergen challenge.

IL-4 in conjunction with IL-3 and IL-9, acts as growth factor for mast cells (Madden *et al.* 1991). Studies looking at a pathogenic link between IL-4 and both the early and late-phase allergic response have demonstrated that blockade of the IL-4 receptor prior to antigen provocation in sensitised mice can inhibit both the BHR and eosinophilic accumulation (Coyle *et al.* 1995; Gavett *et al.* 1997).

An additional mechanism by which IL-4 contributes to airway obstruction in asthma is through the induction of mucin gene expression and the hypersecretion of mucus (Temann *et al.* 1997; Dabbagh *et al.* 1999). Excessive production of airway mucus represents one of the characteristic pathological changes of asthma, in particular in those patients who die in *status asthmaticus* (Aikawa *et al.* 1992). The direct effects of IL-4 on mucus production have been demonstrated both *in vitro*, using cultured airway epithelial cells expressing IL-4R constitutively, and *in vivo* in a mouse model. Under these conditions, exogenous IL-4 was able to induce MUC2 and MUC5 gene expression, respectively, in conjunction with the synthesis of mucus glycol-conjugate. In this study, IL-4 did not increase inflammatory cell numbers in either the airway tissue or BAL, suggesting that IL-4 directly induces the differentiation of epithelium into mucus glycoconjugate-containing goblet cells via its own receptor (Dabbagh *et al.* 1999). Furthermore, when mice were treated with an antibody to the murine IL-4 receptor (anti-IL-4R) three days before intratracheal challenge with antigen, the antigen-induced increases in mucus-containing cells was inhibited, demonstrating that IL-4 is

necessary for *in vivo* development of goblet cell metaplasia (Gavett *et al.* 1997). It has been postulated that some of the actions attributed to IL-4 could be the effect of the interaction of IL-13 with IL-4R, as shown by the observation that airways of STAT6-deficient animals are not able to produce mucus following allergen provocation (Yang *et al.* 2001).

Numerous studies have shown that IL-4 blockade eliminates antigen-induced increases in eosinophils (Lukacs *et al.* 1994; Brusselle *et al.* 1995; Gavett *et al.* 1997). Although the exact mechanisms involved in the ability of IL-4 to induce tissue eosinophilia are unknown, there are several potential mechanisms. IL-4 may mediate pulmonary eosinophilia through its role in Th2 cell differentiation and the subsequent production of IL-5 (Cohn *et al.* 2001; Tomkinson *et al.* 2001). IL-4 increases the expression of inflammatory cytokines from fibroblasts that might contribute to inflammation and lung remodelling in chronic asthma (Dabbagh *et al.* 1999). IL-4 may also regulate eosinophil influx by up-regulating vascular cell adhesion molecule-1 (VCAM-1) expression on the endothelium and/or stimulating the release of eotaxin from resident airway cells (Moser *et al.* 1992; Mochizuki *et al.* 1998). Through its effect on VCAM-1, IL-4 is able to direct the migration of T lymphocytes, monocytes and basophils, and leads to prefential migration of eosinophils to inflamed tissues (Schleimer *et al.* 1992). In support of a role for IL-4/IL-13-mediated VCAM-1 expression in pulmonary eosinophilia, numerous studies have shown that VCAM-1 is necessary for eosinophil recruitment into the lung in response to antigen provocation in mice (Nakajima *et al.* 1994). In addition, IL-4 promotes eosinophilic inflammation by inducing eosinophil chemotaxis and activation through the increased expression of eotaxin (Hoeck *et al.* 2001; Moore *et al.* 2002).

An essential biological activity of IL-4 in the development of allergic inflammation is the ability to drive the differentiation of naïve T helper type 0 (Th0) lymphocytes into Th2 lymphocytes (Hsieh *et al.* 1992; Seder *et al.* 1992). Administration of IL-4 generates Th2-like

lymphocyte clones, whereas incubation with anti-IL-4 blocks this differentiation. While IgE production and the induction of VCAM-1 are activities common to both IL-4 and IL-13, the induction of Th2-like lymphocytes is a unique biological activity of IL-4 because IL-4 receptors and not IL-13 receptors are expressed on T cells (Kopf *et al.* 1993).

A further mechanism by which IL-4 promotes allergic inflammation is through its ability to prevent apoptosis of T lymphocytes. Several cytokines, including IL-2, IL-4, IL-7, and IL-15, are effective in preventing death of activated T cells. Of these, IL-4 seems to be one of the most effective (Vella *et al.* 1997). Inhibition of apoptosis by IL-4 might be mediated partly by maintaining levels of survival-promoting proteins such as Bcl-2 in the cells. The antiapoptotic mechanism appears to be STAT6-independent, suggesting that the IL-4 receptor on resting T cells may use a different signalling pathway to facilitate T cell viability (Vella *et al.* 1997).

Apoptosis of T lymphocytes can be induced through signals mediated by Fas ligand/ Fas (CD95) interaction (Crispe 1994). T cells from asthmatics show defective expression of Fas, and IL-4 downregulates Fas expression on the cell surface, (Spinozzi *et al.* 1998) which might explain the persistence of inflammatory cellular infiltrates in allergic asthma (Duez *et al.* 2001).

Thus, the association of Th2 cells and their effects in the respiratory tract has led to the theory that Th2 cells orchestrate the characteristic inflammatory response that results in asthma. Th1 cells have also been identified in the airways of asthmatics and have been postulated to play a role in the pathogenesis of the disease (Holtzman *et al.* 1996). When activated with a phorbol ester (phorbol 12-myristate 13-acetate, PMA) and a Ca^{2+} ionophore (ionomycin), BAL cells recovered from allergic asthmatics produced IFN-γ. However, bronchoprovocation with allergen did not lead to an increase in IFN-γ production (Kelly *et al.* 1997), indicating that the Th1 cells present in the airways of asthmatics are not allergen-specific.

Animal models of allergic disease, employing sensitisation and allergen challenge, have confirmed the importance of Th2 cells in allergic bronchial inflammation (Robinson 2000).

Furthermore, T cell clones generated from cord blood lymphocytes of newborns with atopic parents produce greater amounts of IL-4 than those from newborns with non-atopic parents, suggesting that altered regulation of IL-4 synthesis develops *in utero* (Piccinni *et al.* 1996).

Our understanding of T helper cell biology has increased substantially over the past few years, but several important questions remain. The molecular mechanisms regulating the initiation of the Th1 or Th2 response and the down-regulatory pathways that restore the Th1/Th2 equilibrium remain incompletely understood (Szabo *et al.* 2003).

1.2.5 Prenatal cytokine environment and postnatal immune maturation

At birth, the immune system is weakly skewed towards a Th2 profile to maintain tolerance and avoid foetal rejection (Holt *et al.* 2000). This weak polarisation is maintained through the secretion of a range of Th2 trophic and Th1 inhibitory molecules by the placenta, including IL-4 (Roth *et al.* 1996), prostaglandine E_2 (PGE_2) and progesterone (Hilkens *et al.* 1995; Szekeres-Bartho *et al.* 1996). As the child is subsequently exposed to infectious, endogenous and environmental antigens, its immune system matures, demonstrating a more balanced T cell profile (Figure 1.2-3).

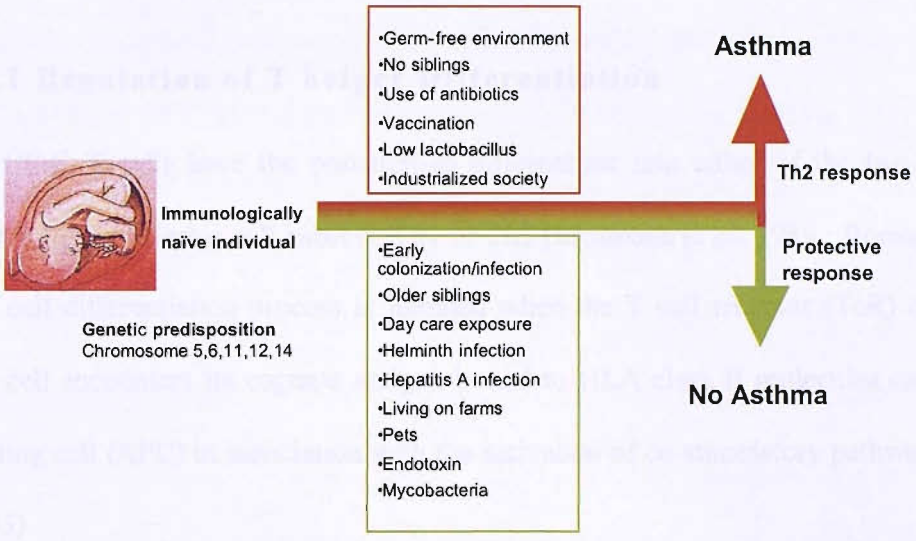


FIGURE 1.2-3: *Effects of environemt on T cell phenotype and development of asthma. (From (Umetsu et al. 2002) modified)*

Studies of the development of the immune system in early life have provided further evidence on cytokine phenotypes and the interaction between infections and atopic disorders. In particular, it has been shown that mononuclear cells obtained from cord blood have markedly decreased cytokine responses to non-specific stimuli (Holt *et al.* 2000). This included both Th1 (IFN-γ) and the Th2 (IL-4) responses. When studied in cord blood and peripheral blood during the first year of life, (Martinez *et al.* 1995; Prescott *et al.* 1999) Th1-like responses were particularly decreased among children with a family history of allergies and among those who subsequently became sensitised to aeroallergens. Since IFN-γ is known to down-regulate Th2-type responses, and these responses are essential for IgE synthesis by B cells, it was suggested that the development of IFN-γ responses could be stimulated by exposure to infectious agents postnatally (Martinez 1994), and that this could be the mechanism by which these infections protect against the development of allergic diseases.

1.2.6 Th2 responses in the respiratory tract

1.2.6.1 Regulation of T helper Differentiation

Naïve $CD4^+$ T cells have the potential to differentiate into either of the two functionally distinct T helper effector cell subsets, Th1 or Th2 (Mosmann *et al.* 1989 ; Romagnani 1997). The T cell differentiation process is initiated when the T cell receptor (TcR) on a naïve T helper cell encounters its cognate antigen bound to HLA class II molecules on an antigen-presenting cell (APC) in association with the activation of co-stimulatory pathways (Szabo *et al.* 2003).

The induction of a Th1 or Th2 response involves a variety of different factors. These include the genetic background, the nature of the antigenic stimulus, the type of APC, the concentration of antigen (duration and strength of signal), and a variety of co-stimulatory signals (CD40L/CD40, CD80,CD86/CD28, ICOS/B7RP-1 (Constant *et al.* 1997; Yoshinaga *et al.* 1999), the CD28-dependent OX40 signalling, (preferentially involved in Th2 differentiation) (Akiba *et al.* 2000; Lane 2000) and B7-H3 (preferentially involved in Th1 immunity) (Chapoval *et al.* 2001).

The house dust mite *Dermatophagoides pteronyssinus*, a major cause of allergic disease in the western world, is well known for its ability to divert the immune response toward the Th2 phenotype. *Der p 1,* the faecal antigen of the house dust mite, is considered to be the most immunodominant allergen involved in the expression of IgE-mediated dust-mite hypersensitivity. Biochemically, *Der p* is a 25-kDa cysteine protease which has been shown to cleave CD25, the α subunit of the IL-2 receptor (Thomas 1993; Schulz *et al.* 1998) and CD23. As a result of cleavage of CD25, peripheral blood T cells show markedly diminished proliferation and IFN-γ secretion. These findings indicate that *Der p* could alter the balance of

Th1/Th2 subset distribution by decreasing growth and expansion of the Th1 subset and, as a consequence, augmenting expansion of the Th2 subset that favours a pro-allergic response. *Der p* may also contribute to the allergic phenotype by selectively cleaving intact CD23 on B cells. CD23 mediates a number of effects including the proliferation and differentiation of B cells and the regulation of IgE synthesis. Binding of IgE to the membrane-bound CD23 transduce an inhibitory signal that prevents further IgE synthesis. Cleavage of CD23 by thereby disrupting the IgE regulatory feed-back mechanism (Shakib *et al.* 1998). Furthermore, the proteolytic cleavage of integral CD23 (iCD23) can produce soluble CD23 (sCD23), which stimulates B-cells to differentiate and secrete IgE (Shakib *et al.* 1998; Gould *et al.* 2003).

Der p has also the ability to disrupt epithelial architecture, enhancing its access to immune cells.

Although allergens, such as *Der p,* can potentially create a microenvironment conducive to Th2 cell expansion, normal individuals do not mount a Th2 responses to these allergens, which suggests that, despite the nature of these antigens, other factors contribute to the allergic outcome following inhalation of aero-allergens in susceptible individuals.

Dendritic cells (DC) have potent antigen-processing capabilities. They are present at sites that facilitate naïve T cell encounters and represent the most efficient APC involved in presenting antigenic peptides to naïve T cells (Jenkins *et al.* 2001). Phenotypically distinct subsets of dendritic cells seem to possess unique functions in T helper development and differentiation (Moser *et al.* 2000). In the mouse, $CD8^{+}$ dendritic cells secrete the T cell factor Eta1/osteopontin that favours Th1 differentiation via simultaneously inducing IL-12 and inhibiting IL-10 secretion by APC (Ashkar *et al.* 2000). A second subset, CD8α dendritic cells, are thought to stimulate Th2 differentiation, perhaps through IL-6-dependent mechanisms (Rincon *et al.* 1997). Dendritic cells instruct the T cells, via levels of secretion of

cytokines such as IL-12, to differentiate along the Th1 or the Th2 pathway. The environment in which the DC have been stimulated, the type of stimulus, and the origin of the DC all play a part in the fate of the T cell response. Studies indicate that respiratory tract DC have an immature phenotype, expressing a low level of MHC class II molecules on their surface and producing minimal IL-12, thus favouring Th2 differentiation (DC2) (Stumbles *et al.* 1998; Rissoan *et al.* 1999). In contrast, mature DCs isolated from peripheral lymphoid organs produce elevated levels of IL-12 and thus stimulate the generation of Th1 cells (DC1). Th1 cells can be generated in the respiratory tract when the appropriate stimulus is provided. For example, pathogenic organisms that require macrophage activation for host defence, such as *Mycobacterium tuberculosis*, stimulate IL-12 production by APC, resulting in Th1-dominated, cell-mediated immune response. In addition to the maturation state of the DC, *in vitro* and *in vivo* data suggest that IL-12 production by DC can also be modulated by microenvironmental tissue factors, as well as pharmacological agents. Prostaglandin E_2 (PGE_2) (van der Pouw Kraan *et al.* 1995), β_2-adrenoceptor agonists (Panina-Bordignon *et al.* 1997), $1,25(OH)_2$-vitamin D3 (D'Ambrosio *et al.* 1998), histamine (van der Pouw Kraan *et al.* 1998) and nitric oxide (Huang *et al.* 1998) have all been identified as inhibitors of IL-12 production and thus as favouring Th2 differentiation.

Taken together, these findings support the theory that induction of either Th1 or Th2 differentiation depends on the DC maturation state and on the influence of several factors on the ability of DC to produce IL-12. It is clear, however, that the most important factor influencing this process is the type of cytokines present in the T cell microenvironment during antigen presentation and initiation of the T cell response (Figure 1.2-1). The cytokines IL-4 and IL-12 (together with IFNγ, IL-18, IL-23, and IL-27) are the principal cytokines influencing Th2 and Th1 differentiation from naïve precursor T cells, respectively.

1.2.6.1.1 Interferon-γ (IFN-γ)

Interferon gamma (IFN-γ) is a pleiotropic cytokine that plays an essential role in both the innate and adaptive phases of an immune response. Natural killer (NK), CD8 and Th1 cells are the most potent sources of IFN-γ, although macrophages, dendritic cells, naïve CD4 T cells and even B cells seem able to secrete IFN-γ (Szabo *et al.* 2003). IFN-γ binds the IFN-γ receptor, composed of the IFNR1 and R2 chains (Boehm *et al.* 1997). The IFNR complex utilizes the Janus kinase (Jak)/signal transducer and activator of transcription 1 (STAT1) signalling pathway (Bach *et al.* 1997). Defects in IFN-γ, IFNR1, IFNR2 or the signalling molecule STAT1 have been associated with increased susceptibility to intracellular microbial pathogens and uncontrolled mycobacterial infections, both in mice and humans (Szabo *et al.* 2003). IFN-γ plays an important role in Th1 differentiation, both acting alone, independently of IL-12, or by maintaining expression of the IL-12Rβ2 chain (Szabo *et al.* 1997). In the absence of IFN-γ (or IFN-α in humans) during the Th2 differentiation process, developing Th2 cells lose the ability to respond to IL-12 by downregulating the IL-12Rβ2 chain (see 1.2.6.1.2) (Szabo *et al.* 1997). This provides a potential mechanism for Th2 lineage stabilization via the modulation of responsiveness to the Th1-inducing cytokine, IL-12.

If IFN-γ is included during Th2 development, IL-12Rβ2 expression is maintained and the ability to produce IFN-γ is restored. Forced expression of the IL-12Rβ2 chain on Th2 cells by transgenic expression or retroviral transduction restores a functional IL12R complex and the ability of these cells to proliferate and phosphorylate STAT4 in response to IL-12 (Heath *et al.* 2000; Nishikomori *et al.* 2000). However, these Th2 cells fail to produce significant levels of IFN-γ and fail to repress IL-4. Thus, IL-12R expression alone is insufficient to induce IFN-

γ production or to alter Th2 development. Clearly, the inclusion of IFN-γ in developing Th2 cultures must cause other alterations in addition to the induction of IL-12Rβ2 expression.

1.2.6.1.2 IL-12

IL-12 is a heterodimer composed of two subunits, p35 and p40, and is secreted by activated APC, including macrophages, monocytes and dendritic cells (Trinchieri 1998). IL-12 signals through the IL-12 receptor complex composed of the IL-12Rβ1 and IL-12Rβ2 chains (Chua *et al.* 1994; Presky *et al.* 1996). Naïve $CD4^+$ T cells are unresponsive to IL-12, owing to a lack of IL-12R expression. Activation through the TcR is necessary to induce both the IL-12Rβ1 and IL-12Rβ2 chains, leading to the formation of a functional IL-12R complex (Presky *et al.* 1996). IL-12Rβ2 expression is maintained on differentiating Th1 cells and is extinguished on developing Th2 cells (Rogge *et al.* 1997; Szabo *et al.* 1997). The IL-12R complex is coupled to Jak/STAT1-3-4 (Gately *et al.* 1998). In humans, STAT4 phosphorylation is induced by IFN-γ, IL-12 (Szabo *et al.* 1997) and, to a lesser extent, IL-23. Individuals with naturally occurring mutations in one of the components of the IL-12R signalling pathway have severely impaired immune responses, especially to microbial infections (Altare *et al.* 1998; de Jong *et al.* 1998).

1.2.6.1.3 IL-12 and development of allergy

The low levels of IL-12 observed in atopic patients lead to aberrant Th2 development. A number of previous studies have suggested that a deficiency in IL-12 may skew the phenotype of the inflammatory cells infiltrating the airways towards a Th2 type immune reaction. Naseer *et al* showed that bronchial biopsies from atopic asthmatic subjects exhibit lower expression

of IL-12 mRNA when compared to those from normal controls (Naseer *et al.* 1997). Similarly, van der Pouw Kraan *et al*, using whole blood cultures with different stimuli found a 2.6-fold reduction in IL-12 in patients with allergic asthma compared to controls (van der Pouw Kraan *et al.* 1997).

The mechanisms for the impaired IL-12 production in allergic patients are not fully elucidated, but may result from a defect of the genes encoding for IL-12, mutations of IL-12Rβ2 gene (Matsui *et al.* 1999), an imbalance between factors able to stimulate or inhibit IL-12 production (e.g. IFN-γ *vs.* IL-4) (van der Pouw Kraan *et al.* 1995; van der Pouw Kraan *et al.* 1998) or may a loss in IL-12 responsiveness and therefore a deficit in the IFN-γ/IL-12 loop (Matsui *et al.* 2000). IL-12 responsiveness of Th1 cells has been shown to correlate with the mRNA encoding for IL-12β2R, and various allergic conditions have been associated with altered IL-12β2 gene expression. A study by Janefjiord *et al* showsed that another mechanism involving CD2-CD58 interaction can account for the observed deficit in IL-12 (Janefjiord *et al.* 2001).

Further evidence for the importance of IL-12 in regulating allergic immune responses has been provided by *in vivo* studies using a murine model of allergic airway inflammation where exogenous IL-12 has shown an anti-allergic activity when administered systemically. In particular, IL-12 was able to prevent antigen-induced increases in airway hyperresponsiveness, lung eosinophilia and Th2 cytokine expression (Gavett *et al.* 1995) and to inhibit IgE synthesis (Kips *et al.* 1996). Moreover, experiments employing mucosal gene transfer resulting in IL-12 expression in the lung have shown inhibition of the local Th2 cytokine production, thus preventing the development of allergic disease and BHR (Hogan *et al.* 1998). Unfortunately, these data have not yet been confirmed in a phase-I trial of IL-12 in human asthma. In this trial subcutaneous injections of IL-12 caused a significant reduction of

peripheral blood and sputum eosinophils, but no effects were observed on the allergen-induced early and late-phase responses (see 1.3.3) (Bryan *et al.* 2000).

A different approach to studying the relevance of IL-12 in modulating the immune response *in vivo* comes from models where the production of IL-12 is induced by stimuli like bacteria or bacterial products. These stimuli are known to boost the innate immune response by stimulating IL-12 in addition to up-regulating costimulatory molecules on the surface of APC and T cells.

1.2.6.1.4 IL-18, IL-23 and IL-27

IL-18, IL-23 and IL-27 are potent cofactors for IL-12-induced Th1 development and enhance IFN-γ production from effector Th1 cells. IL-18 is primarily produced by macrophages and dendritic cells. The IL-18R consists of a ligand-binding subunit (IL-18Rα) and a signal-transducing subunit (IL-18β) that are absent on naïve $CD4^+$ T cells and induced on Th1 cells by IL-12 (Yoshimoto *et al.* 1998; Sareneva *et al.* 2000).

Although IL-18 is not essential for Th1 differentiation, it can facilitate IL-12-induced Th1 development by optimizing IFN-γ production (Robinson *et al.* 1997).

IL-23 is a heterodimer of the p40 subunit of IL-12 and a novel subunit designated p19 (Oppmann *et al.* 2000). IL-23 binds to the IL-23R complex composed of the IL-12Rβ1 chain and a novel receptor chain (IL-23R) related to IL-12R2 and gp130. The IL-23R complex is not expressed on naïve CD4 T cells; thus, IL-23 may not mediate initiation of Th1 differentiation but rather may be involved in sustaining IFN-γ production in the later stages of T helper cell development.

IL-27 mediates its effects through a novel member of the type I cytokine receptor family – identified by two groups and named WSX-1 (Sprecher *et al.* 1998) or T cell cytokine receptor (TCCR) (Chen *et al.* 2000) – with a strong homology to the IL-12Rβ2 chain. WSX-1/TCCR expression is predominantly lymphoid specific, with the highest levels of expression found in NK cells and resting $CD4^+$ T cells while it declines during T helper cell differentiation of both Th1 and Th2 cells.

1.2.6.2 Transcription factors involved in T Cell development

T helper cells are classified by their distinct patterns of cytokine gene expression. As cytokine gene expression is transcriptionally regulated, understanding how the hallmark Th1 cytokine IFN-γ and the corresponding signature Th2 cytokine IL-4 are controlled at the level of transcription has led to insights into the regulation of Th1 and Th2 differentiation (Figure 1.2-4).

Commitment to a Th1 or Th2 lineage is determined, at intracellular level, by numerous transcription factors (Figure 1.2-4). The signal transducer and activator of transcription (STAT) proteins play an important role in the selective response of cells to particular cytokines (Kaplan *et al.* 1998). The STAT factors STAT4 and STAT6, induced respectively by IL-12 and IL-4, play a crucial role in mediating the differentiation of naïve T cells. Developmental commitment to the Th2 lineage results from rapid loss of IL-12 signalling in Th2 cells (Szabo *et al.* 1995). The inability of Th2 cells to respond to IL-12 appears to be due to a selective down-regulation of the IL-12Rβ2 subunit (Rogge *et al.* 1997).

Th2 cell fate is governed by the transcription factors GATA-3, c-Maf and NFAT. Signalling via GATA-3 results in the production of Th2 cytokines. Modulation of chromatin structure

also regulates cytokine gene expression during T cell differentiation (Agarwal *et al.* 1998). Differentiation of naïve helper T cells into mature Th2 cells is associated with chromatin remodelling of the IL-4 and IL-13 genes, whereas differentiation into Th1 cells involves selective remodelling of the IFN-γ gene.

IL-4 locus remodelling is accompanied by demethylation, with the acquisition of a characteristic open chromatin structure. This could lead to occupancy of the accessible DNA by specific transcription factors such as GATA-3 (Zheng *et al.* 1997) and c-Maf (Ho *et al.* 1998) in Th2 cells. A similar model can be envisaged for Th1 gene expression, although much less information is presently available for Th1-specific transcription factors that could operate downstream of STAT4.

In contrast to Th2 differentiation, very little is known about the molecular basis of Th1 differentiation. The signalling pathway for Th1 cell differentiation involves IL-12, IFN-γ, STAT4, (Kaplan *et al.* 1996; Thierfelder *et al.* 1996; Kaplan *et al.* 1998) and interferon regulator factor 1 (IRF-1) (Lohoff *et al.*, 1997; Taki *et al.*, 1997), neither of which is Th1 restricted. More recently, T-bet has been identified and seems to be expressed selectively in thymocytes and Th1 cells (Szabo *et al.* 2000). IL-12 is required for the activation of STAT4, which then induces IFN-γ gene expression via T-bet. The resulting IFN-γ forms part of an auto-regulatory circuit with T-bet, whereby IFN-γ stimulates T-bet gene expression and *vice versa* (Neurath *et al.* 2002; O'Shea *et al.* 2002). IFN-γ also antagonises the differentiation of Th0 to Th2 cells, thus reinforcing the original lineage decision.

During the developmental stage, the induction and maintenance of sustained levels of the Th1- or Th2-specific T helper regulators – T-bet or GATA-3 – is necessary for T helper lineage commitment. While specifically inducing the Th1 or Th2 lineages, T-bet and GATA-

3 may also be involved in silencing the opposing T helper lineage, perhaps by inhibiting expression of the opposing T helper regulators (Szabo *et al.* 2003).

1.2.6.3 Transcription factors involved in Th1 cell development

T-bet is a Th1-specific transcription factor for Th1 development. T-bet trans-activates the IFN-γ gene, induces IFN-γ production and redirects polarized Th2 cells into the Th1 into the Th1 pathway (Szabo *et al.* 2002). Although T-bet is expressed in $CD8^+$ and NK cells, it is required for the control of IFN-γ production in $CD4^+$ and NK cells, but not in $CD8^+$ cells. Thus, the regulation of a single cytokine, IFN-γ, is controlled by distinct transcriptional mechanisms within the T cell lineage (Mullen *et al.* 2001)

In $CD4^+$ T cells, T-bet is rapidly and specifically induced in developing Th1 but not Th2 cells. T-bet expression appears to be controlled by both the T-cell receptor (TcR) and the IFNR/STAT1 signal transduction pathways (Lighvani *et al.* 2001) and not by the IL-12/STAT4 pathway (Lighvani *et al.* 2001; Afkarian *et al.* 2002). Furthermore T-bet seems to be able to induce its own expression (Szabo *et al.* 2003). This results in an increase in local IFN-γ and creates a positive feedback loop driving Th1 differentiation. T-bet also induces IL-12Rβ2 chain expression allowing IL-12/STAT4 signalling to optimize IFN-γ production, thereby completing the Th1 developmental commitment process (Szabo *et al.* 2003).

Transfection of T-bet into developing fully polarized Th2 cells resulted in a dramatic induction of IFN-γ expression, accompanied by a striking reduction of IL-5 and IL-4 production. Mice lacking T-bet fail to mount a Th1 response *in vivo*, while the excess of Th2-type cytokines is responsible for the spontaneous development of the pathological airway features similar to those seen in patients with asthma (Finotto *et al.* 2002).

The lungs of both T-bet$^{+/-}$ and T-bet$^{-/-}$ animals demonstrate significant baseline airway hyperresponsiveness, which is normally seen in wild-type animals only after allergen sensitisation. Furthermore, the airways of these animals are significantly hyperresponsive to methacholine and express increased levels Th2 cytokines. In asthma patients T-bet expression in the lung, primarily in CD4^{+} T cells, is significantly diminished.

STAT4 is an essential component of the IL-12 signalling pathway and plays an important role in Th1 differentiation (Kaplan *et al.* 1996; Thierfelder *et al.* 1996) and Type I IFNs (Rogge *et al.* 1998).Whilst TcR-activated IFN-γ production from effector Th1 cells appear to be independent of STAT4, the IL-12/IL-18 pathway for IFN-γ production in effector Th1 cells relies heavily on STAT4 (Szabo *et al.* 2003).

1.2.6.4 Molecular events controlling Th2 generation

GATA-3 is a Th2-specific transcription factor, essential for T-cell development (Ting *et al.* 1996; Zheng *et al.* 1997). GATA-3 belongs to a subfamily of zinc finger transcription factors that interact with specific deoxyribonucleic acid (DNA) binding sequences in the regulatory regions of genes encoding Th2-like cytokines. GATA-3 has been shown to be differentially expressed in Th2 and Th1 cells, and expression of this gene is sufficient to drive Th2 differentiation (Zhang *et al.* 1997; Das *et al.* 2001; Macaubas *et al.* 2001). Altered regulation of GATA-3 expression may be important in atopy, in as much as GATA-3 expression has been shown to be elevated in cells in BAL fluid and bronchial biopsy specimens of asthmatic patients in comparison with normal controls (Caramori *et al.* 2001; Christodoulopoulos *et al.* 2001) and GATA-3 correlated significantly with IL-5 expression and BHR (Nakamura *et al.* 1999). Furthermore, blockade of this gene with a dominant negative mutant of GATA-3 inhibits allergic inflammation in mice (Zhang *et al.* 1999).

GATA-3 mRNA is expressed at low levels in naïve $CD4^+$ T-cells. It is markedly up-regulated in cells differentiating along a Th2 lineage, and is down-regulated in Th1 cells (Zhang *et al.* 1997). It seems that GATA-3 expression is alone sufficient for $CD4^+$ Th2 cell function. GATA-3 autoregulates its own promoter and appears to control the accessibility of the IL-4 and IL-5 gene for transcription. In developing Th1 cells, GATA-3 overexpression blocks IFN-γ production (Ouyang *et al.* 1998; Lee *et al.* 2000) and IL-12Rβ2. Taken together, GATA-3 appears to be essential for Th2 cytokine production, transactivating the IL-5 promoter and acting as a chromatin remodelling factor, allowing the transcription of IL-4 and IL-13.

Studies in humans have clearly shown increased GATA-3 expression in asthma, and inhibition of GATA-3 activity causes a severe blunting of Th2 effects, both locally in the lung (eosinophil influx and mucus production) and systemically (IgE production) (Ray *et al.* 1999).

STAT6 is required for Th2 differentiation (Kaplan *et al.* 1996; Takeda *et al.* 1996). IL-4 engagement with its receptor leads to phosphorylation of STAT6 by Janus family kinase, Jak1 and Jak3, and results in the activation of IL-4–regulated genes such as IL-4R, IgE, FcεR, and MHC class II molecules. Once translocated to the nucleus STAT6 can interact directly with GATA-3 to activate Th2 promoters and enhancers (Luft *et al.* 2004).

The nuclear factor of activated T cells, (NF-AT) and the B cell lymphoma 6 (BCL-6) protein appear to be repressors of Th2 responses *in vivo*. BCL-6 is a potent transcriptional repressor, and has been shown to bind the STAT6 DNA-binding sequence in the CD23 promoter and to repress IL-4–induced activation of CD23 expression (Dent *et al.* 1997)

The proto-oncogene c-Maf is a Th2-specific transcriptor factor that was shown to transactivate the IL-4 promoter (Ho *et al.* 1996). Unlike GATA-3, c-Maf is not sufficient for the initiation of IL-4 transcription and it does not regulate the expression of all Th2 cytokines (Kim *et al.* 1999). C-maf knockout mice have impaired IL-4 production and a skewed T cell

response toward a Th1 phenotype. The concomitant expression of other more general factors, such as the member of the NF-AT family including NFATc1, NFATc2, NFATc3, NF-κB and activating protein 1 (AP-1) is required for high-level expression of the Th2 cytokine genes (Ray *et al.* 1999). NFATc1 and NFATc2 seem to exert a differential effect on Th1/Th2 differentiation as demonstrated by the fact that NFATc2-deficient mice develop lymphoproliferative responses associated with an increase in Th2 responses and NFATc1-deficient mice produce low levels of Th2 cytokines (Luft *et al.* 2004).

In conclusion, the initiation of Th2 differentiation in antigen- and cytokine-stimulated naïve $CD4^+$ T cells appears to be orchestrated by STAT6, which pre-exists in the cell cytoplasm. NF-ATp and NF-ATc also exist in the cytoplasm of naïve $CD4^+$ T cells and undergo rapid nuclear translocation upon stimulation of the cells. Neither GATA-3 nor c-Maf is sufficient, but both are critical for IL-4 gene expression *in vivo*. However, GATA-3 plays an essential role in the control of production of all key Th2 cytokines implicated in asthma pathogenesis. The concomitant activation of more general factors, such as NF-κB, AP-1, NF-AT and C/EBPβ, is required for high-level expression of the Th2 cytokine genes (Figure 1.2-4).

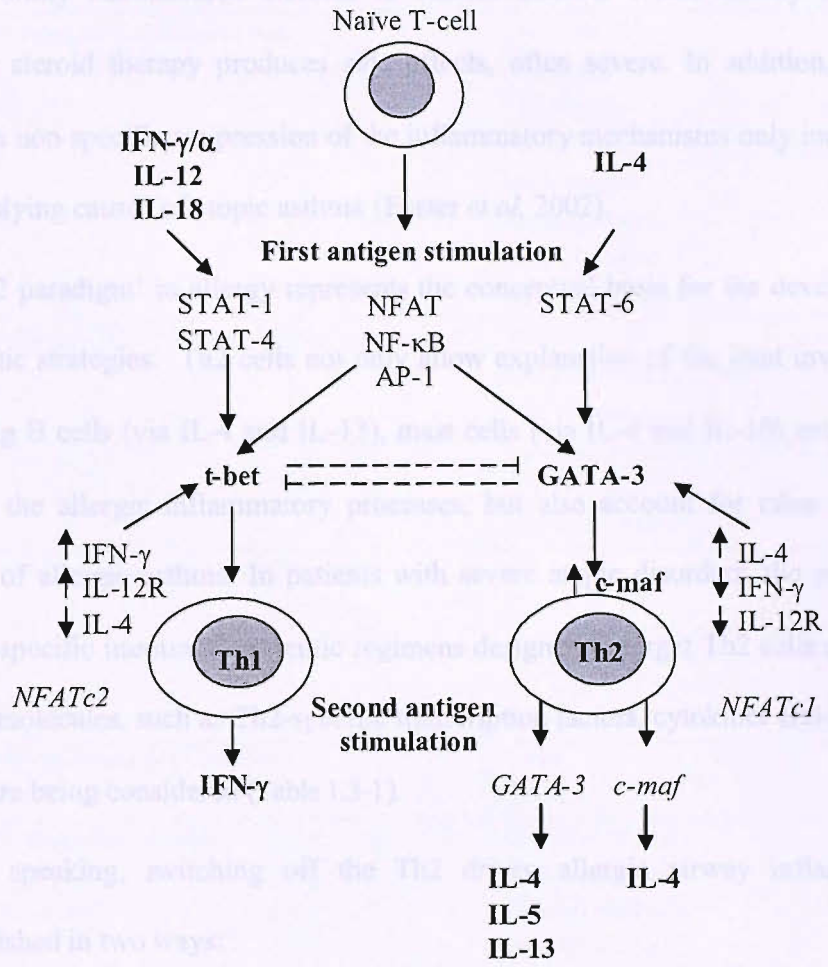


FIGURE 1.2-4 : *Signalling pathways and transcription factors in T cell differentiation (adapted from (Rao et al. 2000))*

1.3 POSSIBLE IMMUNOLOGICAL INTERVENTION IN THE TREATMENT OF ASTHMA

The mainstays of current asthma therapy are agents that induce bronchodilatation (e.g. β_2 agonists) and those that attenuate inflammation (inhaled glucocorticosteroids). Most asthmatic patients find these drugs effective and easy to comply with and do not experience significant side effects. A minority of patients, however, is resistant to this conventional regimen, and

rely on orally administered steroids to control disease. While usually effective, chronic systemic steroid therapy produces side effects, often severe. In addition, steroid therapy, through a non-specific suppression of the inflammatory mechanisms only indirectly addresses the underlying causes of atopic asthma (Foster *et al.* 2002).

The 'Th2 paradigm' in allergy represents the conceptual basis for the development of novel therapeutic strategies. Th2 cells not only allow explanation of the joint involvement of IgE-producing B cells (via IL-4 and IL-13), mast cells (via IL-4 and IL-10) and eosinophils (via IL-5) in the allergic inflammatory processes, but also account for other pathophysiologic features of allergic asthma. In patients with severe atopic disorders, the possibility of non-allergen-specific immunotherapeutic regimens designed to target Th2 cells or Th2-dependent effector molecules, such as Th2-specific transcription factors, cytokines and/or their receptors, or IgE, are being considered (Table 1.3-1).

Broadly speaking, switching off the Th2 driven allergic airway inflammation can be accomplished in two ways:

1. Directly: by inhibiting Th2 cell differentiation and function by directly inhibiting Th2 cytokines (IL-4, IL-5, IL-13) or their receptors employing humanised blocking antibodies, soluble receptors which bind secreted cytokines, small-molecule receptor antagonists or drugs that block the signal transduction pathways activated by cytokines;

2. Indirectly: by stimulating Th1 cells, resulting in the cross-inhibition of Th2 cell differentiation and cytokine secretion via the generation of IFN-γ production. Th1 stimulation can be achieved by administering IL-12 or IFN-γ, or stimulating immune cells to secrete Th1 cytokines employing non-pathogenic bacteria or their components (ISS, CpG, LPS analogues). In addition, some these interventions may induce the generation of regulatory cytokines (IL-10 or TGF-β) with down-regulatory effects on both Th2 and Th1 cytokines.

Target	Agent
Prevention of T-cell activation	Corticosteroids
	CTLA-4Ig
	Cyclosporin
	FK506
	Methotrexate
	Azathioprine
	Mycophenolate
	Suplatast tosilate
Inhibition of Th2 cytokines/phenotype	Soluble IL-4R
	Rhu IL-4 mutant proteins
	STAT6 inhibition
	Anti IL-5 monoclonal antibodies
	GATA-3 inhibition
	Soluble IL-13Rα
Promotion of Th1 cytokines/phenotype	IFN-γ
	IL-12
	IL-18
	BCG
	CpG
Anti-inflammatory cytokines	IL-10
	IL-1Ra
Inhibition of eosinophils	CCR3 antagonists
	VLA4 inhibitors
	ICAM-1 inhibitors
	Met-RANTES
	Met-Ckbeta7
IgE inhibition	Monoclonal anti-IgE (E25)

TABLE 1.3-1: *Novel strategies for the treatment of allergic asthma (Adapted from (Stirling et al. 2000))*

1.3.1 Administration of IFN-γ

Promising results arose from murine asthma models in which IFN-γ, given either during primary sensitisation or during the secondary immune response, decreased IgE production and airway inflammation and normalised airway function (Lack *et al.* 1994; Lack *et al.* 1996). A two-centre randomised, double blind, placebo controlled trial with subcutaneous IFN-γ in asthmatic patients followed. In this study with steroid dependent asthmatic patients, no

beneficial effects were observed (Boguniewicz *et al.* 1993). Studies with aerosolised IFN-γ showed an increase in BALF lymphocyte counts (Martin *et al.* 1993). Despite a significant reduction of eosinophils in the BALF of asthma patients exposed to aerosolised IFN-γ, the symptom scores and pulmonary function did not change after treatment (Boguniewicz *et al.* 1995).

1.3.2 Th1 transfer

Another way of counterbalancing Th2 driven allergic inflammation is to transfer IFN-γ secreting Th1 cells. In an animal model, passive transfer of ovalbumin (OVA) specific Th1 cells was performed either before or after sensitisation (Hansen *et al.* 1999). The results showed that an increase in airway inflammation was observed together with an augmented recruitment of host Th1 and Th2 cells. Accordingly, transfer of OVA specific Th1 cells into severe combined immune deficient (SCID) mice did not attenuate Th2 cell induced airway hyper responsiveness, but rather caused a more severe airway inflammation (Hansen *et al.* 1999). Taken together, these results suggest that antigen specific Th1 cells do not prevent or protect against Th2 mediated allergic disease, but rather initiate additional Th1 driven lung pathology.

1.3.3 IL-12

The ability of IL-12 to stimulate Th1 immune responses has been demonstrated both *in vitro* and *in vivo* using animal models (Kips *et al.* 1996). IL-12 therapy suppressed the allergen-induced airway eosinophilia and hyperresponsiveness. Moreover, a complete inhibition of allergen-specific IgE synthesis was obtained when IL-12 was given during sensitisation but

not during secondary challenge (Kips *et al.* 1996). The protection was accompanied not only by suppression of IL-4 but also by an increase in the IFN-γ levels in the BALF. Recombinant human IL-12 has been administered to humans and has several toxic effects diminished by slow escalation of the dose (Leonard *et al.* 1997). In patients with mild asthma, weekly infusions of human recombinant IL-12 in escalating doses over four weeks caused a progressive fall in circulating eosinophils, a reduction in the rise in circulating eosinophils after allergen challenge and a concomitant reduction in eosinophils in induced sputum. However, there was no reduction in either the early or late response to inhaled allergen challenge and in BHR (Bryan *et al.* 2000).

Both approaches, using IFN-γ or IL-12, are associated with significant toxicity. IL-12-induced toxicity seems to be dependent on the induction of high IFN-γ production (Car *et al.* 1999) as shown by experiments using IFN-γ receptor deficient mice in which the IL-12 toxicity was greatly reduced.

1.3.3.1 Anti-Th2 cytokine therapy

Th2 cytokines and their receptors are considered to be possible therapeutic targets (Tamaoki *et al.* 2000). Cytokine antagonism can be achieved by monoclonal antibodies against the particular cytokine or its receptor, by receptor antagonists, or soluble receptors that act by preventing the binding of the cytokine to its cell-surface receptor. Numerous studies based on the use of knock-out (KO) and transgenic mice have proven the critical role of IL-4, IL-5 and IL-13 not only for eosinophilic airway inflammation but also for airway hyperresponsiveness.

1.3.3.1.1 Anti-IL-5

Eosinophil infiltration into the airways after allergen challenge is a consistent feature of atopic asthmatics (Wardlaw *et al.* 1988). IL-5 is predominantly an eosinophil-active cytokine in the late-phase response to antigen challenge (Robinson *et al.* 1993) and is important for the recruitment and survival of eosinophils (Ohnishi *et al.* 1993). By associating with its receptor, interleukin-5 promotes eosinophil growth and differentiation (Yamaguchi *et al.* 1988; Clutterbuck *et al.* 1989), migration (Warringa *et al.* 1992 ; Resnick *et al.* 1993; Broide *et al.* 2001), activation and effector function, and survival (Giembycz *et al.* 1999).

T-cells are the predominant source of IL-5 in asthmatics, but IL-5 mRNA is also found in activated eosinophils and mast cells in tissues from patients with atopic dermatitis, allergic rhinitis (Bradding *et al.* 1993) and asthma (Broide *et al.* 1992), raising the possibility that IL-5 arises from multiple sources in atopic individuals.

In contrast to IL-3 or GM-CSF, IL-5 alone promotes eosinophil growth and differentiation in the bone marrow. IL-3 and GM-CSF are also less selective than IL-5, stimulating the production of other granulocytes such as mast cells and neutrophils, respectively. Because eosinophils are a dominant cell type in allergic reactions, this exquisite specificity makes IL-5 an excellent target for attenuating these responses. In fact, prolonged eosinophil survival and decreased eosinophil apoptosis caused by IL-5 are reversed by glucocorticoids (Giembycz *et al.* 1999), which accounts for at least some of the efficacy or these agents. IL-5 may also be important for the recruitment of eosinophils from blood vessels into tissues because topical administration of recombinant human IL-5 to the nasal airway of patients with allergic rhinitis induces eosinophil accumulation into the nasal mucosa (Terada *et al.* 1992).

Several studies have demonstrated a correlation between the activation of T lymphocytes, increased concentration of IL-5 in serum and BAL fluid, and increased severity of the

asthmatic response (Gibson *et al.* 1991; Robinson *et al.* 1993; Zangrilli *et al.* 1995). The abolition of the effects of IL-5 with antisense oligonucleotides (Karras *et al.* 2000) or anti-IL-5 blocking antibodies (Menzies-Gow *et al.* 2002; Buttner *et al.* 2003; Flood-Page *et al.* 2003) or IL-5 transcription factor GATA-3 (Finotto *et al.* 2001) are effective in reducing eosinophilic inflammation and BHR in various species. The link between IL-5, eosinophils, and asthma has been investigated using two humanised monoclonal antibodies specific for IL-5. Sch55700 (reslizumab) is a humanised monoclonal antibody with activity against IL-5 from various species (Dent 1999; Egan *et al.* 1999). SB240563 (mepolizumab) is also a humanised antibody with specificity for human and primate IL-5 (Egan *et al.* 1999). A single-dose phase I clinical trial was conducted with Sch55700 in patients with severe persistent asthma who remained symptomatic despite intervention with high-dose inhaled or oral steroids. The two highest doses of Sch55700 significantly decreased peripheral blood eosinophils, with inhibition lasting up to 90 days after the 1 mg/kg dose. SB240563 has also recently been tested in asthmatic persons in a clinical single-dose safety and activity study (Leckie *et al.* 2000). Monoclonal antibody against IL-5 lowered the mean blood eosinophil count and prevented the blood and sputum eosinophilia that follows allergen challenge. However, there was no significant effect of monoclonal antibody to IL-5 on the late asthmatic response or on airway hyper-responsiveness to histamine (Leckie *et al.* 2000). Recently, humanised anti-IL-5 monoclonal antibodies have been studied. Both SB-240563 and Sch 55700 when administered to asthmatic patients have shown high efficacy in reducing eosinophils in the circulation and sputum, but this did not lead to any significant reduction in airway response to allergen or in BHR (Leckie *et al.* 2000; Kips *et al.* 2003). These studies confirm the importance of IL-5 in eosinophilic inflammation in humans, but question the causative role of eosinophils in asthma.

In vitro data suggest that IL-5 effects can also be down-regulated by preventing the cytokine from binding to its receptor (IL-5R) using human soluble receptor analogues or modified receptors (Devos *et al.* 1993; Monahan *et al.* 1997). A variant of the α subunit of the IL-5R has been described (Devos *et al.* 1993). This analogue does not bind to the membrane subunit so that no intracellular signalling takes place upon IL-5 engagement on the α subunit. Covalent modification of the IL-5R by isothiazolones also prevents IL-5 from binding to the receptor (Devos *et al.* 1994).

1.3.3.1.2 Anti-IL-4

The role of IL-4 in asthma and the effects of blocking IL-4 on allergic inflammation are discussed in Chapter three of this thesis.

1.3.3.1.3 Anti-IL-13

The importance of IL-13 in the development of an asthmatic phenotype is underscored by the finding that IL-13 administration in naïve mice induces many of the features of asthma, including BHR and mucous hypersecretion, independently of eosinophilic inflammation. IL-13 synergises with IL-4 in the IgE synthesis and potently induces the secretion of eotaxin from airway epithelial cells. IL-13 KO mice present with a suppressed Th2 development, while anti-IL-13 therapy in wild type mice actively suppresses the allergen-induced airway inflammation and hyperresponsiveness. Overall, IL-13 is thought to play a critical role in secondary immune responses, while IL-4 has its main effects on the initial Th2 cell development. IL-13 also binds to the α-chain of the IL-4R (Lin *et al.* 1995; Zurawski *et al.* 1995). However, IL-13 induces IL-4-independent IgE and IgG_4 class-switching by human B

cells (Punnonen *et al.* 1993). Because T cells lack functional IL-13R, IL-13 (unlike IL-4) does not induce Th2 cell differentiation (de Vries 1998).

IL-13 signals through the IL-4 receptor α-chain but may also activate different intracellular pathways via activation of IL-13Rα1, so that it may be an important target for the development of new therapies. A second specific IL-13 receptor, IL-13Rα2, exists in soluble form and has a high affinity for IL-13, thus acting as a decoy receptor for IL-13. Soluble IL-13Rα2 is effective in blocking the actions of IL-13, including IgE generation, pulmonary eosinophilia, and BHR in mice. No human clinical studies have yet examined the utility of neutralizing IL-13 in allergic disease. However, neutralisation of IL-13 using a soluble form of the IL-13Rα chain (sIL-13Rα2-IgGFc fusion protein) that specifically binds to and neutralizes IL-13 (Donaldson *et al.* 1998) caused a significant reduction in BHR, airway eosinophilia and mucus hyperplasia in mice (Grunig *et al.* 1998; Wills-Karp *et al.* 1998). Humanised IL-13Rα2 is now being developed as a therapeutic approach for asthma.

1.3.4 Regulatory T-cells and the 'Hygiene Hypothesis'

Although the Th1/Th2 dichotomy offers a theoretically attractive explanation of the 'hygiene hypothesis', along with the increase in 'Th2-mediated' diseases like asthma epidemiological studies now also show that the incidence of 'Th1-driven' diseases like type 1 diabetes, multiple sclerosis and inflammatory bowel disease is increasing in parallel with allergic disorders. This suggests that other mechanisms may account for the 'hygiene hypothesis' (Stene *et al.* 2001; Bach 2002; Gale 2002; Sheikh *et al.* 2003) (Figure 1.2-2).

Interestingly, defects in the IL-12 or IFN-γ pathways do not lead to increased manifestations of allergies (Lammas *et al.* 2000), suggesting that Th1 is not a physiological regulator of Th2

function. As discussed in Chapter one, attempts to treat allergic inflammation with Th1-type of cytokines, like IL-12 (Bryan *et al.* 2000) and IFN-γ, have failed to confirm a clinically relevant cross-regulatory properties of Th1 response (Rook *et al.* 2004).

Furthermore, the observation that heavy loads of helminths, which are essentially Th2 inducing, are also correlated with reduced allergic symptoms (Yazdanbakhsh *et al.* 2002; van den Biggelaar *et al.* 2004) questions the validity of Th2/Th1 paradigm (Rook *et al.* 2004). This effect seems associated with production of IL-10 (Yazdanbakhsh *et al.* 2002). A more direct association between helminth infections and atopy was demonstrated in a randomised, controlled trial on Gabonese schoolchildren with a high prevalence of intestinal helminth infestation. In this study, anti-helminthic treatment of chronically infected children resulted in increased atopic reactivity to *Dermatophadoides spp.* (van den Biggelaar *et al.* 2004).

Indeed, there is now evidence to suggest that both allergic and autoimmune diseases represent the result of a defective regulation of T-cell response leading to exaggerated immune activation and disease development. With regard to the airway mucosal system, the mechanisms by which respiratory antigens induce T cell tolerance include T cell clonal deletion, anergy or active suppression mediated by regulatory cells secreting IL-10 or transforming growth factor-β (TGF-β) (Umetsu *et al.* 2002). The major route by which foreign antigens gain access to the body is via mucosal surfaces, such as the intestinal and the respiratory tract. The prevention of a destructive effector T cell response to foreign antigens in mice is dependent on the activity of regulatory T cells (Treg) which are $CD4^{+}CD45RB^{low}$ (Mason *et al.* 1998). Regulatory T cells are a subset of CD4 T cells (approximately 5-10% in normal individuals), and are able to produce IL-10 and TGF-β both *in vivo* and *in vitro*, in mice as well as in humans (Mason *et al.* 1998).

The development of respiratory tolerance is initiated by uptake of antigen in the lungs by immature dendritic cells (DC): pulmonary DC migrate to the draining bronchial lymph nodes where they mature, transiently produce IL-10, and express high amounts of B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules.

Studies in mice show that pulmonary DC from mice exposed to respiratory antigen produced IL-10 and were able to stimulate the development of Treg that, in turn, also produced high amounts of IL-10. In addition, adoptive transfer of pulmonary DC from IL-$10^{+/+}$, but not IL-$10^{-/-}$, mice exposed to respiratory antigen induced antigen-specific unresponsiveness in recipient mice. These studies show that IL-10 production by DC is critical for the induction of tolerance, and that phenotypically mature pulmonary DC mediate tolerance induced by respiratory exposure to antigen as IL-10 monoclonal antibody (mAb) blocks this process (Akbari *et al.* 2001).

Commensal bacteria and bacterial products that colonise the gastro intestinal tract during infancy provide a major stimulus for postnatal regulation and maturation of Th cell function and development of Treg cells (Holt *et al.* 1997). Since Treg cells control immune reactions to commensal and pathogenic bacteria, the establishment of these tolerance mechanisms at mucosal surfaces and the delopment of Treg cells may require the presence of commensal bacteria in the gastrointestinal and upper respiratory tracts (Sudo *et al.* 1997; Umetsu *et al.* 2002; Caramalho *et al.* 2003). This is demonstrated by the observation that immune tolerance induced by mucosal exposure to antigen does not seem to occur in newborn animals maintained in an germ-free environment (Sudo *et al.* 1997; Umetsu *et al.* 2002), which indicates that normal microbial flora in the intestine, and perhaps in the upper respiratory tract, have significant effects on tolerance to mucosally derived allergens (Kohashi *et al.* 1979 ; Sudo *et al.* 1997). Commensal bacteria may enhance production of IL-10 and TGF-β

through mechanisms that involve the innate immune system, downregulating the Th2 response to allergens. It has been observed that monocytes from patients with severe asthma show reduced IL-10 release after stimulation with Lipopolysaccharide (LPS) and significantly less positive staining for IL-10 (Tomita *et al.* 2002), and it seems also that asthmatic patients can carry an IL-10 gene haplotype associated with low IL-10 production (Lim *et al.* 1998) or a polymorphisms of IL-10 and TGF-β1 and TGF-β2 that are associated with elevated IgE production (Hobbs *et al.* 1998). Similarly, children with atopic dermatitis had a high odds ratio of having a low TGF-β1 producer genotype compared with control subjects (Arkwright *et al.* 2001).

In view of the relationship between IL-10, TGF-β and immunoregulation, it may also be relevant that polymorphisms of cytotoxic T-lymphocyte antigen-4 (CTLA-4) have been linked to the severity of asthma and BHR (Lee *et al.* 2002). The genotypes of the CTLA-4 promoter polymorphism was shown to be associated with asthma severity, but not with asthma, atopy, or BHR. (Lee *et al.* 2002). This molecule plays a critical role in the function of Treg cells.

Recently a novel explanatory model for the hygiene hypothesis has been proposed. According to this model mycobacterial infections could inhibit Th2 responses by stimulating the expansion of Treg cells and the consequent production of IL-10 and TGF-β (Zuany-Amorim *et al.* 2002; Zuany-Amorim *et al.* 2002). IL-10 is generally considered primarily anti-inflammatory. It is produced by macrophages and T cells during *M. tuberculosis* infection, possesses macrophage-deactivating properties, including downregulation of IL-12 production, which in turn decreases IFN-γ production by T cells. Macrophages from tuberculosis patients suppress T cell proliferation *in vitro*, and inhibition of IL-10 partially reversed this

suppression (Gong *et al.* 1996). IL-10 directly inhibits $CD4^+$ T cell responses, as well as inhibiting APC function of cells infected with mycobacteria (Rojas *et al.* 1999).

Transforming Growth Factor-β (TGF-β) has also been implicated in the suppression of T cell responses in tuberculosis patients (Hirsch *et al.* 1997; Flynn *et al.* 2001). TGF-β is present in the granulomatous lesions of tuberculosis patients and is produced by human monocytes after stimulation with *M. tuberculosis* or lipoarabinomannan (Toossi *et al.* 1995; Dahl *et al.* 1996; Flynn *et al.* 2001). It appears to inhibit T cell responses to *M. tuberculosis* as well as participating in macrophage deactivation (Hirsch *et al.* 1997; Flynn *et al.* 2001).

TGF-β is also able to promote conversion of naïve peripheral $CD4^+CD25^-$ T cells into anergic/suppressor cells that are $CD25^+CD45RB^{-/low}$ and intracellular CTLA-4^+ (Chen *et al.* 2003). These converted anergic/suppressor cells are unresponsive to TcR stimulation, express TGF-β, and inhibit antigen-specific $CD4^+$ T cell expansion *in vivo*. In a murine asthma model, the co-administration of these TGF-β-induced suppressor T cells prevented *Der p*-induced allergic pathogenesis in lungs (Chen *et al.* 2003).

The factors responsible for the generation of Treg cells from naïve CD4 cells have not yet been fully characterised. However, recent analyses of genetic disorders causing a global immune dysregulation of both Th2 and Th1 immunity, that results in severe atopy associated with autoimmune diseases, have identified the forkhead/winged helix transcription factor *Foxp3* (reviewed in (Hori *et al.* 2004)) as a crucial factor for the generation of Treg cells. TGF-β induces *Foxp3* gene expression in TcR-stimulated $CD4^+CD25^-$ naïve T cells, and *Foxp3*, in turn, mediates the transition of $CD4^+CD25^-$ cells toward a regulatory T cell phenotype (Chen *et al.* 2003).

Foxp3 expressed by Treg cells is fundamental to their development and function (Fontenot *et al.* 2003; Hori *et al.* 2003). *Foxp3* deficient mice lack Treg cells and the disease phenotype can be prevented by the transfer of $CD4^+$ $CD25^+$ Treg cells.

In human allergic disorders, Treg cells are present and functional and are able to inhibit Th1 as well as Th2 cytokine production (Bellinghausen *et al.* 2003). Furthermore, grass pollen immunotherapy has been shown to result in a population of circulating T cells that express the IL-$10^+CD4^+CD25^+$ phenotype in response to allergen restimulation (Francis *et al.* 2003). Similarly, IL-10 and TGF-β suppress the immune response to aeroallergens and control allergic inflammation caused by mucosal allergen in healthy individuals as well as in patients receiving specific immunotherapy (Jutel *et al.* 2003).

A recent study using PBMC of healthy and allergic individuals has shown that Treg cells represent the dominant subset specific for common environmental allergens in healthy individuals; in contrast, there is a high frequency of allergen-specific IL-4–secreting T cells in allergic individuals, indicating that a change in the dominant T-cell subset – frequency of allergen-specific Treg over Th2 -may lead to allergy development or vice versa in recovery (Akdis *et al.* 2004). Treg cells are able to exert both an allergen-specific and bystander suppression of T cell proliferation in response to allergen stimulation *in vitro*. Akdis et al, speculate that, depending on their frequency, the first T cell that contacts the APC may be very critical in the subsequent decision to stimulate or suppress the specific immune response. If the first T cell to contact the APC is a Treg cell, it may silence or regulate the maturation of APC through a variety of mechanisms that include the generation of IL-10, TGF-β or the engagement of inhibitory co-stimulatory molecules like CTLA-4 and PD-1 (Akdis *et al.* 2004).

There is now evidence to suggest that the bacteria involved in the 'hygiene hypothesis' are not those that cause infectious diseases but the harmless commensal species present in the environment throughout human evolution and are now recognised by the innate immune system avoiding triggering a destructive responses. In an elegant review Rook *et al*, (Rook *et al.* 2003) ask why should diminished exposure to microorganisms result in the inadequate priming of Treg cells. They argue that after the effector response has reduced pathogen numbers to low levels, Treg cells may help to terminate the response. Moreover, Treg cells may stop their complete elimination, and so maintain a constant antigen source and immune stimulus, resulting in concomitant immunity to re-infection (Belkaid *et al.* 2002; Rook *et al.* 2003). The organisms most likely to have a highly developed ability to drive Treg cells are harmless 'old friends' and commensals that have been present throughout our evolutionary history, particularly Lactobacilli (Kalliomaki *et al.* 2003), saprophytic environmental mycobacteria (Rook *et al.* 1998; Rook *et al.* 2003), the helminths (Yazdanbakhsh *et al.* 2002; Rook *et al.* 2003) and bacterial DNA.

1.4 Bacterial products and Atopic diseases

Although the currently available treatments for controlling the symptoms of asthma are effective, none of these therapies can cure established asthma. Furthermore, corticosteroids lose their beneficial effects soon after they are discontinued and theoretically, owing to their unselective immunosuppression, this could lead to inhibition of lymphocytes with immunoregulatory properties such as Treg cells with the effect of preventing the development of tolerance to allergens.

The hypothesis that the significant increase in asthma is due to a lack of tolerance to environmental allergens, leads to the speculation that treatments potentially able to induce tolerance to these agents could represent effective treatments for allergic asthma.

Therefore, the use of molecules that cause inhibition of the immune response by activating tolerogenic or regulatory mechanisms could lead to a more effective and possibly more long-lasting protection against the Th2-mediated inflammatory responses to allergens.

1.4.1 Immunostimulatory sequences with CpG motifs

Bacterial DNA (but not mammalian DNA) is known to be mitogenic for mammalian lymphocytes and is an important inducer of IFN-γ (Yamamoto *et al.* 1992). Bacterial DNA has a higher frequency of unmethylated 'CpG motifs' (5'-pur-pur-CpG-pyr-pyr-3') containing oligodeoxynucleotides (ODN) (Krieg *et al.* 1995), while vertebrate and plant DNA has a much lower frequency (Bird 1986). These sequences stimulate elements of anti-bacterial innate immunity, such as antigen-presenting dendritic cells and NK cells that produce IL-12, IFN-α/γ, TNF-α and IL-18 to stimulate a Th1 response (Askenase 2000). In a murine model of asthma, the co-administration of ODN containing the CpG motifs with allergen prevented airway eosinophilia, Th2 cytokine production, IgE production and BHR (Lipford *et al.* 1997; Kline *et al.* 1998; Broide *et al.* 1999). In humans, CpG DNA may potentially suppress Th2 responses, including IgE to allergen, via IFN-γ or IL-10 or via CD8 cells (Cho *et al.* 2000; Horner *et al.* 2000; Marshall *et al.* 2000)

A comparison of the effects of steroids and CpG motif-containing immunostimulatory sequences (ISS) in a mouse model showed that both treatments inhibited IL-5 production, but only ISS treatment induced IFN-γ production, suggesting that only ISS treatment resulted in

immunomodulation (Broide *et al.* 1998). Synthetic ISS, where the immunostimulatory DNA was conjugated to a specific ragweed allergen (*Amb a 1* protein) was tested for immunotherapy in mice. This led to a Th1 biased response to *Amb a 1*, with production of IFN-γ dependent IgG2a and IgG2b antibody isotypes and inhibition of IgE production (Tighe *et al.* 2000). ISS-ODN also led to a significant increase of IFN-γ production by NK cells through an IL-12-dependent mechanism and increased mRNA expression of IL-12 and IL-18 in PBMC and monocyte-derived dendritic cells both in atopic and non-atopic individuals. In PBMC from atopic patients stimulation with ISS-ODN led to IgE inhibition. ISS-ODN induced IL-12, IFN-α, IFN-γ, IL-10 and IL-6 production from hPBMCs from both non-atopic and atopic donors, and increased expression of IFN-γ receptor and decreased expression of IL-4 receptor on B cells from both atopic and non-atopic donors (Horner *et al.* 2001; Matricardi *et al.* 2003). Furthermore, ISS-ODN inhibited IL-4-dependent IgE production *in vitro*. Neutralisation of IL-12, IFN-α, IFN-γ and IL-10 attenuated the inhibitory activity of ISS-ODN on IgE production (Horner *et al.* 2001; Matricardi *et al.* 2003). Although immunomodulation is the primary aim in the treatment of allergic diseases, achieving down-regulation of Th2 cells by means of inducing a strong Th1 immunoresponse may not be the desirable long-term solution and, as previously discussed, it could facilitate the development of autoimmune diseases.

1.5 Immune response to Mycobacteria

Mycobacteria can be classified into slow-growing pathogenic species (e.g. *M. tuberculosis* and *Mycobacterium bovis*) and fast-growing species that are generally non pathogenic (e.g. *Mycobacterium smegmatis* and *Mycobacterium vaccae*) and can be isolated from environmental sources, such as soil and water (van Crevel *et al.* 2002).

It has been suggested that the exposure to environmental non-pathogenic mycobacteria could influence the course of several infectious, inflammatory and allergic diseases (Rook *et al.* 1998). However, the precise mechanisms underlying the effects of environmental mycobacteria on the immune response have not yet been fully elucidated.

Several steps in the interaction between the host immune system and mycobacteria could be responsible for the postulated preventive effect on inflammatory diseases.

A better understanding of the mechanisms linking the immunity to microbial components and allergic response is required in order to elucidate the molecular basis of the 'hygiene hypothesis' and for the identification and development of novel treatment for allergic and other inflammatory diseases.

1.5.1.1 Mycobacterial cell-wall components: lipoarabinimannan and related lipoglycans

Most of the mycobacterial lipid antigens are constituents of the bacterial cell wall and are believed to play important roles in the physiology and virulence of the bacterium as well as in the modulation of the host immune response. The most immunogenic mycobacterial lipoglycans are lipoarabinomannan (LAM), lipomannan (LM) and phosphatidyl-*myo*-inosito mannosides (PIM) that are found ubiquitously in the envelopes of all mycobacterial species (Briken *et al.* 2004). PIM, LM and LAM all share a conserved mannosyl-phosphatidyl-myo-inositol (MPI) that is presumably used to insert these structures into the plasma membrane (Briken *et al.* 2004). It is still unclear which lipids are more frequently immunogenic, the relative importance of the lipid antigens shed by living bacteria compared to those that are covalently attached to the cell wall, and the difference between pathogenic and non-

pathogenic species in the induction of a protective immune-response and induction of tolerance.

The best-studied mycobacterial component is the cell-wall glycolypid lipoarabinomannan (LAM), which can be classified into three major structural families according to the capping motifs present on the non-reducing termini of the arabinosyl side-chains. The arabinan termini in the pathogenic strains are modified with mannosides caps (Nigou *et al.* 1997; Khoo *et al.* 2001; Guerardel *et al.* 2003; Briken *et al.* 2004), resulting in molecules designated ManLAM. In the fast-growing non-pathogenic species branches of the terminal arabinan are terminated by inositol phosphate caps (Briken *et al.* 2004), characterising the PILAM family. A third LAM family, designated AraLAM, recently identified in *M. chelonae*, comprises a LAM molecule devoid of both the manno-oligosaccharide and inositol phosphate caps (Guerardel *et al.* 2002; Briken *et al.* 2004; Koul *et al.* 2004; Quesniaux *et al.* 2004). PILAMs activate macrophages in a TLR2-dependent manner by activating the NF-κB signalling pathway whereas the anti-inflammatory effects of ManLAMs have been attributed to their binding to the mannose receptor or to DC-SIGN (Nigou *et al.* 2001; Geijtenbeek *et al.* 2003; Maeda *et al.* 2003; Quesniaux *et al.* 2004). This suggests an interesting structure-function relationship of LAM, with PILAMs inducing TNF-α and IL-12, and ManLAMs inducing IL-10 and inhibit IL-12 and TNF-α synthesis (Nigou *et al.* 2001; Geijtenbeek *et al.* 2003; Maeda *et al.* 2003; Quesniaux *et al.* 2004). LMs, the biosynthetic precursors of LAMs, can activate macrophages, induce expression of co-stimulatory molecules such as CD40 and CD86 and cytokine synthesis such as TNF-α, IL-8 or IL-12 (Quesniaux *et al.* 2004).

The regulation of the arabinosyltransferases determining the balance between PIM, LM and LAM synthesis by mycobacteria might determine a variety of immunomodulatory signals and the outcome of innate immunity against mycobacteria (Nigou *et al.* 1997; Quesniaux *et al.*

2004). Differences in the structural organisation of the cell wall and the LAM/LM ratio between pathogenetic and saprophytic mycobacteria may lead to different immune responses which involve different receptors and variable interaction with TLR2 (Briken *et al.* 2004).

1.5.1.2 Receptors for pathogen recognition

Recognition of mycobacteria starts with their interaction with APC receptors for conserved molecular structures on bacterial pathogens ("pathogen-associated microbial patterns", PAMP) termed "pattern recognition receptors" (PRR) (Janeway 1992). PPR include different receptors, belonging to multiple structural families which either bind to non-opsonized mycobacteria (macrophage mannose receptor (MR), which recognises terminal mannose residues on mycobacteria) or recognise opsonins on the surface of mycobacteria through complement receptor 1 (CR1), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) (van Crevel *et al.* 2002). The main PRR for mycobateria are the following:

- The Toll-like receptors (TLR), which can recognise glycolipds, lipoproteins, heat-shock proteins and oligodeoxynucleotides (Cook *et al.* 2003);
- C-type lectin receptors (CLR), that contain carbohydrates-recognition domains (CRD) with specificity for sugars expressed on the bacterial surface. CLR family comprise several receptors, including DC-SIGN (DC-specific intercellular adhesion molecule-3 grabbing nonintegrin), (Cambi *et al.* 2005) and mannose receptors, expressed on mature macrophages. Both receptors bind LAM, mediate phagocytosis of whole bacteria, and deliver LAM to endocytic compartments that contain CD1b, thereby facilitating presentation of mycolic acid and lipoglycan antigens to CD4- CD8-T cells or CD8+ T cells (Prigozy *et al.* 1997; Ernst 1998; Geijtenbeek *et al.* 2003). DC-SIGN is frequently co-expressed with mannose receptors, binds mainly to mannose capped

lipoarabinomannans (ManLAM) and seems to be induced by Th2 cytokines such as IL-4. The relationship between IL-4 and the expression of DC-SIGN, may alter the balance between DC-SIGN and TLRs and, ultimately, influence the Th1/Th2 effector phenotype in allergic (prevalence of DC-SIGN signalling) and non allergic (prevalence of TLR) environment.

- CD40 and CD44 (Kaufmann 2003; Quesniaux *et al.* 2004). CD40 is upregulated by mycobacteria and can bind mycobacterial Heat-Shock Proteins (hsp70) (Henderson *et al.* 1997; Lazarevic *et al.* 2003). Soluble CD44 and macrophage-bound CD44 binds to mycobacteria and is important in macrophage recruitment and mycobacterial phagocytosis (Lazarevic *et al.* 2003; Leemans *et al.* 2003).
- Nucleotide-binding oligomerisation domain (NOD) proteins which recognise intracellular pathogen ligands such as peptidoclycans, muramyl dipeptides and diaminopimelate-containing N-acetylglucosamine-N-acetylmuramic acid (GlcNAc-MurNAc) tripeptide (Chamaillard *et al.* 2003; Inohara *et al.* 2003; Quesniaux *et al.* 2004);
- CD14, a phosphatidylinositol glycan-linked membrane protein, can also bind LAM in association with TLR2.

1.5.1.3 Toll-Like Receptors

Cells of the innate immune system can recognise mycobacteria antigens, such as lipoproteins and lipoglycans, through the family of TLRs.

Stimulation via TLR on APC results in rapid activation of innate host defense mechanisms such as secretion of cytokines and production of reactive nitrogen intermediates (RNI).

Several studies have described the interaction of mycobacteria secreted antigens and TLRs (Krutzik *et al.* 2004).

Mycobacterial components are recognised by TLR2, TLR4 and TLR1/TLR6 that heterodimerise with TLR2 (Aliprantis *et al.* 1999; Tsuji *et al.* 2000; Quesniaux *et al.* 2004). Some TLR proteins have been shown to utilise co-receptors that augment TLR-dependent responses and, in some cases, are required for TLR function. For example, both CD14 and the β2 integrin CD11b/CD18 have been shown to augment responsiveness to the TLR4 and also to TLR2 mediated by AraLAM (Heldwein *et al.* 2002).

TLR2 and TLR-4-dependent cell activation seems to be mediated by LAM, LM (lipomannan), phosphatidyl-myo-inositol mannoside (PIM), or the 19-kDa mycobacterial lipoprotein [Reviewed in (Briken *et al.* 2004; Quesniaux *et al.* 2004)]. Different mycobacteria vary in their ability to stimulate TLR4, and although both TLR2 and TLR4 can participate in the activation of macrophages by intact bacteria, the relative contributions of these TLR proteins to cellular responses remains to be determined.

Mycobacterial lipoproteins exist in a diacylated and triacylated form. TLR6/ TLR2 heterodimers recognises diacylated lipopeptides along with TLR2 (Krutzik *et al.* 2004) and TLR1/TLR2 recognises the lipid configuration of the native mycobacterial lipoprotein as well as triacylated lipopeptides (Takeuchi *et al.* 2002).

It seems plausible that heterodimerisation of different TLRs may allow for recognition of different ligands and activation of distinct gene programs based on the specifc composition of the cells wall of different mycobacterial species (Ozinsky *et al.* 2000; Bulut *et al.* 2001). Furthermore, the presence of different mycobacterial antigens such as heat-shock proteins -

which interact with TLR4 - and bacterial DNA may activate different TLR such as TLR9, not otherwise recognised by cell-wall antigens (Cook *et al.* 2003).

Studies have shown that mycobacteria-mediated TLR2 signaling can cause NF-kB-dependent IL-12 synthesis, with consequent generation of mature Dendritic Cells able to stimulate Th1-type immune response, but also can induce the secretion of anti-inflammatory cytokine IL-10 (Jang *et al.* 2004). Consistent with its anti-inflammatory properties, infected mice lacking TLR2 succumb to mycobacterial infections probably because of an exaggerated inflammatory (van Crevel *et al.* 2002; Drennan *et al.* 2004; Jang *et al.* 2004). While both TLR2 and TLR4 signalling induces IL-12p40 expression, only TLR4 stimulation leads to a complete IL-12p70 synthesis by DCs. On the contrary, TLR2 activation leads to the induction of IL-12p40 homodimers (in the absence of IL-12p35) that can act as IL-12 antagonists (Germann *et al.* 1995; Heldwein *et al.* 2002). Furthermore, DC activation via TLR2, but not via TLR4, induces the expression of p19, which forms heterodimers with IL-12p40 to form IL-23. Although IL-12 and IL-23 have structural similarities, the effects of IL-23 seem less dependent on IFN-γ and are related to both the upregulation of pro-inflammatory cytokine IL-17 and the up-regulation of IL-10 (Happel *et al.* 2005; Happel *et al.* 2005) possibly due to proliferation of regulatory T cells (Oppmann *et al.* 2000). These differences could be secondary to the ability of TLR4, but not TLR2, to activate IFN-regulatory factors (IRF 1,2,3) and induce the synthesis of IFN-β (Sabroe *et al.* 2003). There is also evidence that TLRs can desensitise their own signalling, and also that TLR4 and TLR2 can heterologously desensitise each other (Dobrovolskaia *et al.* 2003). Suppressor of cytokine signaling 1 (SOCS-1), a member of the suppressor of cytokine signalling family that suppresses the JAK-STAT signalling cascade activated by proteins such as IFN-γ, also reduces NF-kB activation by LPS

(as TLR4 agonist) and appears to be involved in TLR4 homologous desensitisation [reviewed in (Sabroe *et al.* 2003)].

Finally, TLR2 and TLR4 induce a different set of chemokines with TLR2 favouring the induction of IL-8 expression, whereas TLR4 induces RANTES expression (Re *et al.* 2001; Heldwein *et al.* 2002).

The differential effect on TLR stimulation and ultimately cytokine synthesis seems to be dependent on the virulence of the microorganism, the lipid composition of the cell-wall membrane and the cytokine environment in which antigen presentation takes place.

1.5.1.4 Cross-regulation between C-lectins and Toll-Like receptors

As discussed, the ability of mycobacteria to induce a Th1 response is related to the stimulation of TLR2 and TLR4. C-lectins receptors can counter-regulate TLR-mediated Th1 signals. The best studied molecule is DC-SIGN. DC-SIGN binds strongly to mannose-capped cell-wall component ManLAM (van Kooyk *et al.* 2003), but does not bind to LAM without the mannose cap (AraLAM). This is intriguing because ManLAM is abundant in slow growing virulent mycobacteria, such as *M. tuberculosis* and *M. leprae*, whereas AraLAM is abundant in fast growing atypical, non-virulent mycobacteria, such as *M. smegmatis*, *M. fortuitum* and *M. chelonae* (van Kooyk *et al.* 2003).

The interaction between DC-SIGN and its ligand ManLAM – either membrane bound or secreted - can alter the immune response antagonising the effect of TLRs (Nigou *et al.* 2001; Geijtenbeek *et al.* 2003) and blocking the TLR4-dependent IL-12 secretion (Nigou *et al.* 2001). This can occur in an antigen-specific manner, but can also interfere with the immune function of bystander DCs.

Studies on *M. bovis* BCG (Geijtenbeek *et al.* 2003) have shown that *M. bovis* BCG induce the maturation of DCs and IL-12 synthesis, probably through TLR2 and TLR4 signalling (Tsuji *et al.* 2000). ManLAM specifically blocked the *M. bovis* BCG-induced maturation of DCs and IL-12 synthesis and these effects were dependent on DC-SIGN and mediated by IL-10 secretion (Geijtenbeek *et al.* 2003). The inhibition of DC maturation and the induction of IL-10 might have an important effect on establishing a state of immune tolerance and create the optimal environment for the generation of regulatory cells (Jonuleit *et al.* 2000). The balance between immune activation and immune suppression therefore becomes tightly regulated by the level of TLR activation and the occupancy of C-type lectins.

It is possible that other C-type lectins can recognise non-pathogenic mycobacteria alone or in association with DC-SIGN, regulating DC-induced T-cell responses either to Th1 or Th2 type.

1.5.1.5 T-Cell Subpopulations

Response to mycobacteria involves different T-cell subpopulations (Kaufmann 2003) [reviewed in (Stenger *et al.* 1999)]. These comprise the following:

1. Classical T-lymphocytes expressing the αβ T-cell receptor (TCR), both CD4 and CD8 cells, respectively recognising antigens in the contest of MHC class II, and MHC class I molecules. The effects on the immune response to mycobacteria are mediated by effector cytokines, particularly IFN-γ, and by contact-mediated cytolitic activity which has been demonstrated for both CD8+ and CD4+ cells (Yoneda *et al.* 1998). CTL activity seems dependent on both the Fas-Fas ligand (Fas-L) pathway and the granule exocytosis pathway of cytotoxicity (Lewinsohn *et al.* 1998; Oddo *et al.* 1998). However, as will be described below,

CD4+ cells may acquire a regulatory phenotype and secrete IL-10 and TGF-β, resulting in an attenuated inflammatory response with further expansion of natural Treg cells, which in turn secrete more IL-10 and TGF-β.

2. CD8 αβT cells that recognise bacterial peptides presented by nonclassical non-polymorphic MHC class Ib molecules (Stenger *et al.* 2002).

3.Double-negative (DN), (CD4-, CD8-) αβT cells that recognise bacterial glycolipids presented by the non-classic MHC class I-like CD1 molecules (CD1-restricted). Although the CD1 polypeptides present some homologies with MHC class I or Ib molecules, they are encoded outside of the MHC (Porcelli *et al.* 1999) and this characteristic defines CD1 polypeptides as a distinct family of nonpolymorphic antigen-presenting molecules. In humans, four major types of CD1 molecules can be distinguished (CD1a to CD1d). CD1a, b and c are primarily expressed on conventional antigen-presenting cells, are capable of presenting mycobacterial glycolipids to T cells and also to unconventional αβT cells (Porcelli *et al.* 1999). Moreover, mycobacterial glycolipids have been identified in apoptotic vesicles, where they could be transferred from infected macrophages to bystander DC and subsequently presented to T cells (Schaible *et al.* 2000)).CD1-restricted T cells play a major role in the early recognition of mycobacteria, maturation and instruction of DC towards a type-1 phenotype (Vincent *et al.* 2002; Rook *et al.* 2003). These cells also have both effector and cytotoxic functions. However they seem to rely entirely on a Fas-FasL pathway with no effect on the viability of mycobacteria. This mechanism can contribute to the creation of peripheral tolerance and down regulation of the inflammatory response (Stenger *et al.* 2002).

4.CD4 or DN αβ T cells that generally co-express the NK cell marker and are specific for group 2 CD1 molecules

5.γδ T-lymphocytes cells recognising unusual antigenic ligands independently of specialised presentation molecules. Antigen-specific γδ T play a role in the early response to mycobacteria and they are able to mount both an innate and adaptive immune response (Chen 2005). γδ T cells from the peripheral blood of healthy individuals are strongly reactive to mycobacterial components *in vitro*. The sole mycobacterial antigens recognised by γδ T cells are small-molecular-weight nonproteinaceous phosphorilated molecules (Chen 2005). These phospholigands directly bind and stimulate the major subset of γδ T cells in humans expressing the Vγ2Vδ 2 TCR (Kaufmann 2003) which constitute 60-95% of the total circulating pool of γδ T cells. Increased numbers of γδ T cells have been frequently identified at sites of inflammation and are expanded during mycobacterial infections. The degree of expansion of γδ T cells seems dependent on the virulence, dose and route of infection (Chen 2005). Systemic infections achieve the greatest clonal expansion, whereas pulmonary exposure induce a lower expansion which is detectable in the lung but not in the circulation (Chen 2005). γδ T cells that have undergone clonal expansion after mycobacterial infection show a memory phenotype $CD45RA^{-}CD27^{+}$. Because γδ T cells are activated prior to γδ T cells, they could fill a gap between early, nonspecific resistance mediated by the innate immune system and the later highly specific acquired immune responses mediated by γδ T cells. γδ T cells play also an important role in down-regulating the degree of inflammation (and in so doing possibly contribute to more chronic infections/immunity) by up-regulating Fas and enhancing apoptosis mediated by Fas-FasL (Li *et al.* 1998; Manfredi *et al.* 1998).

1.5.1.6 Cytokines

A variety of cytokines, including TNF-α, IL-1β, IL-6, IL-12, IL-15, IL18, IL-23, are generated following mycobacterial infection.

IL-12 is a key player in host defense against *M. tuberculosis*. IL-12 is produced mainly by phagocytic cells and has a crucial role in the induction of IFN-γ production together with IL-18 and IL-15. IL-15 resembles IL-2 in its biologic activities, stimulating T-cell and NK-cell proliferation and activation (van Crevel *et al.* 2002). Unlike IL-2, however, IL-15 is primarily synthesised by monocytes and macrophages.

Mycobacteria can induce secretion of IL-10 and transforming growth factor beta (TGF-β) with anti-inflammatory properties. IL-10 is produced by macrophages and T cells after phagocytosis of *M. tuberculosis* (Shaw *et al.* 2000) and after the binding of mycobacterial LAM (Dahl *et al.* 1996), IL-10 down-regulates MHC class I, MHC class II, CD40, B7-1, and B7-2 expression (Rojas *et al.* 1999)

T lymphocytes, including *M. tuberculosis*-reactive T cells, are also capable of producing IL-10 (Gerosa *et al.* 1999; Boussiotis *et al.* 2000; van Crevel *et al.* 2002).

Mycobacterial products induce production of TGF--β by monocytes and dendritic cells. Interestingly, LAM from virulent mycobacteria selectively induces TGF-β production. TGF-β and IL-10 seem to synergise: TGF-β selectively induces IL-10 production, and both cytokines show synergism in the suppression of IFNγ- production.

In addition to the TLR-2-dependent pathway, mycobacteria can induce IL-10 synthesis through the interaction between Man-LAM and the C-type lectin DC-SIGN. This blocks the maturation of DC and suppresses T-cell activation (Geijtenbeek *et al.* 2003; Tailleux *et al.* 2003; Koul *et al.* 2004). Interestingly BCG can cause IL-10 secretion even in the absence of

DC-SIGN implying that other receptors may also be involved in the process (Gagliardi *et al.* 2005)

Because of the ability of mycobacteria to powerfully induce a Th1 response and, depending on the species of mycobacteria or the conditions of the host, a regulatory T cell phenotype, mycobacteria and their components have been investigated as a pharmacological tool for the prevention or treatment of Th2-mediated diseases.

In the next section I will discuss the epidemiological links between immunity to mycobacteria and atopy and subsequently I will discuss the characteristics of two immunopreparations derived from a non-pathogenetic mycobacterium – *Mycobacterium vaccae* – which I have used in a clinical trial for the treatment of adult asthma. The results of the study are presented in Chapter three of this thesis.

1.6 Mycobacteria and Atopic diseases

Ecological analyses have repeatedly and consistently found an inverse association between the prevalence of tuberculosis (TB) in a given country and the prevalence of atopic disorders (von Mutius *et al.* 2000; Matricardi *et al.* 2003). One study evaluated retrospectively the occurrence of allergic disease among Finnish adults who had had TB in the first two decades of their life. A TB notification rate of 25 per 100,000 was associated with an absolute decrease in the prevalence of wheeze ever of 4.7% (von Mutius *et al.* 2000).

Recently, Shirakawa *et al.* (Shirakawa *et al.* 1997) demonstrated a strong inverse association between delayed hypersensitivity to *M. tuberculosis* antigen and atopic disorders (asthma and rhinitis) in Japanese school children vaccinated with *M. bovis* BCG. Positive tuberculin responses predicted a lower incidence of asthma, with cytokine profiles biased toward the Th1 type. Total IgE and Th2 responses to a range of environmental allergens are associated with

atopic disease (Pahari *et al.* 2002). Shirakawa *et al.* (Shirakawa *et al.* 1997) suggested that early exposure to mycobacterial antigens could skew this response towards a Th1 profile.

Mycobacterial infections evoke a strong Th1 response in the lung aimed at generating a Th1-driven granulomatous response. Human studies have shown that stimulation with mycobacteria increases IL-12 production (Munk *et al.* 1995; Munk *et al.* 1996). It is likely that infection with mycobacteria sets up an environment rich in IL-12 and IFN-γ. Antigen presentation in this cytokine environment is likely to drive the T-cell towards a Th1 differentiation. IL-12 is a crucial cytokine in controlling *M. tuberculosis* infection. Early administration of IL-12 to *M. tuberculosis*-infected BALB/c mice resulted in significantly decreased bacterial numbers and increased mean survival time (Flynn *et al.* 1995; Flynn *et al.* 2001). Convincing evidence of the importance of IL-12 in resistance to tuberculosis was provided by IL-12p40-gene deficient mice who are susceptible to infection and have a greatly increased bacterial burden, probably due to the substantially reduced IFN-γ production in IL-12p40$^{-/-}$ mice (Cooper *et al.* 1997). Humans with mutations in IL-12p40 or the IL-12 receptor genes present with reduced, but not absent, IFN-γ production from T cells, and are more susceptible to disseminated BCG and *M. avium* infections. IFN-γ is another key cytokine in controlling *M. tuberculosis* infection. To date, IFN-γ knockout mice are the most susceptible to *M. tuberculosis* (Cooper *et al.* 1993; Flynn *et al.* 1993). Individuals defective in genes for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections, including *M. tuberculosis* (Ottenhoff *et al.* 2000).

1.6.1 BCG and allergy

Exposure to pathogenic or environmental mycobacteria, has been associated with a reduced propensity to develop asthma and other allergic diseases (Rook *et al.* 1998).

Mice treated with BCG up to four weeks prior to ovalbumin challenge showed a significant reduction in pulmonary eosinophilia compared to control mice. Aerosolised administration of BCG inhibited a pulmonary Th2 cytokine response and airway eosinophilia (Erb *et al.* 1998). Similarly, BCG immunisation before ovalbumin sensitisation significantly inhibited the development of allergic responses (Herz *et al.* 1998).

Whether immunisation with the commonly used BCG vaccine is associated with protection against development of allergic disease is still a matter of debate. The first epidemiological study showed that within a population of BCG-vaccinated Japanese children, a positive delayed hypersensitivity to *Mycobacterium tuberculosis* reaction at six and 12 years of age predicted a lower incidence of asthma, lower serum IgE levels, and cytokine profiles biased toward Th1 type compared with children with negative tuberculin reactions (Shirakawa *et al.* 1997). A similar study in Guinea-Bissau reported that the prevalence of skin sensitisation to common airborne allergens was lower among Guinea-Bissau children vaccinated with BCG in infancy in comparison with those never vaccinated with BCG (40%; adjusted OR 0.19) (Aaby *et al.* 2000).

In contrast, several retrospective studies have failed to demonstrate a negative correlation between BCG vaccination (as indicated by tuberculin responses) during early childhood and the subsequent development of atopy or asthma (Table 1.6-1). A recent study from Norway did not find a significant relationship between a positive tuberculin reaction and atopy (Omenaas *et al.* 2000). This young adult population had their BCG vaccination at the age of 14, which may indicate that timing of immunization may play a role in the protective effect on

allergy. However, in another study, immunisation soon after birth with BCG vaccine, a common procedure in some countries, also did not appear to confer protection against the development of atopic disorders (Alm *et al.* 1997). A separate Swedish study also did not find any protection by BCG immunisation on asthma development and these findings compare with a study from Turkey where the authors did not find any relationship between tuberculin response and atopic status later in life in BCG immunised subjects (Yilmaz *et al.* 2000).

No inverse association between tuberculin responses and asthma, allergic diseases, atopic responses and total IgE levels has been reported in any of 11 studies (Alm *et al.* 1997; Strannegard *et al.* 1998; Aaby *et al.* 2000; Kroger *et al.* 2000; Omenaas *et al.* 2000; Yilmaz *et al.* 2000; Wong *et al.* 2001; Gruber *et al.* 2002; Jang *et al.* 2002; Ozmen *et al.* 2002; Pahari *et al.* 2002; Matricardi *et al.* 2003). Differences in study design, population examined, BCG preparations and, above all, the pattern of natural exposure to *M. tuberculosis* and mycobacteria other than tuberculosis (Rook *et al.* 1999) can partially explain failure to confirm previous findings. Studies that tested whether immunisation with BCG can protect from atopic diseases did not find a significant protective effect of BCG administered early in life against the development of asthma or allergy later in childhood (Alm *et al.* 1997; Strannegard *et al.* 1998; Gruber *et al.* 2002; Pahari *et al.* 2002; Krause *et al.* 2003; Matricardi *et al.* 2003). Ota *et al.* (Ota *et al.* 2003) reported on a population of 507 children (age 8-12) living in Gambia, 360 of whom bore evidence of a BCG scar. The prevalence of positive immediate skin test responses to locally relevant allergens and the serum levels of total IgE were not significantly different among children with or without a BCG scar (Ota *et al.* 2003).

The relevant implications of the revised immunological model for the issues treated here can be summarised as follows. First, according to this model, a healthy or an allergic outcome of the interaction between allergens and humans is not fixed early in life, but retains some

flexibility until adulthood. This represents a dynamic and quantitative process, which is extended to adulthood and is subjected to genetic factors that regulate how early and intense exposure to infections must be to afford permanent protection from atopic sensitisation and its inflammatory consequences (Matricardi *et al.* 2003). Secondly, a single intradermal administration of BCG, even if very early in life is not able to mimic the natural exposure to environmental mycobacteria and the persistent immune stimulation provided by an intense and protracted natural exposure to pathogenic and non-pathogenic mycobacteria to be able to prevent the development of atopy.

Finally, as T regulatory cytokines, rather than Th1 cytokines, seem to protect from allergy, then the response to tuberculin would not be the best parameter to evaluate the putative allergy-protective effect of natural or artificial exposure to mycobacteria.

	Country	BCG	Age (yrs)	Wheezing /asthma	Allergic diseases	Atopy	Total IgE
Tuberculin response							
Shirakawa (1997)	Japan	867[a]	12-13	Inverse	Inverse	Inverse	Inverse
Stannegard (1998)	Sweden	6497[e]	4-9		None		
Yilmaz (2000)	Turkey	736[b]	2.5-16	None	None	None	None
Omenaas (2000)	Norway	574[e]	20-44			None	None
Kroger (2000)	Finland	201[a]	10-12		None		
Wong (2001)	Hong Kong	980[a]	10	None	None	None	
Gruber (2001)	Germany	774[d]	birth cohort	None	None	None	None
Jentolf (2002)	Norway	386[c]	20-44	None			
Pahari (2002)	UK	329	11-18	None			
Jang (2002)	Korea	486[c]	10-13	None		None	
Ozmen (2002)	Turkey	206[b]	1-14			Direct	
Ota (2003)	Gambia	507[†]	8-12			None	None
BCG immunisation							
Alm (1997)	Sweden	574[c]	3-7	None	None	None	
Stannegard (1998)	Sweden	6080[d]	4-9		None		
Aaby (2000)	Guinea-Bissau	400	3-14			Inverse	
Gruber (2001)	Germany	774[d]	birth cohort	None	None	None	None
Pahari (2002)	UK	329	11-18	None	None		None
Gruber (2002)	Germany	38808[a]	6	Inverse	None		
Krause (2003)	Greenland	1575[a]	8-16			None	
Ota (2003)	Gambia	507[†]	8-12			None	None
Tuberculosis							
Von Hertzen (1999)	Finland	2324[¶]	Adults				
Von Mutius (2000)	23 countries	235477	13-14	Inverse	Inverse		
Shirtcliffe (2002)	38 countries		6-7	Inverse	Inverse		
Shirtcliffe (2002)	55 countries		13-14	Inverse			

TABLE 1.6-1: *Studies on the relation among tuberculin response, BCG immunisation, asthma and atopy. Age at first BCG immunization: a=neonatal period; b=2months; c<6months; d<12 months; e: at 14 years; ¶ With tuberculosis; † Evidence of BCG scar. (From (Matricardi et al. 2003), adapted)*

1.7 GENES AND THE HYGIENE HYPOTHESIS

Several molecules shown to play a crucial role in the interaction with mycobacterial antigens (including toll-like receptors, Nramp1, CD14) are also relevant to asthma. In particular, several of these molecules have polymorphisms that relate to susceptibility both to mycobacterial disease and to allergic disorders (Rook *et al.* 2003).

1.7.1 Toll-like Receptors

The recent discovery of the importance of the 'toll like receptors' (TLR) protein family in immune responses has provided new insight into the link between innate and adaptive immunity. TLR serve as pattern-recognition receptors for a variety of microbe-derived molecules but also 'self' components (Rook *et al.* 2003). Extensive work has suggested that a number of endogenous molecules, such as heat shock proteins (hsp), may be potent activators of the innate immune system via the Toll-like receptors (TLR) signal-transduction pathways in a manner similar to LPS (via TLR4) and bacterial lipoproteins (via TLR2) (Vabulas *et al.* 2002; Tsan *et al.* 2004).

Ten TLR types are known, and these may act as homodimers or as heterodimers with other TLR. Moreover they are situated within complex 'rafts' involving CD14, CD11b-CD18, CD16a, CD32, CD36, CD64, hsp70, hsp90, CXCR-4 and growth-differentiation factor 5 (Rook *et al.* 2003). Signalling via TLR may depend on the composition of this 'cluster', the activation state of the cell, and the cell type.

Transfection of a constitutively active human mutant TLR results in the activation of NF-κB, as well as the expression of various NF-κB-controlled cytokines and the costimulatory molecule B7.1. The TLR-dependent activation of the NF-κB pathway is mediated by the adapter protein MyD88, which is also required for signal transduction through IL-1R (Hultmark 1994; Medzhitov *et al.* 1997; Medzhitov *et al.* 1998; Flynn *et al.* 2001).

TLR play an important role in the activation of immune cells by pathogens, including *M. tuberculosis*. Brightbill *et al.* (Brightbill *et al.* 1999) demonstrated that induction of IL-12 *in vitro* by the 19-kDa mycobacterial lipoprotein is dependent on human TLR2. However, it would appear that distinct mycobacterial components may interact with different members of

the TLR family such as TLR2 or TLR4 both in a CD14-dependent or independent manner (Means *et al.* 1999).

1.7.2 Natural resistance-associated macrophage protein-1-NRAMP1 (SLc11a1)

Natural resistance-associated macrophage protein-1 (Nramp1) is an integral membrane protein that functions as an ion transporter (Gunshin *et al.* 1997; Blackwell *et al.* 1999). Functional studies have shown that Nramp 1 plays an important role in macrophage function and activation with many pleiotropic effects on macrophage function, including regulation of the CXC chemokines, IL-1, inducible nitric oxide synthase, MHC class II molecules, tumour necrosis factor and nitric oxide release (Blackwell *et al.* 1999). Macrophages carrying the mutated infection-susceptible Nramp1 allele also have a defect in their ability to process antigen (Lang *et al.* 1997). This results in an *in vivo* bias towards development of a Th1 response in mice bearing the wild-type (infection resistant) allele, while a Th2 response is elicited in Nramp1 mutant (infection susceptible) mice (Kramnik *et al.* 1994; Soo *et al.* 1998; Blackwell *et al.* 1999).

Nramp1 variant alleles were found to influence susceptibility or disease type in various studies of leprosy and tuberculosis (reviewed in (Rook *et al.* 2003)). A functional Nramp1 polymorphism associated with tuberculosis correlated with higher lipopolysaccharide-induced production of IL-10, a cytokine associated with Treg cells activity (Awomoyi *et al.* 2002). Treatment of OVA-sensitized Nramp1s mice with *M. vaccae* suppressed airway hyperresponsiveness, airway eosinophilia, antigen-specific IgE, IL-4 and IL-5 production after ovalbumin aerosol challenge. In contrast, *M. vaccae* hardly affected these parameters in Nramp1r mice. In addition, the Nramp1 gene affected both T cell-mediated responses to *M.*

vaccae in vivo and the level of macrophage activation after stimulation with *M. vaccae in vitro* (Smit *et al.* 2003).

Stratification by BCG vaccination unmasked a potential genetic risk factor for atopy in the region of the Nramp1 locus (Alm *et al.* 2002). Moreover, inflammatory bowel diseases, multiple sclerosis and rheumatoid arthritis have been associated with various polymorphisms of this gene (Kojima *et al.* 2001; Kotze *et al.* 2001; Alm *et al.* 2002; Rodriguez *et al.* 2002). In a mouse model of allergy induced by immunization with ovalbumin in alum, Nramp1-resistent animals showed less release of Th2 cytokines, IgE and mast cell granules into the airways after aerosol challenge than did Nramp1-sensitive mice (Smit *et al.* 2003).

1.7.3 CD14

CD14 is expressed by myeloid cells, and secreted by these cells and by the liver. It is not a signalling molecule, but it plays a crucial role in binding many microbial components (notably lipopolysaccharide and liporabinomannan) and facilitating their interaction with cell membrane-associated signalling molecules such as TLR. *M. leprae* signals via TLR2, and this crucial interaction is enhanced by CD14 (Bochud *et al.* 2003). In some studies (Baldini *et al.* 1999) but not others (Sengler *et al.* 2003) a polymorphism in the promoter of CD14 has been related to the levels of circulating IgE. However, low CD14 in amniotic fluid or breast milk has been associated with an increased risk of atopic eczema and of allergic sensitisation (Jones *et al.* 2002). The blood cells of children of farmers, who have a reduced risk of allergies associated with their exposure to the farming environment, expressed higher levels of mRNA encoding CD14 (Jones *et al.* 2002).

1.7.4 T-cell Immunoglobulin Mucin 1(Tim-1)

As previously discussed, the TIM family, particularly *tim-1*, plays a critical role in immune responses that regulate the development of atopic diseases.

In humans, certain polymorphic variants of *tim-1* are strongly associated with protection against atopy, and this association occurs only in individuals who have had past infection with hepatitis A virus (HAV) (Umetsu *et al.* 2002; McIntire *et al.* 2004).

1.8 *MYCOBACTERIUM VACCAE*: CHARACTERISATION AND PROPERTIES

Mycobacteria are environmental saprophytes, present in soil, mud, water and dust. Since the exposure to these bacteria is dependent on lifestyle, the exposure to mycobacteria may be significantly reduced in Western societies. Mycobacterial infections usually have a very strong capacity to elicit Th1 responses (Huygen *et al.* 1992; Marchant *et al.* 1999). As a result, mycobacteria have attracted much attention in the contest of the 'hygiene hypothesis', as bacteria able to modulate the balance between Th1 and Th2 (Smit *et al.* 2004).

Some mycobacteria, such as BCG or its components have been utilised in humans as immunostimulators, but not as therapy against allergic diseases. The non-pathogenic mycobacterium, *M. vaccae*, has been proposed for the prevention and therapy of allergic diseases (Rook *et al.* 1998). *Mycobacterium vaccae* is one of about 60 named species within the genus *Mycobacterium* and is one of a number of rapidly growing, yellow pigmented mycobacteria that normally occur as environmental saprophytes. Natural exposure to environmental mycobacteria has been suggested as being able to regulate T-cell cytokine responses. It has, therefore, been hypothesised that administration of saprophytic

mycobacteria in childhood could prevent Th2 activation in response to common allergens and that this would result in the prevention and treatment of allergic disorders (Rook *et al.* 1998).

1.8.1.1 Characteristics of the strain

The final drug product is a sterile, heat-killed suspension of the non-pathogenic environmental saprophyte *Mycobacterium vaccae* NCTC 11659. The species *Mycobacterium vaccae* was described by Bonicke and Juhasz in 1964 (Boenickse *et al.* 1964) (SRPharma; SRPharma).

1.8.1.2 Isolation

Mycobacterium vaccae strain R877 was isolated from a sample of soil taken from around the roots of grass pulled from a swamp bordering the eastern bank of Lake Kyoga in the Sorote District of Uganda in 1971 (Stanford *et al.* 1973). The soil sample was decontaminated by a modification of the method of Kubica and inoculated on to Lowenstein-Jensen medium without added starch. The medium was incubated at 32° C and the colony was picked off after two weeks incubation. The strain is scotochromogen and the initial isolate of R877 produced mainly smooth colonies on culture media but occasional rough colonies were seen. On subculture, smooth colonies produced occasional rough colonies, whereas the rough coloniws were stable. One rough colony, designated R877R, was selected for subculture and deposisted in the National Collection of Type Cultures under the Budapest Convention in 1988. It bears the number NCTC 11659 (SRPharma; SRPharma).

1.8.1.3 Cultural studies and staining characterisics

Growth of NCTC 11659 is sustained on a range of simple bacteriological media, including Sauton's medium solidified with agar. This medium has the advantage of supporting the growth of most mycobacterial species but of relatively little else. Its most important characteristics is that it contains no antigenic substances and is therefore the basis of media used in the production of BCG vaccine. The optimum temperature range for growth 32-35 ^{o} C. Rapid visible growth commences the day after inoculation and continues for the first 2 weeks of incubation. On further incubation, the culture slowly becomes thicker and more rugose and the initial yellow colour slowly changes into orange-brown. Little further visible change occurs after 4 weeks of incubation and the culture is considered to be in the stationary phase. Eventually, after several months, the bacteria becomes difficult or impossible to subculture, the thickness of the bacterial mass diminishes and the colour fades to an off-white cream as the culture enters its involution phase. When stained by the Ziehl-Neelsen technique in the first few days of incubation, the bacilli appear as short, well stained rods with numerous weakly staining forms. During the later stage of rapid growth and in the early stationary phase almost all the bacteria are short, strongly stained rods. In involution, as is common in mycobacteria, the stained cytoplasm appears to be brocken into regular clumps an polymorphism increases with the appearance of many small round forms of unstained 'gosts' (SRPharma; SRPharma).

1.8.1.4 Culture identity

Bright yellow colonies with a dry, wrinkled appearance are evident on Löwenstein-Jensen medium and on Middlebrook and Sauton agar. Microscopically, the bacilli are acid fast . The

grwth is observed on Löwenstein-Jensen medium at 32 ° C, 37 ° C and 42 ° C but not at 45 ° C (SRPharma; SRPharma).

1.8.1.5 Utilisation of carbohydrates and organic acids as carbon sources

In the system of Gordon (Gordon *et al.* 1953) NCTC 11659 utilised arabinise, benzoate, erythritol, glucose, inositol, lactate, mannose, mannitol, rhamnose sorbitol, succinate, trehalose and xylose as carbon sources. It failed to utilise citrate, dulcitol, raffinose, malonate, mucic acic and oxalate as carbon sources in the test system (SRPharma; SRPharma).

1.8.1.6 Amidase activity

Amidases as hydrolases that split amides with the liberation of ammonia which is detected by use of Russel's reagent which gives a blue colour in the presence of free ammonia. Ten amides are used in the amidase row (Bonicke 1962). Those not hydrolysed, or weakly hydrolised are acetamide, benzamide, nicotinamide, pyrazinamide, succinamide, malonamide, isonicotinamide and salicylamide (SRPharma; SRPharma).

1.8.1.7 Nitrate reductase

This activity is detected by a reagent giving a red colour in the presence of nitrite ions. NCTC 11659 is negative (SRPharma; SRPharma).

	Pigmentation in		Temperature for growth (°C)				Carbohydrate utilisation		Amidase	Nitrate reductase
	Light	Dark	32	37	42	45	Ben	Rha		
M. vaccae	+	+	+	+	+	-	+	+	3,8	-
M. parafortuitum	+	+	+	+	+	-	+	-	3,5,6	-
M. avium	+	+	+	+	-	-	-	-	3,5,6	+
M. Obuense	+	+	+	+	-	-	-	-	3,5,8	-

TABLE 1.8-1: *Variable cultural and biochemical properties of Mycobacterium vaccae and closely related species and sub-species:* Ben= benzoate; Rha= rhamnose. Bonicke's amides: 3= urea; 5=nicotinamide; 6=pyrazinamide; 6=allantoin

1.8.1.8 Secreted and total proteins

The unique patten of mycobactins produced by *M. vaccae* may be used to differentiate this species from other rapdly growing mycobacteria (Barclay *et al.* 1992). *M. vaccae* produces copious amounts of water soluble exochelins that resemble those of *M. leprae* (SRPharma; SRPharma).

1.8.1.9 Antigenic analysis by immunodiffusion

Sonicated preparations of a wide range of mycobaterial species grown on Sauton's medium have been used to raise antibodies in rabbits. NCTC 11659 possesses the same species specific antigens as the type strain of *M. vaccae*. In common with *M. leprae*, it possesses no growth speed-related antigens (SRPharma; SRPharma).

1.8.2 *Mycobacterium vaccae* preparations: SRL172 and SRP 299

Mycobacterium vaccae is commercially available as two preparations: SRL172 and SRP299. The two preparations have similar immunological properties as both have been derived from the same seedlot. They are the same species and same strain and share the same genetic material. The two preparations are grown in the same medium under the same GCP/GMP conditions and are considered equivalent preparations by the Medicines and Healthcare products Regulatory Agency (MHRA). The only difference is that SRL172 is grown on solid a medium and SRP299 on a liquid medium; the reason for this is that larger quantities can be grown on solid medium. SRL172 is a sterile suspension of killed *M. vaccae* grown on Sauton's medium solidified with agar and formulated to GMP at a concentration of 10 mg/ml in borate buffered saline at pH 8. SRP299 is grown in liquid Sauton's medium and formulated to GMP at a concentration of 10 mg/ml in phosphate buffered saline at pH 7.3.

Both preparations are administered by intradermal injection and have been used interchangeably in animal studies. For example, in a series of papers, both preparations have been shown to inhibit periodontal disease in rats, showing identical immunomodulatory properties (Breivik *et al.* 2000; Brelvik *et al.* 2002; Breivik *et al.* 2003). Similarly, in a mouse model of asthma both SRL172 (Smit *et al.* 2003) and SRP299 (Zuany-Amorim *et al.* 2002; Hunt *et al.* 2005) have been used and have been able to ameliorate allergic inflammation. Furthermore, in two papers (Zuany-Amorim *et al.* 2002; Zuany-Amorim *et al.* 2002) the authors have considered the two preparations equivalent and they have used, in the same *in vivo* experimental model, SRL172 for some experiments and SRP299 for others, and no differences between the two preparations were noted (G. Rook, personal communication).

The fact that SRL172 is autoclaved in borate buffer, and the fact that the final suspension for injection contains this buffer, makes this preparation suitable for *in vivo* injection but unsuitable for *in vitro* experiments in view of the extreme toxicity to T cells in culture (G. Rook, personal communication). Therefore, SRL172 cannot be run in parallel with SRP299 *in vitro*.

For reasons concerning their production and their clinical use, the two *M. vaccae* preparations have been separated and SRP172 is now produced as immunotherapy for the treatment of cancer and tuberculosis, whereas SRP299 is produced for the treatment and prevention of allergic disorders. However, the immunoregulatory properties of *M. vaccae* are robust and independent of the method of preparation, probably because they are primarily due to the presence of major lipids that are consistent in the two preparations.

In this thesis, I have used SRL172 for the *in vivo* clinical trial and SRP299 for the *in vitro* study. Although the two preparations are considered to have identical immunomodulatory properties, it would have been desirable to be able to run parallel *in vitro* experiments with the two preparations in order to demonstrate their equivalence and establish a firmer link between the *in vivo* findings and the *in vitro* results. It is of course fundamental to good scientific practice to compare different lots of the same compound in a standard test assay to confirm bioequivalence. Unfortunately, as mentioned above, this was not possible. As direct *in vitro* comparison of the two preparations cannot be made, it will not be possible to make an irrefutable link between the observations made in this thesis between the *in vivo* challenge experiment and the *in vitro* effects, although it is likely that the mechanisms observed *in vitro* account, at least, partly, for the *in vivo* effects, especially in view of the observations made with the two preparations in the animal model.

The mechanisms by which Heat-Killed *M. vaccae* retains its immunomodulatory properties are not clear. It is possible that the effects on the immune cells do not require the intact organism or that components, such as glicolipids, that are relatively heat-stable and retain antigenic properties after autoclaving. Other species of mycobacteria, such as *M. bovis* BCG have been used as heat-killed preparations for the treatment of allergic disease, both in animal models and human asthma (Shirtcliffe *et al.* 2001; Major *et al.* 2002; Shirtcliffe *et al.* 2003). Since it is likely that the factors that determine immunomodulation or confer virulence may be different for pathogenic and saprophytic species, further studies will be necessary to identify and possibly separate immunity from virulence (SRPharma; SRPharma).

1.8.3 Safety

Data from clinical studies suggest that both SRL172 and SRP 299 have good safety profiles. Most people develop local erythema and swelling at the site of injection. This is dose-dependent but does not worsen with continued administrations. The local reactions are similar to those following BCG vaccination of tuberculin-negative children. A small ulcer may develop, but this usually heals in one to three weeks, resulting in a residual scar. A minority of people report mild influenza-type symptoms in the 48 hours following the injection (Investigator's Brochure SRP299) (SRPharma; SRPharma).

1.8.4 Pre-Clinical and Clinical studies

M. vaccae has been used in clinical studies involving patient with pulmonary tuberculosis (Prior *et al.* 1995), neoplastic diseases such as melanoma (Maraveyas *et al.* 1999) prostate cancer (Hrouda *et al.* 1998; Hrouda *et al.* 1998) and lung cancer (Webb *et al.* 1998), where the aim has been to promote Th1 cytokine-dependent immune defences. Similarly, it has been

employed in the treatment of diseases, such as tuberculosis and allergic diseases, where a depressed Th1 immune response is though to contribute to the pathogenesis of the diseases.

1.8.4.1 Cancer

SRL172™ has been studied as an adjunct to standard treatments for the treatment of a variety of cancers.

Lung Cancer

In a phase II study patients with small cell lung cancer (SCLC) were randomized to receive chemotherapy (platinum-based or anthracycline-based) with (n=14) or without (n=14) intradermal SRL172 on day 0, after 4 and 8 weeks and then 3-6 monthly. The median survival was 8.6 months and 12..9 for patients treated with chemotherapy alone and with the combination respectively, which, although not statistically significant, the study showed a trend to improved median survival in SCLC with the combination of chemotherapy and SRL172 with no increased toxicity. A phase III study examining chemotherapy in combination with SRL172 in SCLC is now underway (Assersohn *et al.* 2002).

Other studies have confirmed similar survival trends and safety profile of SRL172 in inoperable non-small cell lung cancer (NSCLC) and mesothelioma. In the group of patients randomized to receive chemotherapy combined with SRL172, there was a trend towards improved response rate (54% vs. 33%) with more patients in the combined arm receiving radical surgery and radiotherapy, improved median survival (9.7 months vs. 7.5 months) and improved 1 year survival (42% vs. 18%). There was no detectable change in serum cytokine levels for IFN-γ and TNF-α before and after treatment (O'Brien *et al.* 2000). A subsequent open-label, randomised phase III study in non-small-cell lung cancer (NSCLC) where patients

were randomised to receive platinum-based chemotherapy, with (210 patients) or without (209 patients) monthly SRL172, showed that SRL172 significantly improved patient quality of life without affecting overall survival times (O'Brien *et al.* 2004). The data from this trial have been reanalysed taking into account full compliance with the SRL172 treatment phase. In the case of patients with adenocarcinomas, treatment with four or more injections of SRL172 induced a significant improvement in median survival (p=0.012) compared to patients on chemotherapy alone (SRPharma). SRL172 was proven to be safe when given as intrapleural injection in addition to intradermal SRL172 and standard chemotherapy (mitomycin-C, vinblastine and cisplatin) in patients with malignant mesothelioma. This result was thought to be secondary to improved type 1 immunity as documented by an increase in activation of NK cells and a decrese in of IL-4 producing T cells in all tested patients post-treatment (Mendes *et al.* 2002).

Melanoma

Encouraging results were also obtained from a phase I/II trial of SRL172™ monotherapy in patients with advanced malignant melanoma.

A phase I-II study was designed to assess safety and clinical efficacy of intradermal SRL 172 in 24 patients with advanced stage IV (AJCC) malignant melanoma initially at 15-day intervals for three vaccinations and then at monthly intervals. The vaccination schedule resulted in improved survival and documented increase in intracellular IL-2 in PBMC of patients who received at least three doses (Maraveyas *et al.* 1999).

Prostate cancer

Pre-clinical animal studies have suggested SRL172 significantly delayed the growth of established prostate tumours (Hrouda *et al.* 1998).

Subsequent clinical studies have shown that intradermal SRL172 is safe in patients with advanced hormone-refractory prostate cancer, and can modulate cytokine production by PBMC with increase in IL-2 and reduction in IL-4. These changes in cytokine profile have been associated to a reduction in the levels of prostatic specific antigen (PSA) in one study (Hrouda *et al.* 1998) but not confirmed in a larger trial with patients with more advanced disease (Eaton *et al.* 2002).

1.8.4.2 Tuberculosis

Studies, both in animals and in humans have shown that *M. vaccae* is an effective immonomodulator, promoting IL-12 and IFN-γ synthesis (Dlugovitzky *et al.* 1999), particularly from CD8+ T cells, (Skinner *et al.* 1997) while down-redulating IL-4 production (Skinner *et al.* 1997; Dlugovitzky *et al.* 1999).

A number of studies have been carried out on the treatment of newly diagnosed pulmonary tuberculosis, usually with a single dose regiment of *M. vaccae* together with chemotherapy. Overall these studies have shown no survival or cure benefit using *M. vaccae* (1999; de Bruyn *et al.* 2000; de *et al.* 2001; de Bruyn *et al.* 2003) and no change was reported in the Xray appearance (Johnson *et al.* 2004). The lack of efficacy was independent of the HIV status (1999; Mwinga *et al.* 2002). T

Despite these negative trials, *M. vaccae* showed faster rate of cure in newly diagnosed tuberculosis in Romania (Corlan *et al.* 1997) and, in chronic or relapsed disease, was effective

in improving bacteriological success, chest X-ray, recovery of body weight in comparison with patients receiving chemotherapy alone. These changes were seen even in those failing bacteriological cure (Corlan *et al.* 1997).

Treatment of multi-drug resistant tuberculosis (MDRTB) with SRL172 in suggest a possible role of the treatment of this disease (Stanford *et al.* 2001).

1.8.4.3 Allergic diseases

Animal data strongly suggest beneficial immunological changes of allergic inflammation following *M. vaccae* treatment. Less clear are the results derived from clinical trials where *M. vaccae* seems effective in allergic dermatitis but ineffective in rhinitis or asthma.

1.8.4.3.1 Asthma

Studies using *Mycobacterium vaccae* for the prevention and the treatment of allergic diseases in rodents, have shown that treatment with *M. vaccae* prior or during allergic sensitisation can inhibit the generation of Th2-type cytokines in response to allergen, reduce aiway inflammation, eosinophilia and airway hyperesponsiveness. However, not all studies have conclusively demonstrated that *M. vaccae* is effective in the eshablished phase of allergic response. This may be partially explained by differences in the study protocol, the timing and the route of administration of *M. vaccae.*

The first study examining the effects of *M. vaccae* as a potent inducer of Th1 immunity on allergic responses in a murine model, used a single injection of *M. vaccae* into ovalbumin (OVA)-preimmunized BALB/c. A single injection of *M. vaccae* inhibited IgE synthesis, however, repeated injections (two and four) of 10^7 *M. vaccae* were also effective in

preventing the ovalbumin-induced IL-4 and IL-5 production by spleen cells, without affecting IFN-γ production and IgG1 and IgG2a synthesis (Wang *et al.* 1998).

In a similar animal model, *M. vaccae,* in the period pre-sensitisation, was able to reduce the late airway response (LAR) following allergen challenge, BHR to methacholine, airway eosinophilia and Th2 response, when administered alone (Hopfenspirger *et al.* 2002)or with *M. bovis* (Tukenmez *et al.* 1999). However, other authors have suggested that the down-regulatory effect on IgE, BHR and IL-5 levels occurs only when animals receive *M. vaccae* before or during allergen sensitisation and challenge (Smit *et al.* 2003) but not after sensitisation (Smit *et al.* 2003).

Besides the effects on the inflammatory component following allergen challenge, studies in animal models of asthma showed that heat-killed *M. vaccae* could prevent the development of the structural airway changes seen in chronic asthma. Increase in goblet cell numbers, thickness of basement membrane and airways smooth muscle hyperthrophy seen in a murine model of asthma were all prevented by *M. vaccae* administration (Ozdemir et al. 2003).

These studies advocated that the induction of a Th1 response was the mechanism responsible for the effects of *M. vaccae* on allergic inflammation. However, no significant increase in the levels of IL-12 and IFN-γ was noted. Interestingly, a significant increase in plasma IL-12 was observed with BCG as compared to *M. vaccae* administration, suggesting a stronger type 1 response to BCG (Hopfenspirger et al. 2001). *M. vaccae* may down regulate Th2 response by mechanisms other than Th1 activation, as demonstrated by data showing that mycobacteria exert a therapeutic effect also when administered to mice with Th1-mediated diseases, such as diabetes or encephalomyelitis (Lehmann *et al.* 1992; Qin *et al.* 1993; Martins *et al.* 1999; Walker *et al.* 2003). Indeed, more recent studies have demonstrated that the effects of *M. vaccae* on T cells are more complex and may be independent of IFN-γ synthesis (Zuany-

Amorim *et al.* 2002). Treatment of mice with *M. vaccae* stimulates allergen-specific $CD4^{+}CD45RB^{Low}$ regulatory T cells (Treg), which inhibits airway inflammation. This specific inhibition was mediated through interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), as antibodies against IL-10 and TGF-β completely reversed the inhibitory effect of $CD4^{+}CD45RB^{Low}$ (Zuany-Amorim *et al.* 2002).

Furthermore, in murine models, *M. vaccae* seems to be able to down-regulate effector immune response not only directed against the specific antigen to which mice were sentitised by inducing allergen-specific Treg cells but also to different antigens through a 'bystander' mechanism. *M. vaccae* can stimulate Tregs either directly or through the activation of CD11c+ dendritic cells which secrete the immunoregulatory cytokines IL-10, TGF-β and IFN-α (Adams et al. 2004).

Both the direct stimulatory effect of *M. vaccae* on CD4 Treg and the induction of APC to produce IL-10, TGF-β and IFN-α, which in turn, induce proliferation and maturation of Treg are indicative of regulatory T-cell effects might explain the observed suppressive Th2 effect without up-regulation of Th1 cytokines. In addition, recent work has shown that SRP299 given orally can significantly reduce the symptoms of allergic pulmonary inflammation including eosinophilia and total cellular infiltration associated with an increase of IL-10 and IFN-γ (Hunt *et al.* 2005). The ultimate mechanism of action or the component of M. vaccae responsible for the improvement in airway inflammation is not clear, particularly considering that in some studies an the reduction in BHR and airway eosinophilia occureed in the absence of detectable changes in IL-4, IL-5, IL-13 and IFN-γ as well as IgE levels (Zuany-Amorim *et al.* 2002; Zuany-Amorim *et al.* 2002; Hunt *et al.* 2005)

A limited number of studies have so far been conducted in asthma. Different preparations of *M. vaccae* and different schedules and routes of administration have been tested. In a clinical study, forty-two patients with stable moderately severe asthma (corticosteroid dose <1200 mcg/day of beclomethasone dipropionate or equivalent), who were skin-prick test positive to house dust mite, were randomised to receive two intradermal injections of phosphate-buffered saline (PBS, placebo), heat-killed *M. vaccae* (HKMV) (0.5 mg), or delipidated deglycolipidated *M. vaccae* (DDMV) (0.05 mg). DDMV is delipidated, deglycolipidated and arabinogalactan-depleted derived from autoclaved, acid-treated *M. vaccae.* It comprises peptidoglycans, sugars, proteins and residual lipids and is formulated in saline and 0.03% polysorbate 80. In *in vitro* assay, weight for weight, DDMV is 10-fold more effective than *M. vaccae* in stimulating IL-12 production. Markers of asthma severity (mean morning PEFR, symptom-medication score) were measured for three months and blood eosinophils, IgE levels and T cell proliferative and cytokine responses were monitored. There were no significant differences between either treatment group and the placebo group for any of the outcome variables. There was also no difference between the treatment groups and placebo for eosinophils, IgE levels or the T cell proliferative and cytokine response and the study concluded that low dose intradermal DDMV or *M. vaccae* had no effect on asthma severity in patients with established asthma (Shirtcliffe et al. 2001).

In a subsequent study, thirty-seven adults with stable moderately severe asthma (corticosteroid dose < 1000 mcg/day of beclomethasone dipropionate or equivalent),who were skin prick test-positive to house dust mite were randomized to receive two doses 2 weeks apart of delipidated acid-treated *M. vaccae* (first dose 0.4 mg and second dose 0.8 mg) or placebo, given as intranasal drops. Safety, tolerability and markers of asthma severity (including peak flow, FEV_1, major and minor exacerbations, symptom scores and beta-agonist

use), blood eosinophil and IgE levels were monitored for 8 weeks. The change in route of administration was based on previous animal studies demonstrating the superiority of BCG administered intranasally compared to intraperitoneal and subcutaneous route in preventing airway eosinophilia (Erb et al. 1998). Delipidated acid-treated *M. vaccae* was safe and well tolerated although there was an occasional mild local reaction. However, there was no evidence of efficacy in reducing the severity of asthma.

The reasons why mycobacterial-based vaccines have not shown equivalent efficacy in human trials compared with animal models is not clear. The role of factors such as duration of disease, route of administration and the active component of mycobacteria need to be addressed (Shirtcliffe et al. 2003).

It is possible that components of mycobacterial cell walls such as lipoglycans (including lipoarabinomannan, LAM; lipomannan, LM; and phosphatidylinositol mannan, PIM) may play a major role in the effects of mycobacteria on allergic response and explain differences between delipidated preparations of *M. vaccae* and preparations containing the whole organism. A recent study has shown that LAM from BCG and *M. bovis* and PIM from *M. bovis* and *M. smegmatis* (a non-pathogenic mycobacterium similar to *M. vaccae*) is able to suppress airway eosinophilia by stimulating IL-10 production without affecting Th1 cytokines (Sayers *et al.* 2004).

Sakai Chemical Industry Co. Ltd has funded a multi-centred phase II, randomized, placebo-controlled study of the safety and efficacy of SRP299 the treatment of 178 adults with moderate persistent asthma. Treatment with SRP299 showed only a trend towards improvement in asthma symptoms. However, exploratory analysis showed that patients receiving 2 high doses of SRP299 had a significant reduction in asthma symptoms score and asthma exacerbations compared to placebo (SRPharma).

1.8.4.3.2 Atopic dermatitis

A pre-clinical study in a mouse model of atopic dermatitis showed that a single intradermal administration of a heat-killed *Mycobacterium vaccae* reduced pruritus, a major clinical symptom of eczema, although did not prevent the development of eczema (Arkwright *et al.* 2005).

Clinical studies in children aged 5 to 18 years with moderate to severe atopic dermatitis showed that a single injection of SRL172 (0.3 ml of a suspension containing 10^{10} heat-killed *M. vaccae* per ml) caused a 48% reduction in surface area affected by dermatitis compared with a 4% reduction for the placebo group and a 68% reduction in dermatitis severity score compared with 18% for the placebo group at three months after treatment. None of the children, however, had complete resolution of their dermatitis (Arkwright *et al.* 2001). A second study in younger children aged 2-6 years showed that although a 38-54% reduction in surface area affected by dermatitis was noted at all time points after *M. vaccae* administration, this improvement was not significantly different from that observed in the placebo group. Meta-analysis of this and the previous cohort (97 children aged 2-18 years) showed that *M. vaccae* may offer greater benefit in children over 5 years old, whose AD appears less likely to regress spontaneously (Arkwright *et al.* 2003).

The immunological mechanism underlying the improvement in the severity of dermatitis is not clear since no changes were noted in the levels of IgE and eosinophils (Arkwright *et al.* 2001) and a study on PBMC from the same cohort of patients showed no difference in the synthesis of IL-4, IL-5 and TGF-β. The transient increase in IFN-γ noted at 1 month was considered insufficient to explain the effect of *M. vaccae* on disease severity since the eczema improved at a later time when Th1-type response had returned to baseline (Hadley *et al.* 2005).

1.9 Hypothesis and Aims of the Thesis

The overall purpose of the work presented in this thesis has been to employ different strategies to inhibit the generation of Th2 cytokines in response to allergen in subjects with bronchial asthma both *in vivo* and *in vitro*. Based on the cross-regulatory characteristics of T helper subtypes, I have employed the soluble IL-4 receptor (sIL-4R) to block the key Th2 effector cytokine IL-4 in an *in vitro* model. As an alternative to direct inhibition of the cytokine IL-4, I have sought possible clinical applications of the 'hygiene hypothesis' by studying the effects of non-pathogenic bacteria (*Mycobacterium vaccae*) on the lung function and T cell phenotype both *in vivo* and *in vitro* and the effects of the bacterial component LPS on the Th2 response to allergen *in vitro*, based on prior recognition in animal models that these two approaches can inhibit Th2 responses.

My specific hypotheses were:

- Inhibition of IL-4 using soluble IL-4 receptor (sIL-4R) can inhibit Th2 responses as shown by an effect on the production of Th2 cytokines IL-5 and IL-13 by PBMC of atopic asthmatic individuals stimulated with allergen;
- *Mycobacterium vaccae* can attenuate allergen-induced airway response in asthmatics as evidenced by a reduction in the late asthmatic response (LAR) following allergen challenge;
- Th2 responses to allergen can be inhibited by the non-pathogenic *Mycobacterium vaccae* and lipopolysaccharide through the induction of IFN-γ and regulatory T cells (Treg).

1.9.1 IL-4 Inhibition by sIL-4R

Using an *in vitro* model, I have sought to inhibit allergen-stimulated Th2 responses using a human recombinant soluble IL-4 receptor (sIL-4R), containing the extracellular domain of the α-chain that avidly binds to IL-4 and thus blocks its effects.

1.9.2 Mycobacterium vaccae

The first strategy employed to elucidate the effects of *M. vaccae* was based on the concept that administering a preparation of *M. vaccae* to asthmatic patients prior to allergen challenge might reduce the allergic inflammation within the airways and down-regulate the Th2 cytokine production and IgE synthesis. Using this approach, I have tested the hypothesis that *M. vaccae* can regulate T cell responses following allergen challenge.

I have measured the outcome of these studies by investigating the following parameters:

1. Changes in the EAR, LAR and BHR following allergen challenge;

2. Sputum cellularity and eosinophilia;

3. *Ex vivo* IL-5 production by allergen-stimulated peripheral blood mononuclear cells (PBMC);

4. PBMC proliferation in response to allergen.

I have also sought to elucidate mechanisms involved in the down-regulation of Th2 cytokine response, by blocking cytokines such as IL-10, IL-12 and IL-18, which are known to be induced by bacterial stimuli; or prostaglandins, which are produced by activated APC following. Furthermore, I have compared the effects on T-cell activation induced by *M. vaccae* with the effects induced by recombinant IL-12, a powerful Th1 stimulator secreted by APC in response to various stimuli, most importantly bacteria.

1.9.3 Lypopolisaccharide

Lipopolysaccharide (LPS) or endotoxin has been implicated in the hygiene hypothesis as a natural inhibition of Th2 responses. Lipopolysaccharide is a potent pro-inflammatory agent present in the cell wall of Gram-negative bacteria. It is continuously shed into the environment and is abundant in occupational organic dust, house dust, and in human oral and nasal cavities (Di Luzio *et al.* 1973; Hasday *et al.* 1999). It is also found in domestic water, which can be inhaled in bathrooms and from air humidification systems.

Endotoxin levels in samples of dust seem inversely related to the occurrence of atopic diseases and atopic sensitization (Braun-Fahrlander *et al.* 2002). One intriguing hypothesis is that LPS is recognised by the innate immune system in the absence of overt infection, and stimulates the maturation of the immune response, towards the development of tolerance to other environmental components, such as pollen and animal dander (Braun-Fahrlander *et al.* 2002).

The understanding of the underlying mechanisms responsible for protection from developing allergic diseases could provide the necessary information for a targeted immunological intervention, such as modified non toxic LPS analogues, to restore the skewed cytokine profile in response to allergen exposure.

CHAPTER TWO

General Materials and Methods

2.1 MATERIALS

2.1.1 Cell culture media and tissue culture ware

All plastic ware was from Falcon (Becton Dickinson, Oxford, UK) unless otherwise stated. Stock solutions of chemicals were stored at room temperature unless otherwise indicated. Chemicals used and their abbreviations are listed below.

2.1.1.1 Reagents

[^{3}H]thymidine	Amersham Pharmacia Biotech, Little Chalfont, Bucks.
2-mercaptoethanol (2-ME)	Sigma Ltd, Poole, Dorset, UK
Culture plates	Costar, Corning, NY, USA
Diff-Quick staining kit	Harleco, Gibbstown, NY, USA
DTE	Sigma
Filters	Becton Dickinson Ltd, Cowley, Oxfordshire
Foetal Calf Serum (FCS) (Heat-inactivated)	Gibco BRL Life Technologies, Paisley
Fungizone® (amphotericin B 250 µg/ml solution	Gibco
Gentamicin	Sigma
HEPES 100 ×	Gibco
Histamine acid phosphate solutions (0.03 - 64 mg/ml in normal saline)	

Human AB serum	Sigma
Indometacin	Sigma
Killed *M. vaccae* suspension – SRL172	SR Pharma, London, UK
Killed *M. vaccae* suspension- SRP299	SR Pharma
L-glutamine 100× (200 mM solution	Gibco
Lipopolysaccharide (Escherichia coli, 055:B5)	Sigma
Lymphoprep™ [Ficoll-isopaque]	Nycomed, Asker, Norway
Methanol	Sigma
Microscope Slides with frosted ends	neoLab, Germany
Penicillin/Streptomycin 10,000-U/ml, 10,000-µg/ml solution	Gibco
PGI_2 (Iloprost)	Schering Health Care Limited, Burgess Hill, West Sussex, UK
Phosphate-buffered saline (PBS)	In-house
Phytohemagglutinin (PHA-L from Phaseolus vulgaris, red kidney bean)	Sigma
Prostaglandin E_2	Cayman Chemical, Ann Arbor, MI, USA
Recombinant human-IL-4	R&D systems, Abingdon, Oxfordshire
RPMI-1640 medium	Gibco
Scintillation fluid (Optiphase)	Wallac Bertold, Milton Keynes, Buckinghamshire
Scintillation vials (Pony vial)	PerkinElmer LAS (UK) Ltd., Beaconsfield, Buckinghamshire

Sodium pyruvate 100×	Gibco
Soluble IL-4 receptor	Nuvance®, Immunex, Seattle, WA, USA
Trypan blue 0.4% solution	Sigma
Vacutainers (heparin)	Becton Dickinson

2.1.1.2 Antibodies, cytokines, bacteria, soluble Receptors

Anti-Human IL-18 mAb	R&D
Anti-Human IL-12 p70 mAb	R&D
Anti-Human IL-10 mAb	R&D
Recombinant IL-12	R&D
Anti-IL-4 antibodies	R&D
Anti- IL-4R antibodies	R&D
IgG2a antibodies Isotype control	R&D

2.1.1.3 Equipment

Beta scintillator counter	Wallac Bertold, Milton Keynes, Buckinghamshire
Centrifuges: Mistral 3000i, Biofuge IR	BDH, Heraeus, Brentwood, Essex
Microscope	CETI Belgium
Pipettes	Pipetaman®, Gilson, Villiers Le bel , France
Multiskan Ascent, ELISA reader	Labsystem, Helsinki, Finland
Cytospin centrifuge 3	Shandon Southern Products, Runcorn, Cheshire
Electric pipettes	Biohit Helsinki ,Finland
Lab-counter	The Denominator Company, Woodbury, USA

ELISA reader	Titerteck Multiskan, Helsinki, Finland
Vitalograph spirometer	Vitalograph Ltd, Maids Moreton, Buckinghamshire
Cell Harveste	Tomtec Harvester 96
Ultrasonic Nebulizer	DeVilbiss Ultra Neb 99, Heston, Middlesex
Resuscitation trolley	Southampton General Hospital NHS Trust
Inhalation dosimeter	Spira® Electro, Spira Respiratory Care Center Ltd., HaÈmeelinna, Finland)

2.1.1.4 Allergens

Cat hair and dander	Aquagen SQ – ALK, Horshølm, Denmark.
Dog hair and dander	Aquagen SQ
House dust mite (*Dermatophadoides pteronyssinus*)	Aquagen SQ
Grass mix	Aquagen SQ

2.1.1.5 Kits

ELISA kits: IL-5, IL-10, IL-13, IFN-γ, IL-12,	CytoScreen™, BioSource International Inc., Camarillo, CA, USA
ELISA for IgE	Regional Immunology Laboratory, Southampton General Hospital

2.1.1.6 Software packages

Office®	Microsoft
SPSS 8.0	SPSS Inc.
Instat®	GraphPad™ Corp.
SigmaPlot 8.0	SPSS Inc.
EndNote 7.0	ISI ResearchSoft

2.1.2 Subjects

Adult atopic asthmatics with mild to moderate disease were recruited for the studies. Individuals with a history of smoking during the previous 12 months, alcohol or drug abuse, or serious adverse reaction to medications were excluded. None of the subjects had reported a respiratory infection within 14 days of the study, required prescription medication (other than that for asthma) during the same period, nor had used oral corticosteroids within the previous six months.

All the studies presented in this thesis were approved by the Southampton University and Hospitals Ethics Committee and subjects gave their written informed consent.

2.2 METHODS

2.2.1 Lung Function Tests

All subjects in the clinical trial employing the preparation of *Mycobacterium vaccae*/placebo were investigated with basic spirometry including forced expiratory volume (FEV_1), forced

vital capacity (FVC) and peak expiratory flow (PEF). At least three acceptable tracings were obtained and each tracing was examined. The best reading was accepted and recorded.

2.2.2 Histamine inhalation Challenge

Histamine challenge is a standard technique used to determine the degree of non-specific bronchial reactivity of an individual. The method used in this thesis is a technique modified from Chai H, et al., 1975 (Chai *et al.* 1975). Subjects were asked not to take salbutamol (or other bronchodilator) for at least six hours before histamine challenge. Immediately prior to saline inhalation, spirometry was recorded. FEV_1 had to be >70% of predicted. For each timepoint at least two technically satisfactory FEV_1 measurements were recorded, and the largest of these used in the calculations. Inhaled solutions were administered via a nebuliser driven by compressed air at a flow of 8 L/min to produce a mass median particle diameter of <5 μm at a delivery rate of 0.25–0.50 ml/min. After recording baseline spirometry and ensuring that the subject was suitable to continue, the subject inhaled five breaths of nebulised normal saline. This involved forced inhalation from functional residual capacity (FRC) to total lung capacity (TLC). Measurements of FEV_1 were made one minute and three minutes after the inhalations and the smaller reading was used for calculation. If a fall in FEV_1 of greater than 10% occurred after saline, the challenge was aborted, as the subject was considered too reactive. If there was no fall in FEV_1 (or less than 10%) after the saline, then the first dose of histamine was delivered, starting with 0.03 mg/ml solution. Doubling concentrations of histamine (0.03–64 mg/ml) were administered every five minutes, and FEV_1 recorded at one minute and three minutes after each exposure. These steps were followed through the histamine concentrations until a fall in FEV_1 from the post-saline value of >20% occurred, or the highest concentration of histamine was reached.

The degree of airways responsiveness to histamine was expressed as a non-cumulative PC_{20} (provocative dose producing a 20% fall in FEV_1) calculated using the following equation (Equation 2.2-1)

$$PC_{20} = anti\log\left[\log C_1 + \frac{(\log C_2 - \log C_1)(20 - R_1)}{(R_2 - R_1)}\right]$$

(EQUATION 2.2-1)

C1 = CONCENTRATION IMMEDIATELY BEFORE 20% FALL IN FEV_1
C2 = CONCENTRATION CAUSING > 20% FALL IN FEV_1
R1 = % FALL IN FEV_1 AFTER C1
R2 = % FALL IN FEV_1 AFTER C2

2.2.3 Allergen Inhalation Challenge

Allergen challenge was performed using a modification of an established protocol (Twentyman *et al.* 1990). If subjects were taking regular inhaled beclomethasone they stopped treatment five days before allergen challenge to enable a LAR to develop. Allergens which produced the greatest cutaneous wheal response were used. After taking five breaths of physiological saline to ensure the absence of non-specific airways irritability, subjects inhaled increasing concentrations of allergen solution (Aquagen, ALK, Denmark), beginning with 1 SQ unit/ml and increasing 10-fold at 15 minute intervals up to 10,000 SQ units/ml or until a fall of at least 25% from post-saline baseline had been achieved (EAR). FEV_1 measurements were recorded at 20, 30, 45 and 60 minutes and thereafter, hourly for eight hours. A LAR was defined as a fall in FEV_1 >15% from post-saline baseline between three and eight hours post-challenge. Subjects were then routinely given 2.5 mg of nebulised salbutamol and allowed home. They returned the following morning for histamine challenge using a previously reported protocol (Twentyman *et al.* 1990).

For safety considerations, arbitrary minimum acceptable values for FEV_1 and FVC were set prior to the challenge:FEV_1 >70% predicted and FVC >80% predicted.

2.2.3.1 Allergen Preparation

Allergen was prepared freshly each study day by dilution of the 'neat' commercial solution (100,000 SQ-U/ml) with normal saline. Concentrations of 10,000 SQ-U/ml, 1,000 SQ-U/ml, 100 SQ-U/ml, 10 SQ-U/ml, and 1 SQ-U/ml were used as specified in individual studies.

2.2.3.2 Administration of allergen

Immediately prior to saline inhalation, lung function was recorded. For all time points throughout this procedure, at least two technically satisfactory FEV_1 measurements were recorded, and the greatest of these used for comparisons with other time points.

After recording baseline spirometry the subject (using a nose peg) inhaled five breaths of nebulised normal saline, from functional residual capacity (FRC) to total lung capacity (TLC), taking each inspiration over five seconds. The time of the first inhalation was recorded. If a fall in FEV_1 of >10% occurred after saline, the challenge was aborted for that day. If there was no fall in FEV_1 (or less than 10%) after saline, then the first dose of allergen was delivered, starting with the 1 SQ-U/ml dilution. FEV_1 was recorded after five, 10 and 15 minutes.

The subsequent doses of allergen were administered based on the following guidelines:

⇒ If the fall in FEV_1 was less than 10% from the post saline value, then the next dose of allergen was delivered

⇒ If the fall in FEV_1 was between 10% and 15% of the post-saline baseline, a concentration of allergen two to five times higher was given

⇒ If the fall in FEV_1 was >15% but <25% from the post-saline value, a further five breaths of the same concentration were administered

These steps were followed through the allergen concentrations until a fall in FEV_1 from the post-saline value of >20% was achieved.

A late asthmatic reaction (LAR) was defined as a fall in FEV_1 of >14% from post-saline baseline at two to eight hours post challenge. At the conclusion of the study period inhaled salbutamol was administered. The degree of airways responsiveness to allergen is expressed as a cumulative PD_{25} (provocative dose producing a 25% fall in FEV_1) using the following equation (Equation 2.2-2).

$$PD_{25} = anti\,log\left[log\,D_1 + \frac{(log\,D_2 - log\,D_1)(25 - R_1)}{(R_2 - R_1)} \right]$$

EQUATION 2.2-2: CALCULATION OF $PD_{25}FEV_1$ FOLLOWING ALLERGEN CHALLENGE.

D1 = Cumulative dose immediately before 25% fall in FEV_1

D2 = Cumulative dose causing > 25% fall in FEV_1

R1 = % fall in FEV_1 after D1

R2 = % fall in FEV_1 after D2

2.2.4 Sputum Induction and Processing

2.2.4.1 Sputum induction

Induction of sputum production involved the subject inhaling saline solution. Before induction, subjects were asked to blow their noses and rinse their mouths. The subject was instructed to expectorate into a sterile plastic container.

Subjects were pre-medicated with 200 μg salbutamol via a spacer. A peak expiratory flow (PEF) was recorded. Subjects were seated in an induction chamber and hypertonic saline (4.5%) was aerosolised by an ultrasonic nebuliser (DeVilbiss Ultra Neb 99, Middlesex, UK) with an output set at 3 ml/min. Subjects wore a noseclip and inhaled aerosol for up to four five-minute periods.

After five minutes the nebulisation was stopped and PEF noted. The procedure was discontinued when there was a fall in PEF of >15% or respiratory discomfort. After each inhalation period, or whenever they felt the need to expectorate, patients rinsed their mouths with water to minimise contamination with saliva, and expectorated the sputum into a Petri dish which was placed on ice immediately.

2.2.4.2 Sputum processing

The total amount of sputum obtained was weighed and placed in a 50-ml Falcon tube. An equal weight of 10-mM dithioerythritol (DTE, Sigma) was added to the sputum, the samples were gently mixed and placed on a rocker for 30 minutes to allow mucus breakdown. The contents were then filtered through a 70-μm filter to remove mucus. The filtrate was centrifuged for 10 minutes at 400 *g* and 4°C to obtain a cell pellet.

The supernatants were aspirated and aliquoted into labelled Eppendorf tubes and stored at -80°C for future assays of inflammatory mediators: tryptase and eosinophil cationic protein (ECP). The cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) and total cell count and viability were determined in a Neubauer's chamber after staining with trypan blue.

Cytospins were obtained using 40–70 µl of sputum/PBS solution. Slides were fixed with methanol and allowed to dry. Subsequently the slides were stained with Diff-Quick staining. Differential cell counting was performed by counting 600 cells in each cytospin.

2.2.5 Skin-Prick Test

All subjects were investigated with skin-prick testing to a panel of common aeroallergens including *Dermatophagoides pteronyssinus*, to which subjects had to be allergic if they were to take part in the allergen challenge studies and/or donate their blood for stimulation of PBMC with this allergen. The testing was carried out on the inner forearm with the arm coded with a marker pen for the allergens to be tested. A drop of the allergen solution (Soluprick SQ, 10 HEP, ALK, Denmark) or positive (histamine dihydrochloride, 10 mg/ml) or negative (solvent) control was placed by each code and the skin was then pricked through the drop using the tip of a lancet. The reactions were read at 15 minutes and compared to positive and negative control. A subject was classified as atopic if any allergen caused a weal three mm or more in diameter.

2.2.6 *In vitro* cytokine response to allergen

2.2.6.1 Preparation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by Ficoll-Hypaque gradient centrifugation (Lymphoprep®, Nycomed Pharma AS, Asker, Norway). Heparinised blood was taken, shaken and diluted in an equal volume (1:1) of phosphate-buffered saline (PBS) with 2% foetal calf serum (FCS). PBMC were then separated using Lymphoprep density centrifugation. Ten ml of Lymphoprep were pipetted into a 25 ml universal tube and the same volume of peripheral blood was layered on top and centrifuged for 25 minutes at 800 *g* and at room temperature (20°C). The lymphocyte/monocyte interface was aspirated, collected into a 50 ml polypropylene tube and washed twice in PBS 2% FCS, centrifuging for 10 minutes at 400 *g*, and 4°C to remove excess platelets.

The cell pellet was resuspended in two ml PBS 2% FCS and the concentration of the cells adjusted to 1×10^7 cells/ml. All cell counts were performed using 0.4% trypan blue in PBS to exclude dead cells. Cells were resuspended at 2×10^6 cells/ml in RPMI 1640 medium, supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME (all from GibcoBRL Life Technologies, Paisley) and 5% heat-inactivated human AB serum, and cultured for 48 hours at 37°C in 5% CO_2 in the presence or absence of house dust mite allergen extract (*Dermatophagoides pteronyssinus*, ALK, Horshølm, Denmark) at a concentration of 5,000 SQ units/ml. The dose of allergen was chosen from pilot experiments as causing the greatest increase in IL-5 synthesis.

2.2.6.2 Mycobacterium vaccae in vivo

Twenty ml of heparinised venous blood were collected from each study subject before and after *M.vaccae*/placebo administration as described in 4.2.1.5. PBMC were isolated by Ficoll-Hypaque gradient centrifugation as described above. Culture supernatants were assayed by commercial ELISA kit for IL-5 levels (CytoScreen™, BioSource International Inc., Camarillo, CA, USA) with sensitivity at 4 pg/ml.

2.2.6.3 Mycobacterium vaccae in vitro – Lipopolysaccharide

PBMC were isolated from 40 ml of heparinised venous blood by Ficoll-Hypaque gradient centrifugation. Cells were washed twice and resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented as described above (2.3.6.1). Cells were cultured for seven days at 37°C in 5% CO_2 in the presence or absence of house dust mite allergen extract (*Dermatophagoides pteronyssinus; Der p*) at a concentration of 5,000 SQ units/ml. The dose of allergen and duration of culture were chosen from pilot dose-response experiments which showed these to be optimal for IL-5 and IL-13 production. To assess responsiveness to non-specific stimuli, PBMC were stimulated at day six with phytohaemoagglutinin (PHA) at a concentration of 10 mg/ml and harvested after 24 hours. Culture supernatants were assayed by commercial ELISA kits for IL-5, IL-13 and IFN-γ (CytoScreen™, BioSource International Inc.) with sensitivity at 4 pg/ml.

In order to study the effects of *M. vaccae*, PBMC were stimulated with 10 μg/ml of a preparation of heat-killed *M. vaccae*, SRP299 (SR Pharma, London, UK). To study the mechanisms involved in any modulation of allergen-induced Th2 cytokine responses by *M. vaccae*, PBMC stimulated with allergen in the presence of *M. vaccae* were also incubated

with single neutralising antibody or a combination of neutralising antibodies to IL-18 (mouse IgG_1), IL-12 p70 and IL-10 (all from R&D Systems). All the neutralising antibodies were used at a concentration of 1 μg/ml. IL-12 was used at a final concentration of 1 ng/ml. Parallel control wells contained the same concentration of a control isotype abtibody (IgG2a).

2.2.6.4 Proliferation assay of PBMC

Cells undergoing proliferation increase their rate of protein and DNA synthesis; this can be measured by adding [^{3}H]thymidine, a radioisotope-labeled DNA precursor, to the cell culture medium. The amount of ^{3}H taken up by the dividing cells is correlated to the level of cellular proliferation.

The proliferation assay protocol was as follows:

- Proliferation responses were set up in triplicate. 96-well U-bottom tissue-culture plates were labelled.
- Antigen was prepared in RPMI 1640 (supplemented as described in 2.2.6.1) in 100 μL per well.
- Each assay included the medium control for background proliferation; i.e. complete RPMI 1640 without antigen; positive control (PHA 10 μg/mL). One hundred μL of the antigen/RPMI was aliquoted per well. Each well contained 2×10^5 PBMC made up in complete RPMI and 5% heat-inactivated autologous serum, to give a final concentration of 5% serum in 200 μL.
- Cells were incubated at 37°C, 5% CO_2, in a humidified incubator for seven days. Cells were pulsed with 20 μL containing 0.5 μCi [^{3}H]-thymidine (1 μL = 1 μCi) 16

hours prior to harvest and then were re-incubated at 37°C, 5% CO_2, in a humidified incubator for 16 hours.

- Cells were harvested onto glass fibre filters. Filter was allowed to dry. Cells adsorbed onto glass filter were transferred into Pony scintillator vials and scintillant liquid was added (Optiphase Supermix, Wallac) and gently mixed until filter was dissolved.
- Radioactivity was counted in a scintillation counter using the [^{3}H]-thymidine programme.
- Data were printed and transferred to Microsoft Excel.

2.2.7 Solid phase Enzyme-Linked Immunosorbance Assay (ELISA)

Enzyme-linked Immunosorbance assay (ELISA) for cytokines was performed according to the manufacturers' instructions. In brief, a standard curve of the appropriate antibody was added to the pre-coated ELISA plates, along with the same volume of cell culture supernatants (either neat or diluted). Plates were incubated at room temperature and then washed three times with the appropriate washing solution included in the kit.

Plates were then incubated with a biotinylated polyclonal antibody. During the first incubation, the cytokine antigen binds simultaneously to the immobilised (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site.

After removal of excess secondary antibody, streptavidin-horseradish peroxidase (HRP) conjugate was added. This bound to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a

substrate solution [tetramethylbenzidine (TMB) plus hydrogen peroxide (H_2O_2)] was added, which was acted upon by the bound enzyme to produce colour. Plates were then incubated for 30 minutes in the dark until colour developed. The intensity of this colour product was proportional to the concentration of cytokine present in the original culture supernatant. The colour reaction was terminated with a stop solution provided by the manifacturer. The plate was then analysed by an ELISA reader.

The minimum detectable dose of the cytokine measured was 4 pg/ml.

Total serum IgE was measured by the Regional Immunology Laboratory, Southampton General Hospital by enzyme-linked immunosorbent assay (ELISA) using a monoclonal anti-IgE antibody developed in-house and a commercially available antibody (Dako, High Wycombe, UK).

CHAPTER THREE

IL-4 and allergic inflammation – Effects of IL-4 inhibition on cytokine profile

3.1 IL-4 AND ALLERGIC INFLAMMATION

One of the most important cytokines involved in the pathogenesis of allergic inflammation is represented by IL-4. Because of its pleiotropic effects on both inflammatory and structural cells in the airways, IL-4 has been viewed as a potential target for treatment. A particularly desirable effect of such treatment would be the inhibition of differentiation of uncommitted Th0 cells into Th2 cells (Hsieh *et al.* 1992; Seder *et al.* 1992) and survival of Th2 cells (Vella *et al.* 1997).

3.1.1 IL-4 receptor (IL-4R)

Human studies and experimental animal models clearly show the importance of Th2-derived cytokines in the pathophysiology of allergic asthma (Barnes *et al.* 1998; Chung *et al.* 1999). Therapeutic strategies that target the key Th2 cytokines have been the subject of intensive investigation (Anderson *et al.* 1994). Although many of the described Th2 cytokines contribute to the pathogenesis of allergic inflammation, several studies to date have identified IL-4, IL-5 and IL-13 as pivotal in the orchestration of the allergic inflammation, and the blockade of their action may represent useful therapeutic targets in the management of atopic conditions (Tournoy *et al.* 2002).

Signalling by IL-4 involves its interaction with receptors consisting of a heterodimer of high-affinity (Kd 20-300 pM) IL-4Rα and either the gamma common (γc) chain or the IL-13 receptor α chain (Jiang *et al.* 2000).

IL-4 receptors are found on T cells, B cells, mast cells, basophils, eosinophils, macrophages, fibroblasts, endothelial cells, hepatocytes, keratinocytes, stromal cells and neuroblasts (Ohara *et al.* 1987; Lowenthal *et al.* 1988; Dubois *et al.* 1998; Nelms *et al.* 1999). Although homodimerisation of the IL-4Rα chain can result in the generation of biochemical signals

within the cell (Lai *et al.* 1996; Fujiwara *et al.* 1997), physiological signalling depends upon IL-4-mediated heterodimerisation of the IL-4Rα chain with γc (Russell *et al.* 1993) (Figure 3.1-1).

The IL-4Rα chain also functions as a component of the IL-13 receptor (IL-13R) (Hilton *et al.* 1996; Murata *et al.* 1998), although IL-13 appears not to utilize the γc chain. Rather, its receptor employs other cell surface polypeptides, the IL-13Rα and IL-13Rα1 chains, in place of γc (Hilton *et al.* 1996)(Figure 3.1-1).

Structural alterations in the IL-4R extracellular region may result in altered receptor signalling capabilities, as shown by a variant of the human IL-4Rα chain isolated from atopic individuals containing Ile50Val substitution that has been shown to enhance signal transduction resulting in the increased production of IgE (Mitsuyasu *et al.* 1999).

3.1.1.1 Activation of Signal Transduction by the IL-4R

Ligand-induced dimerization (or multimerization) of cytokine receptors results in the activation of tyrosine kinases that initiate signalling cascades (Reichel *et al.* 1997). Neither the IL-4Rα nor the γc chain has endogenous kinase activity; rather, the IL-4R requires recruitment of non-receptor tyrosine kinases for the initiation of signal transduction such as the Janus-family (Jak) tyrosine kinases (Ihle 1995). Three members of the Janus kinase family, Jak-1, Jak-2, and Jak-3, are activated in response to IL-4R engagement and associate with components of the IL-4R complex. Jak-1 associates with the IL-4Rα chain, while Jak-3 associates with the γc chain (Figure 3.1-1) (Miyazaki *et al.* 1994).

Activation of IL-4R-associated kinases leads to the tyrosine phosphorylation of the IL-4Rα chain itself. The conserved tyrosine residues in the cytoplasmic region of the IL-4R are

potential sites of phosphorylation and interaction with downstream signalling proteins through Src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domains within these molecules (Nelms *et al.* 1999). The IL-4Rα chain cytoplasmic region has three functionally distinct domains, one that acts as an interaction site for Jak, required for activation of proliferative pathways, one that confers protection from apoptosis, and a third domain involved in the activation of pathways leading to induction of gene expression (Nelms *et al.* 1999)(Figure 3.1-2).

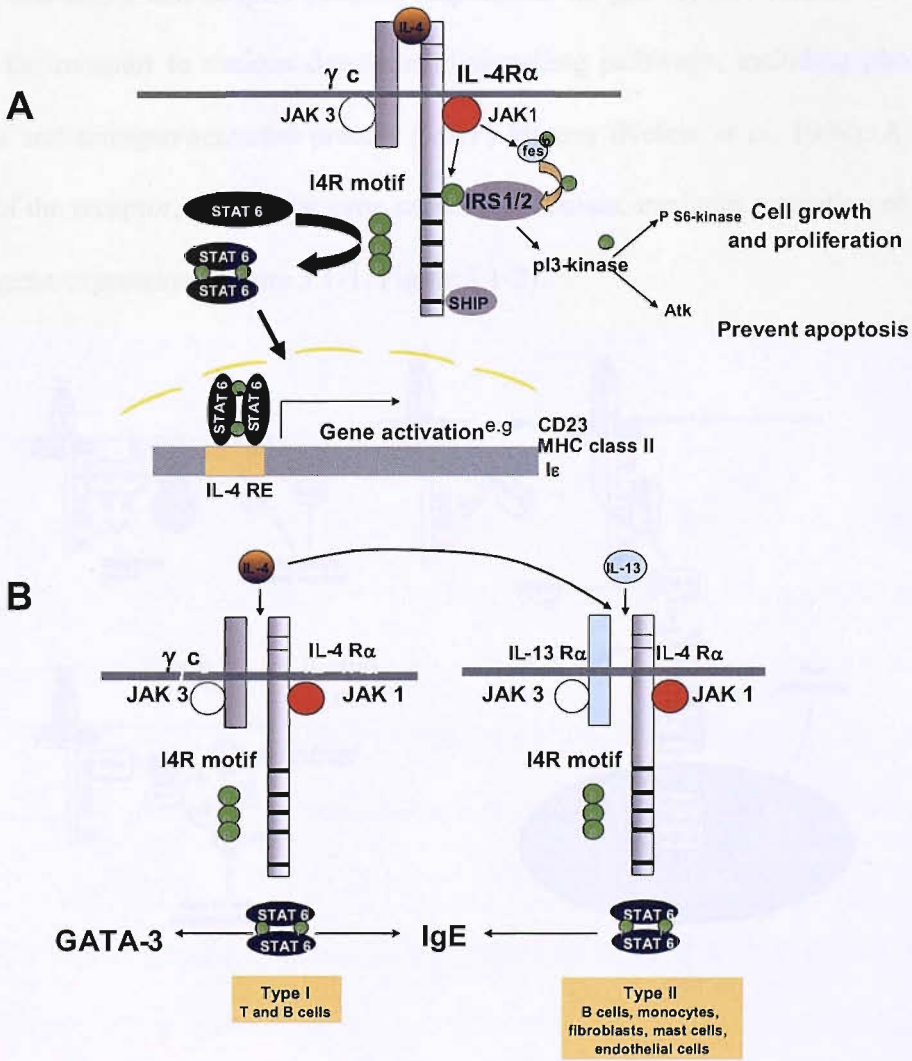


FIGURE 3.1-1: *IL-4 receptor: a) effects of IL-4 on cell proliferation, cytokine production; b) cross-binding with IL-13R.*

3.1.1.2 IL-4R Signalling Pathways

Signal transduction through IL-4R uses multiple signalling pathways to induce cell growth and survival, cell proliferation, and differentiation.

The region of the receptor proximal to the membrane contains sequences, which bind Jak-1 (bound to IL-4Rα) and Jak-3 (bound to γc). Distal to this region is the I4R motif, a region of sequence homology found in the insulin-like growth factor-1(IGF-1). This motif and its central tyrosine are essential for the tyrosine phosphorylation of insulin receptor substrates (IRS)-1 and IRS-2 and adaptor proteins responsible for growth activities of the receptor by linking the receptor to various downstream signalling pathways, including phosphoinositol 3-kinase and mitogen-activated protein (MAP) kinases (Nelms *et al.* 1999). A more distal region of the receptor, termed the gene expression domain, mediates activation of STAT6 and related gene expression (Figure 3.1-1, Figure 3.1-2).

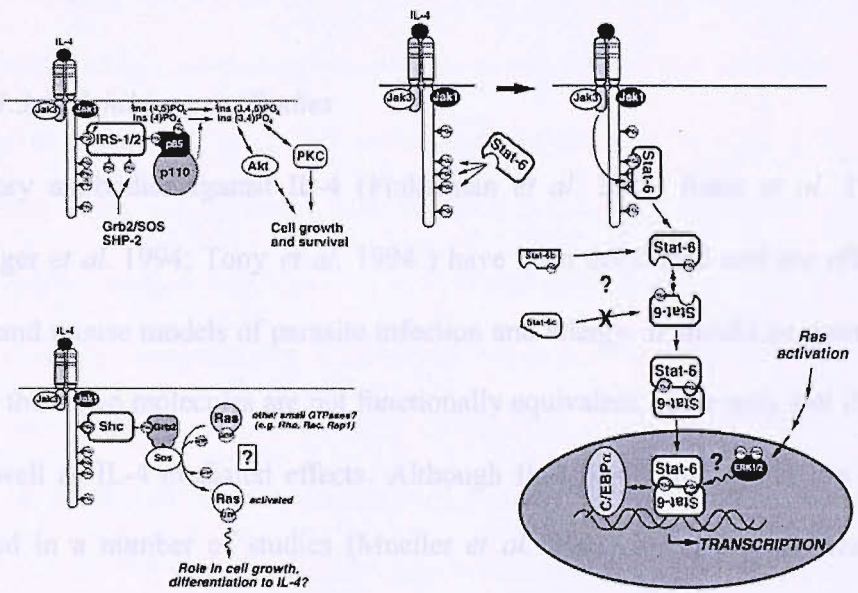


FIGURE 3.1-2: *Activation of signalling pathways through the I4R motif of the IL-4R and STAT 6. (Left panel) Activation of IRS leads to 1. Cell growth and proliferation 2. inhibition of apoptosis. (Right panel) Activation of STAT6 leads to trascription of IL-4-specific genes. From (Wills-Karp 1999).*

3.1.1.3 Inhibitors of IL-4: antibodies and IL-4 antagonists

Once IL-4 and IL-13 have been produced during allergic sensitisation or upon allergen challenge, the inhibition of the receptor–ligand interaction may offer the most straightforward approach to interfere with the biological effects of IL-4. Either the ligand or the receptor subunits could be targets. Blocking the receptor γc is problematic since this receptor chain is shared by IL-2, IL-7, IL-9, IL-15, and IL-21 (Sugamura *et al.* 1996), which implies that severe side effects can be anticipated. In fact, the γc chain gene, which is localised to human chromosome Xq13, plays a vital role in thymic maturation of T cells and mutations cause human X-linked severe combined immunodeficiency (XSCID) characterised by a complete or profound T cell defect (Noguchi *et al.* 1993; Russell *et al.* 1993). Among the cytokines sharing the γc chain, at least IL-7 is essentially involved in early T cell development (Noguchi *et al.* 1993; Russell *et al.* 1993).

3.1.1.3.1 Inhibitory antibodies

Inhibitory antibodies against IL-4 (Finkelman *et al.* 1988; Renz *et al.* 1996) and IL-4R (Fluckiger *et al.* 1994; Tony *et al.* 1994) have been developed and are efficient in cellular assays and mouse models of parasite infection and allergy. It should be noted that antibodies against these two molecules are not functionally equivalent, since only anti IL-4R inhibits IL-13 as well as IL-4 mediated effects. Although IL-4/IL-4R interaction has been effectively inhibited in a number of studies (Mueller *et al.* 2002), there are concerns that anti-IL-4 antibodies can potentially increase the biological effects of IL-4. Indeed, injection of mice with mixtures of IL-4 and neutralizing anti-IL-4 mAb, at a cytokine/anti-cytokine mAb molar ratio of approximately 2:1, has been shown to enhance and prolong the *in vivo* IL-4 activity. The stimulatory effect of IL-4-containing complexes could be blocked by increasing the ratio

of anti-IL-4 mAb to IL-4 by injection of anti-IL-4R mAb. These observations are most consistent with the possibility that anti-IL-4 mAb acts as carrier protein that increases the *in vivo* half-life of IL-4 by different means such as preventing its excretion, slowing down its renal clearance, protecting the cytokine from proteolytic digestion, and possibly by preventing modification of its active site (Finkelman *et al.* 1993). In contrast, blocking IL-4R is always inhibitory unless a conscious effort is made to induce homodimerization of the receptor, which may lead to non-physiological activation (Lai *et al.* 1996; Reichel *et al.* 1997).

Blocking IL-4 could introduce a completely new range of treatment for allergy affecting the cytokine network. In a murine model of asthma, IL-4 blocking antibodies inhibit allergen-induced BHR (Corry *et al.* 1996), goblet cell metaplasia, and pulmonary eosinophilia (Gavett *et al.* 1997). Studies employing a mouse-human chimera have been useful in allowing the *in vivo* study of the components of the human immune system relevant to asthma. Mouse anti-IL-4 monoclonal antibodies (mAb) have been shown to be capable of preventing both eosinophilia and IgE production when given during the allergen sensitisation phase (but not when given during the secondary immune responses) (Coyle *et al.* 1995; Tournoy *et al.* 2002). This effect was confirmed with human anti-IL4 mAb and following administration of an IL-4 mutant protein, which acts as an IL-4 and IL-13 receptor antagonist in HuPBLSCID (mice created by intraperitoneal injection of human peripheral blood lymphocytes into SCID mice) models (Kilchherr *et al.* 1993; Spiegelberg *et al.* 1994; Carballido *et al.* 1995; Carballido *et al.* 1995). Accordingly, IL-4 receptor blockade with a monoclonal antibody inhibits allergen-induced airway responses. No changes in IFN-γ concentrations and, thus, in Th1 activity were observed, suggesting a selective inactivation of the Th2 driven inflammatory response (Gavett *et al.* 1997).

Antibodies against IL-4R are a promising option. In a mouse model of allergen-induced BHR, anti-IL-4R antibody blocked IgE production, prevented development of BHR, and attenuated airway eosinophilia in some (Gavett *et al.* 1997) but not in other studies where the combined administration of anti-IL-4 and anti-IL-5 mAb was necessary to block IgE production, airway eosinophilia and BHR (Kurup *et al.* 1997; Tanaka *et al.* 1998).

The administration of murine antibodies in humans, however, almost always elicits an immune response resulting in the production of neutralising antibodies against the administered antibodies (Wong *et al.* 2000), necessitating the reduction of immunogenicity by protein engineering (humanisation) or production of non-immunogenic human antibodies by phage display technology.

3.1.1.3.2 Cytokine antagonists

Cytokine antagonist analogues are designed by mutating a binding site with the intention to create a variant that is still able to bind one receptor subunit but not the other. Antagonistic mutants cannot activate the receptor and are highly specific inhibitors. In the case of IL-4, mutation of amino acids in the binding site for γc leads to the creation of antagonistic variants. An antagonist of human IL-4 was discovered after Tyr124 of the recombinant IL-4 protein had been substituted by an aspartic acid residue (Kruse *et al.* 1992; Kruse *et al.* 1993). This IL-4 variant, Y124D, bound with high affinity to the IL-4 receptor (K_d = 310 pM) but retained no detectable proliferative activity for T-cells and inhibited IL-4-dependent T-cell proliferation competitively (K_i = 620 pM). The loss of efficacy in variant Y124D was estimated to be greater than 100-fold on the basis of a weak partial agonist activity for the very sensitive induction of $CD23^+$ B-cells (Kruse *et al.* 1992; Kruse *et al.* 1993). Initially, three amino acids located close to the C-terminus were identified as particularly sensitive

toward exchanges: R121, Y124 and S125 (Kruse *et al.* 1992; Kruse *et al.* 1993). Simultaneous mutation of R121 and Y124 to aspartic acid residues (R121D/Y124D) creates a protein that has a high binding affinity for IL-4R, no detectable biological activity, and which completely inhibits both IL-4 and IL-13 signalling (Tony *et al.* 1994).

The analogous amino acids in mouse IL-4 are Q116 and Y119. The variant Q116D/Y119D corresponding to the human antagonist is a complete antagonist for IL-4 and IL-13 (Grunewald *et al.* 1997; Grunewald *et al.* 1998). Inhibitory activity is also created by the single point mutation Y119D or by truncating mouse IL-4 after residue 118. Mutations in the C-terminus region of the IL-4 protein produce IL-4 mutants that bind to the IL-4R alpha-chain with high affinity, but do not induce cellular responses. A murine IL-4 mutant (C118 deletion) protein (IL-4R antagonist) inhibited IL-4- and IL-13-induced STAT6 phosphorylation as well as IL-4- and IL-13-induced IgE production *in vitro*. Administration of murine IL-4R antagonist during allergen (ovalbumin) challenge inhibited the development of allergic airway eosinophilia and BHR in mice previously sensitized with OVA (Tomkinson *et al.* 2001). The inhibitory effect on airway eosinophilia and BHR was associated with reduced levels of IL-4, IL-5 and IL-13 in the BAL fluid as well as reduced serum levels of OVA-IgE. These observations demonstrate the therapeutic potential of IL-4 mutant protein receptor antagonists that inhibit both IL-4 and IL-13 in the treatment of allergic asthma (Tomkinson *et al.* 2001).

BALB/c mice sensitised to ovalbumin have also been treated with a mutated IL-4 variant (Q116D, Y119D) during the sensitisation phase or alternatively after ovalbumin allergy was established (Hahn *et al.* 2003). The inhibition of the IL-4/IL-13 system during allergic sensitisation resulted in a dose-dependent reduction of OVA-specific IgE and inhibition of airway eosinophilia together with decreased IL-5 levels and decreased numbers of IL-4-secreting $CD4^+$ T cells. Moreover, goblet cell metaplasia and airway responsiveness to

methacholine could be reduced significantly by the IL-4/IL-13 inhibitor. However, the inhibition of the IL-4/IL-13 system at various time points after allergy was established showed only little effect on all measured allergic parameters (Hahn *et al.* 2003).

Another approach is blockade of IL-4 receptors with a mutated form of IL-4 (BAY 36-1677), that binds to IL-4Rα and IL-13Rα1, thus blocking both IL-4 and IL-13 (Shanafelt *et al.* 1998; Srivannaboon *et al.* 2001). Indeed, IL-4.Y124D, a macromolecular IL-4 mutant resulting from the replacement of Tyr124 by aspartic acid, has been found to bind with high affinity to the IL-4 receptor and inhibit IL-4-dependent T-cell proliferation (Kruse *et al.* 1992) as well as IL-4/IL-13-induced IgE synthesis (Aversa *et al.* 1993).

Alternative splicing of mRNA can generate protein isoforms that are preferentially expressed in different tissues or during different states of cell differentiation or activation. Protein isoforms may have different functions. A naturally occurring splice variant of human IL-4, called IL-4δ2 is characterised by the lack of the second exon of IL-4 by alternative splicing, with exons 1, 3, and 4 joined in an open reading frame. IL-4δ2 is preferentially expressed in the thymus and airways and, in contrast to IL-4, does not act as a costimulator for T cell proliferation, in fact, it can inhibit T cell proliferation function of complete IL-4. The balance between IL-4 and IL-4δ2 may be important in the regulation of IL-4 effects (Atamas *et al.* 1996).

3.1.1.3.3 Animal studies and clinical trials

Mutant IL-4 molecules have been developed which act as antagonists of IL-4-induced Th2 cell proliferation, differentiation and signal transduction (Kruse *et al.* 1993; Grunewald *et al.* 1997; Grunewald *et al.* 1998; Shanafelt *et al.* 1998; Tournoy *et al.* 2002). The value of IL-4 antagonists has been demonstrated in mice, where the application of antagonists created by

mutating murine IL-4 prevented the development of allergic reactivity (Grunewald *et al.* 1998). Allergic responses (e.g. antigen-specific IgE and IgG_1, skin response to intradermal antigen, and systemic anaphylactic shock upon intravenous application of the antigen) were absent if mice had been treated with the antagonistic mouse IL-4 variant Q116D/Y119D at the time of sensitisation (Grunewald *et al.* 1998). Similar effects of inhibition of IL-4/IL-13-induced STAT6 phosphorylation, IgE production, airway eosinophilia, Th2 cytokine production and BHR were achieved in studies using another murine IL-4 antagonist created by deleting the C-terminal amino acids following Q119 (Tomkinson *et al.* 2001).

These data confirm the findings of decreased bronchial hyperresponsiveness and reduced infiltration of eosinophils following the application of a human IL-4 antagonist in a monkey *(Macaca fasciculata)* model of allergic asthma (R. Gundel, W. Sebald *et al.*, unpublished observations) (Mueller *et al.* 2002).

The antagonistic IL-4 mutant R121D/Y124D has now entered a phase II clinical trial where it is being evaluated for the treatment of allergic asthma.

3.1.1.4 Inhibitors of IL-4: soluble IL-4R

Soluble cytokine receptors naturally arise from genes encoding membrane-bound receptors or are direct derivatives of the receptors themselves (Fernandez-Botran 2000). The extracellular domains of cytokine receptors are produced biologically by shedding (Jung *et al.* 1999) or through specific mRNAs generated by differential splicing (Heaney *et al.* 1996). In many cases, soluble receptors appear to play an integral part in the dynamic interaction between ligands and their membrane-bound receptors, and dysregulated expression of soluble receptors can contribute to certain human diseases (Chilton *et al.* 1997; Heaney *et al.* 1998).

Soluble forms of cytokine receptors normally participate in the control of cytokine activity *in vivo* by inhibiting the ability of cytokines to bind to their membrane receptors, thereby preventing them from generating a biological response. The ability of soluble cytokine receptors to act as cytokine inhibitors, coupled to their specificity, high affinity and low immunogenicity, has prompted considerable interest in their use as immunotherapeutic agents (Fernandez-Botran 2000). Although early clinical trials of soluble receptors have shown unexpected toxicities, their application in medicine continues to advance, and it is likely that soluble receptors will join hormones, cytokines and growth factors as established biological therapies as soluble receptor-derived binding proteins offer the advantage that these are physiological proteins that should not elicit an immune response.

Studies in mice have shown that after stimulation of naïve lymphoid cells with IL-4, soluble IL-4 receptor (sIL-4R) release was dependent on up-regulation of spliced IL-4R mRNA, as shown by inhibition with specific antisense oligonucleotides. In contrast, TcR stimulation with anti-CD3 mAb of IL-4-deficient mice led to IL-4-independent sIL-4R production through proteolytic shedding of membrane-bound IL-4R, as demonstrated by the absence of an increase in the spliced IL-4R mRNA and no inhibitory influence of antisense oligonucleotides (Blum *et al.* 1996).

Soluble IL-4R occurs naturally in both animals (Chilton *et al.* 1997; Hannen *et al.* 1999; Kruse *et al.* 1999) and humans (Sato *et al.* 1993; Hannen *et al.* 1999; Jung *et al.* 1999). The soluble binding protein is, therefore, able to compete with the cell-bound receptor for its ligand, and is highly efficient in blocking the effects of IL-4. However, soluble IL-4R does not bind IL-13 to a measurable extent, and under some circumstances may potentiate IL-4 effects due to the same mechanism as observed for inhibitory anti-IL-4 antibodies (Mueller *et al.* 2002). Secreted forms of IL-4Rα occur naturally and are expressed in allergic

inflammation. Soluble IL-4R is capable of interacting with IL-4 and because it lacks the transmembrane and cytoplasmic domains (Figure 3.1-3), it does not induce cellular activation but instead sequesters IL-4, serving as an anti-inflammatory mechanism that can counter the effects of IL-4 (Figure 3.1-3). This mechanism might represent an endogenous autoregulatory or homeostatic mechanism. *In vivo*, exogenous sIL-4R has been shown to have both agonistic and antagonistic effects on IL-4 responses, depending on the relative concentration ratios of sIL-4R to IL-4 (Sato *et al.* 1993; Jung *et al.* 1999).

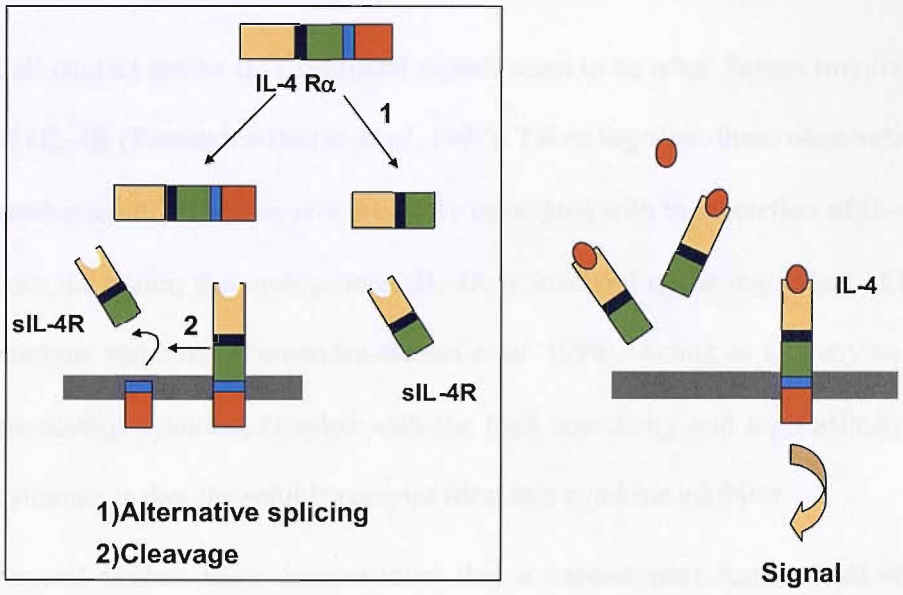


FIGURE 3.1-3: *Generation of cytokine soluble receptors*

Soluble receptors are formed by alternative mRNA splicing that gives rise to a polypeptide lacking a transmembrane region that is then secreted from the cell or by proteolitic cleavage of membrane bound receptor proteins. SIL4R appears to be generated by both mechanisms.

In an effort to elucidate the potential role of endogenous sIL-4R in the regulation of IL-4 responses, the mechanisms controlling the production of sIL-4R have been investigated. Although many cell types are able to constitutively secrete low levels of sIL-4R, its

production is significantly up-regulated *in vitro* upon T cell activation and stimulation with IL-4 (Blum *et al.* 1996; Fernandez-Botran *et al.* 1997). Serum levels of sIL-4R have consistently been found to be increased during a Th2 immune responses as consequence of the IL-4-mediated up-regulation of sIL-4R transcription (Chilton *et al.* 1997), both in the presence and absence of antigenic stimulation (Fernandez-Botran *et al.* 1997). However, inhibition of endogenous IL-4 with a neutralising anti-IL-4 mAb achieved only a modest (~ 20%) inhibitory effect on sIL-4R production, and the stimulation of Th1 clones with rIL-4 resulted only in a two-fold increase in sIL-4R levels, suggesting that IL-4 is not the only factor that regulates sIL-4R production.

Cell contact and/or IL-1 mediated signals seem to be other factors involved in the production of sIL-4R (Fernandez-Botran *et al.* 1997). Taken together, these observations suggest that the production of sIL-4R *in vivo* is closely associated with the secretion of IL-4 and are consistent with the notion that endogenous sIL-4R is involved in the regulation of IL-4 activity during immune responses (Fernandez-Botran *et al.* 1996). Acting as a decoy to bind and neutralise circulating cytokine, coupled with the high specificity and high affinity of binding for the cytokine, makes the soluble receptor ideal as a cytokine inhibitor.

Several studies have demonstrated that a recombinant form of sIL-4R acts as an IL-4 inhibitor, regulating cytokine production in experimental models of Th2-mediated infectious diseases. In a *Leishmania major* model, *L. major*-specific Th2 clones could be inhibited by recombinant murine sIL-4R in a concentration-dependent fashion; and treatment of *L. major*-infected BALB/c mice with recombinant sIL-4R rendered the animals clinically resistant to *L. major* and shifted the pattern of cytokines towards a Th1 type, providing resistance against reinfection (Gessner *et al.* 1994). Similarly, in a model of systemic candidiasis, mice treated with sIL-4R showed persistent ablation of circulating IL-4 detected by ELISA which was

associated with a cure rate of >90% in otherwise lethally infected mice and a shift from a predominant Th2 to a Th1 pattern of reactivity (Puccetti *et al.* 1994).

Soluble IL-4R has also been studied in mouse allograft models, where it has been shown to prevent rejection and IgE response to anti-IgD treatment (Maliszewski *et al.* 1992; Maliszewski *et al.* 1994), and more extensively in allergic diseases both in animal models and recently in human asthma.

In mouse models of allergic diseases, sensitized mice treated with sIL-4R administered in parallel to the sensitisation protocol developed significant suppression of anti-OVA IgE and anti-OVA IgG_1 antibody production. Allergen-specific immediate cutaneous hypersensitivity responses in mice was inhibited in a dose-dependent manner. Treatment with intraperitoneal sIL-4R also prevented expansion of Vβ8.1/8.2 T cells in OVA-treated animals (Sato *et al.* 1993; Renz *et al.* 1995; Renz *et al.* 1996). Local administration to the lungs of sIL-4R by aerosolization not only decreased the IgE/IgG1 responses to OVA but also reduced total serum IgE levels (Renz *et al.* 1996). *In vitro* studies have also demonstrated that murine sIL-4R and dimeric sIL-4R fusion protein significantly reduce allergen-specific polyclonal IgE production by lymphocytes obtained from allergen-sensitized mice (Renz *et al.* 1995). These effects were comparable to the findings following treatment with monoclonal anti-IL-4 antibody (Renz *et al.* 1995; Renz *et al.* 1996).

In similar models, elevating airway levels of sIL-4R through the administration of exogenous sIL-4R is effective in blocking the late phase pulmonary inflammation and prevents the development of airway inflammation (Henderson *et al.* 2000). However, sIL-4R did not reduce BHR in response to methacholine challenge. These findings were confirmed in human *in vitro* studies using PBMC from atopic donors where sIL-4R suppressed the IL-4-induced IgE synthesis and sCD23 secretion of PBMC (Konig *et al.* 1995).

Soluble IL-4R seems to play an important role in human allergic diseases. It has been shown that sIL-4R levels are increased in BAL of asthmatic patients when compared to controls (Fitch *et al.* 2003) and its levels in the nasal fluid are increased during the pollen season in patients with seasonal allergic rhinitis (Benson *et al.* 2000). A weak positive correlation was also found with IgE and eosinophils (Benson *et al.* 2000).

Soluble recombinant human IL-4 receptor (rhuIL-4R; Nuvance™; Immunex) is the extracellular portion of human IL-4Rα. Because the amino acid and the glycosylation sequences are identical to those of human IL-4R, soluble receptors are relatively nonimmunogenic. This is in contrast to chimerised or humanised monoclonal antibodies, which retain some murine sequences, or IL-4 muteins that are not authentic. However, it is not known whether and to what extent sIL-4R can inhibit allergen-induced T cell responses in humans.

Preclinical studies have shown that sIL-4R is safe and effective in the treatment of patients with asthma (Borish *et al.* 1999; Borish *et al.* 2001). A single nebulised dose of sIL-4R prevents the fall in lung function induced by withdrawal of inhaled corticosteroids in patients with moderately severe asthma (Borish *et al.* 1999). Subsequent studies have demonstrated that weekly nebulisation of sIL-4R improves asthma control over a 12-week period (Borish *et al.* 2001).

In a phase I study, subjects with mild or moderate persistent asthma treated with sIL-4R had significantly better FEV_1, improved symptom scores and reduced β_2-agonist use and BHR to methacholine. From these studies, it may be speculated that the action of sIL-4R in inhibiting inflammation at a key regulatory point, may ameliorate the long-term disease progression in asthma.

3.2 AIM

I hypothesised that IL-4 is critical for the differentiation, proliferation and survival of Th2 cells and that its presence is required within days of contact of T cells with allergen. To address this hypothesis, I first studied the direct effects of recombinant IL-4 (rIL-4) on IL-5 generation by peripheral blood mononuclear cells (PBMC) from allergic asthmatics. Focusing thereafter on allergen-driven responses, the kinetics of IL-5 and IL-13 synthesis by PBMC stimulated with lipolysaccharide (LPS)-free house dust mite allergen, *Dermatophagoides pteronyssinus (Der p)*, was investigated. I sought to inhibit the synthesis of these two cytokines and reduce T cell proliferative responses to allergen by neutralising IL-4 using the soluble recombinant human IL-4 receptor (rh-sIL-4R) obtained by cloning and expression in a mammalian system and consisting of the extracellular portion of human IL-4Rα (Sato *et al.* 1993). The effectiveness of sIL-4R was compared with that of neutralising antibodies against soluble IL-4 and its surface expressed receptor, respectively.

3.3 METHODS

3.3.1 Study Design

In the first experiments, which aimed at studying the direct effects of IL-4, PBMC from atopic asthmatics donors were stimulated with IL-4 for seven days, at which point IL-5 production was measured. In subsequent experiments, PBMC were stimulated with *Der p* for three or seven days in the presence or absence of human recombinant sIL-4R to investigate whether allergen-induced production of IL-5 and, additionally, IL-13 was also dependent on IL-4, and to define the kinetics of the response (Figure 3.3-1). In addition, the effects of sIL-4R on the production of IL-12 and the Th1 cytokine, IFN-γ, as well as T-cell proliferative responses

were studied. The inhibitory effects of sIL-4R were compared with those of neutralizing antibodies against both IL-4 itself or IL-4R expressed on cell surfaces.

The study was conducted in accordance with good clinical practice (GCP) and in keeping with the Helsinki Declaration. Approval was obtained from the Southampton and South-West Hampshire Local Research Ethics Committee, and subjects gave their written informed consent.

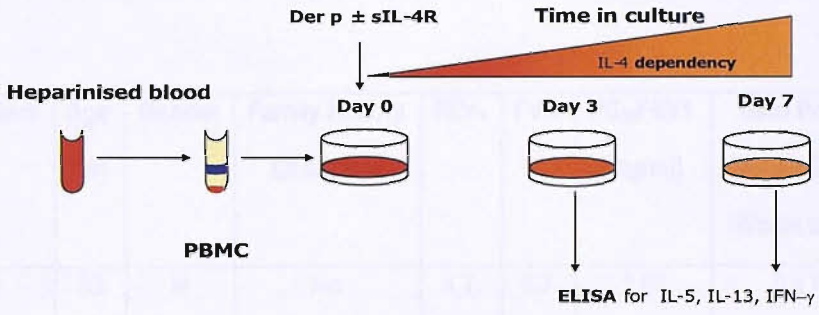


FIGURE 3.3-1: *Experimental design used for culturing allergen-stimulated PBMC with sIL-4R.*

3.3.2 Subjects

Eight atopic asthmatics (mean age 35 years, range 22-52 years) with mild asthma participated in the study. The subjects were non-smokers and had asthma requiring inhaled $\square_2$-agonists only. All were sensitive to *Der p* (Table 3.3-1). None of the subjects had suffered from a respiratory infection within 14 days of the study, required prescription medication, other than that for asthma, during the same period, or had used oral or inhaled corticosteroids within the previous six months.

Subject	Age (yr)	Gender	Family History Of asthma	FEV_1	FVC	$PC_{20}FEV1$ (mg/ml)	Skin Prick test House Dust Mite (Wheal area, mm)	Total IgE (IU/ml)
1	33	M	No	4.7	6.7	1.62	5 x 7 (35)	10
2	22	M	No	4.7	5.3	2.72	5 x 5 (25)	59
3	29	M	No	4.1	5.5	1.25	12 x 7 (84)	984
4	36	M	Yes	4.3	5.6	1.24	7 x 5 (35)	112
5	52	M	Yes	3.4	4.9	1.18	9 x 8 (72)	64
6	37	M	Yes	3.9	4.9	0.35	10 x 7 (70)	160
7	38	M	Yes	3.9	6.2	0.62	15 x 15 (225)	37
8	34	M	Yes	2.5	3.7	2.12	5 x 6 (30)	284

TABLE 3.3-1: *Subjects' description*

3.3.3 Peripheral blood mononuclear cells (PBMC)

Blood was drawn into heparinized tubes. PBMC were isolated, as described in 2.2.6.1. and 2.2.6.3.

Cells were cultured either in medium alone or in medium supplemented with LPS-free *Der p* extract (ALK) at a concentration of 5,000 SQ units/ml in the presence or absence of sIL-4R or

neutralizing anti-IL-4 or anti-IL-4R antibodies (both from R&D systems), at concentrations ranging from 0.1 to 10 μg/ml. Both antibodies and the sIL-4R were LPS-free as declared by the manufacturer. Culture supernatants were harvested and analysed for IL-5, IL-13, IL-12 and IFN-γ using commercial ELISA (Cytoscreen®; BioSource) whose detection limit was 4 pg/ml.

3.3.4 Proliferation assay

Proliferative responses of PBMC were studied in seven-day cultures by [^{3}H]-thymidine incorporation, as described in 2.2.6.4.

3.3.5Solid phase sandwich Enzyme Linked-Immunosorbance Assay (ELISA)

ELISA was performed as described in 2.2.7.

3.3.6 Statistics

All statistical analyses were performed using InStat® version 3 for Windows (GraphPad™ Software Inc.). Data were analysed for distribution with the Kolmogorov-Smirnov test. Data on cytokine levels passed the normality test and, therefore, repeated-measures ANOVA was used for comparisons among the groups. When differences were statistically significant, Dunnett's test for multiple comparisons was used for *post hoc* between-group analysis. For between-group analysis I also used Bonferroni's test for multiple comparisons. When multiple comparisons were not required, the paired Student *t*-test was employed. The Pearson's

correlation test was used for correlation analysis. For all tests $p<0.05$ was considered statistically significant.

3.4 RESULTS

3.4.1 Kinetics of IL-5 and IL-13 generation by allergen-stimulated PBMC

In order to determine the kinetics of cytokine generation, supernatants from PBMC stimulated with allergen were harvested at day two, three, five and seven post incubation. Optimal culture conditions for cytokine synthesis had been established in preliminary experiments where I studied the influence of type of medium (serum-free medium or medium supplemented with human AB serum), cell density and type of culture plate (*e.g.* flat versus round bottom). These preliminary experiments were largely based on more extensive previous work on PBMC cultures carried out as part of a PhD degree by Dr C. Promwong.

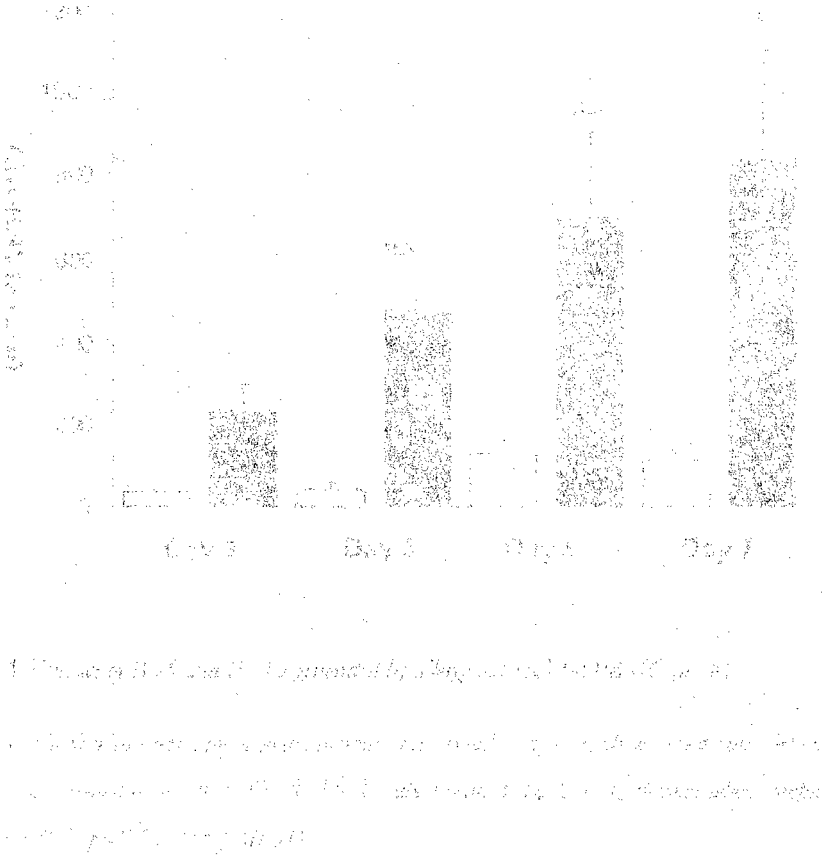
IL-5 and IL-13 synthesis increased with time, reaching statistically significant concentrations compared to day one after five days of culture ($p < 0.05$) and even more so after seven days ($p<0.01$). IL-13 levels reached statistical significance after seven days ($p<0.05$). From these preliminary experiments I chose day seven as the best time point for cytokine measurement (Figure 3.4-1).

3.4.2 Effects of rIL-4 on IL-5 synthesis

To test the hypothesis that IL-5 synthesis was directly induced by IL-4, I stimulated PBMC from five atopic asthmatic donors with recombinant human IL-4 (rIL-4) and assayed the culture supernatants for IL-5. Recombinant human IL-4 caused a concentration-dependent

increase in IL-5 production by PBMC which peaked at 20 nM. There was no further increase at 200 nM; in fact there was a slight reduction when compared with 20 nM ($p<0.05$) (Figure 3.4-2).

The ability of rIL-4 alone to induce IL-5 synthesis in the absence of allergen could be explained by the fact that this is an ex-vivo model where PBMC could be already primed. In this context, IL-4 may potentiate IL-5 transcription and prevent apoptosis without necessarily having an effect on clonal expansion and cell proliferation.



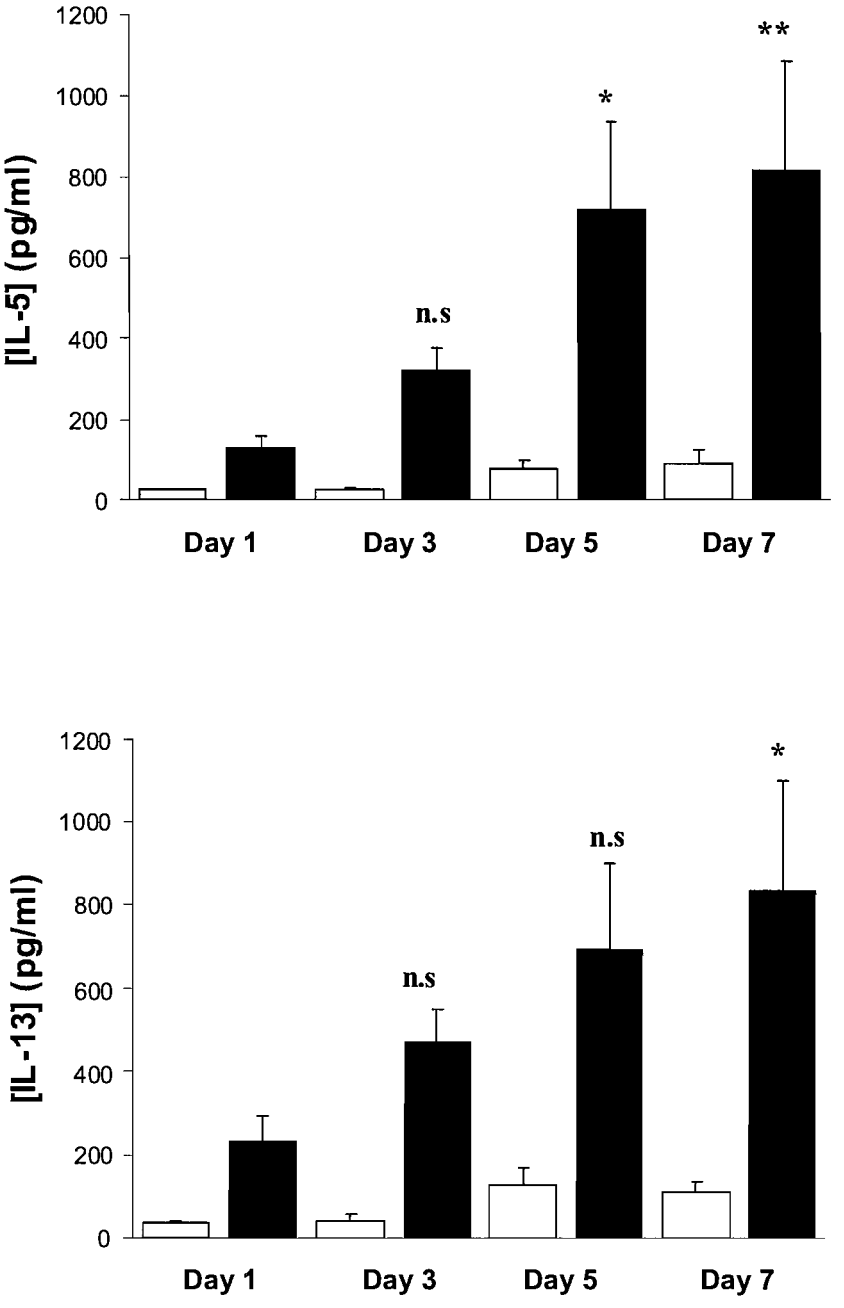


FIGURE 3.4-1: *Kinetics of IL-5 and IL-13 generated by allergen-stimulated PBMC (n=8).*

PBMC were stimulated with Dermatophagoides pteronyssinus (solid bars) or medium (open bars) for one, three, five and seven days. The results are shown as mean ± SEM. The P values indicate levels of significance when compared to day one Der p-stimulated culture (= $p<0.05$; **=$p<0.01$).*

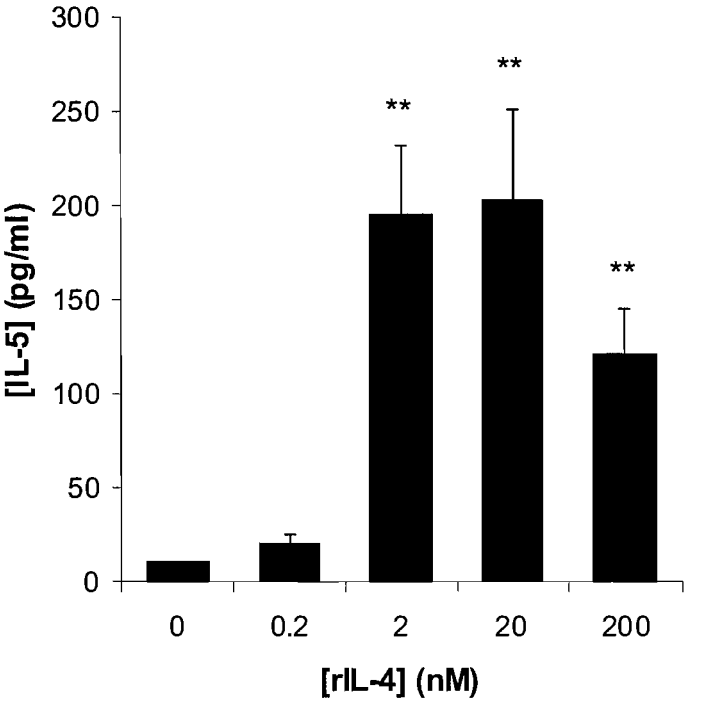


FIGURE 3.4-2: *The effect of rIL-4 on IL-5 synthesis (n=8). PBMC were stimulated for seven days with increasing concentrations of rIL-4. The results are shown as mean ± SEM. The P values indicate levels of significance when compared to control, Der p-stimulated culture (**=p<0.01).*

3.4.3Effects of sIL-4R on IL-5 and IL-13 synthesis induced by allergen

Stimulation with allergen caused a significant increase in IL-5 production after three and seven days ($p<0.001$ and $p<0.05$, respectively) (Figure 3.4-3, Figure 3.4-4). sIL-4R was ineffective at inhibiting IL-5 synthesis during the first three days of stimulation with allergen, but caused a significant concentration-dependent inhibition over the next four days which was maximal at 10 μg/ml (Figure 3.4-3, Figure 3.4-4). In contrast to IL-5, the production of IL-13

was significantly inhibited by sIL-4R already at day three, although the extent of inhibition was significantly greater over the whole seven-day period (Figure 3.4-5).

I investigated whether there was any correlation between IL-5 or IL-13 synthesis in response to allergen and the degree of inhibition induced by sIL-4R, with the aim of assessing whether individuals who produce more of either Th2 cytokine are more sensitive to IL-4 inhibition. There was a strong and highly significant positive correlation between the allergen-induced IL-13 measured in the absence of sIL-4R and the relative change in IL-13 seen in cultures to which sIL-4R was added ($r=0.9$; $p=0.004$) (Figure 3.4-7). In contrast, there was no such correlation for IL-5 (Figure 3.4-7).

3.4.4 The effects of neutralising antibodies for IL-4 and IL-4 receptor on IL-5 production

The effect of blocking free IL-4 or its membrane-expressed receptor with neutralising antibodies was compared with the degree of inhibition achieved with sIL-4R in seven-day cultures. Anti-IL-4 significantly blocked *Der p*-induced IL-5 synthesis ($p<0.05$), with mean inhibition of 61%, 69% and 75% at antibody concentrations of 0.1, 1 and 10 μg/ml, respectively (Figure 3.4-8). Anti-IL-4R caused comparable, concentration-dependent inhibition of the *Der p*-induced IL-5 synthesis ($p<0.05$), with mean inhibition of 45%, 54% and 60% at concentrations of 0.1, 1 and 10 μg/ml, respectively (Figure 3.4-8). The extent of inhibition with blocking antibodies was not significantly different from that achieved with sIL-4R.

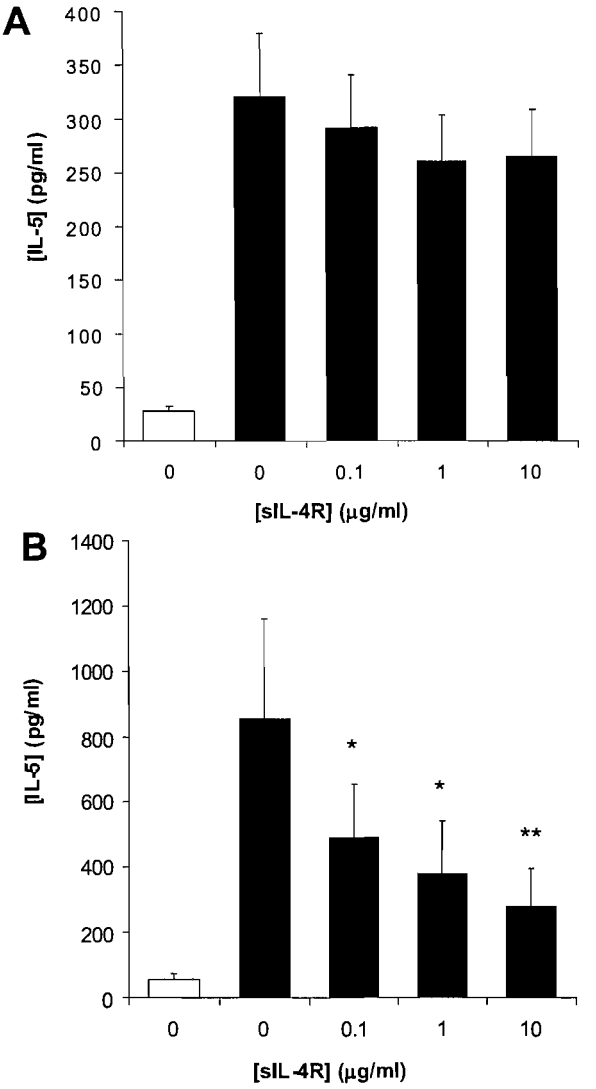


FIGURE 3.4-3: *The effect of sIL-4R on allergen-induced production of IL-5 (n=8). PBMC were either stimulated with 5,000 SQ units/ml Der p (closed bars) or cultured in medium alone (open bar) for three (A) and seven days (B). The results are shown as mean ± SEM. The P values indicate levels of significance when comparing each condition with control culture, i.e. stimulation with Der p in the absence of sIL-4R (*P<0.05; **P<0.01).*

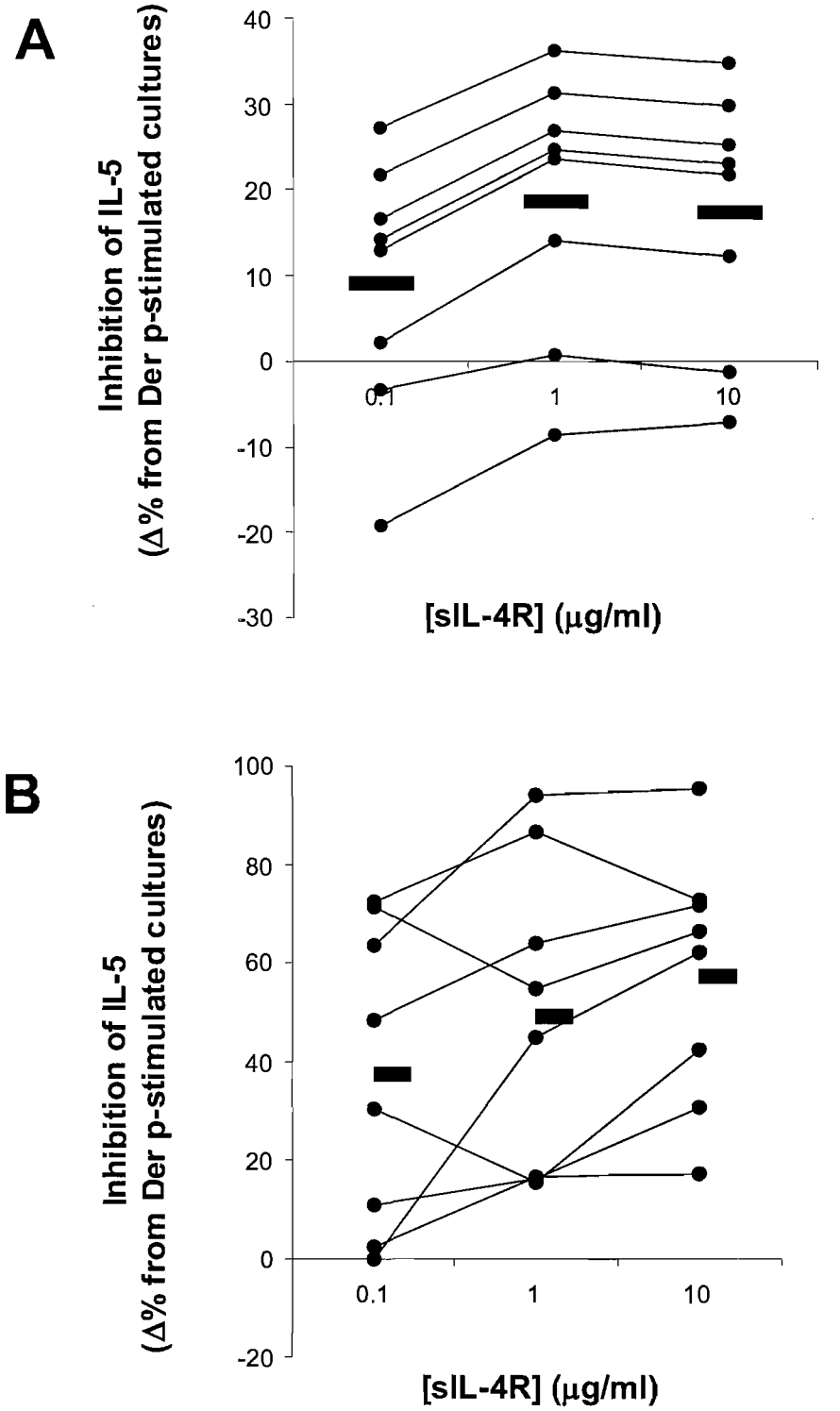


FIGURE 3.4-4: *Inhibition of IL-5 post treatment with sIL-4R (n=8). The Individual Δ% changes post-sIL-4R treatment at three days (A) and seven days (B) are shown as filled circles. Means are shown as horizontal bars.*

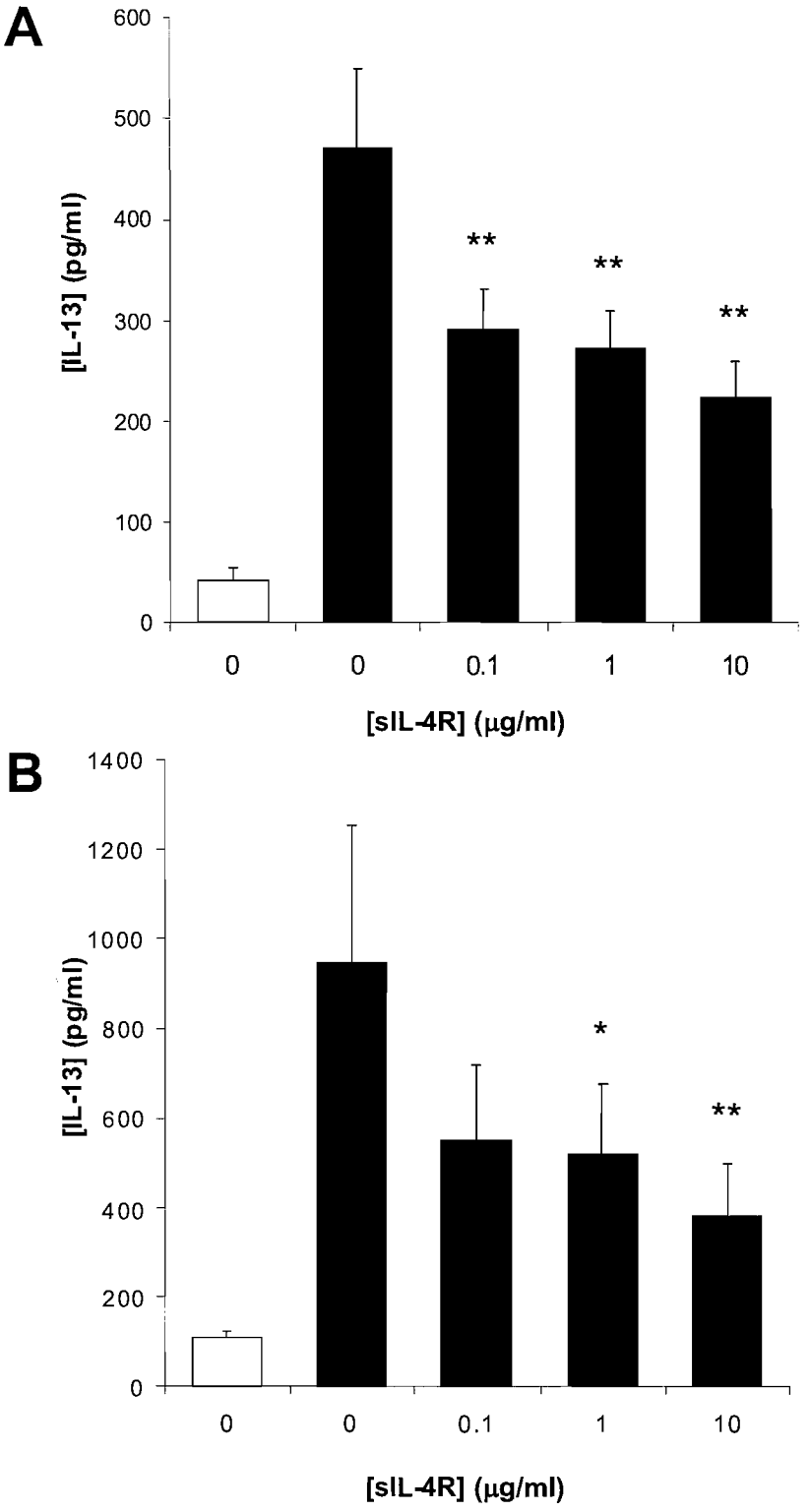


FIGURE 3.4-5: *The effect of sIL-4R on allergen-induced production of IL-13 (n=8). PBMC were stimulated with 5,000 SQ U/ml Der p (closed bars) or cultured in medium alone (open bar) for three (A) and seven days (B). The results are shown as mean ± SEM. The P values indicate levels of significance when comparing each condition with control culture, i.e. stimulation with Der p in the absence of sIL-4R (*P<0.05; **P<0.01).*

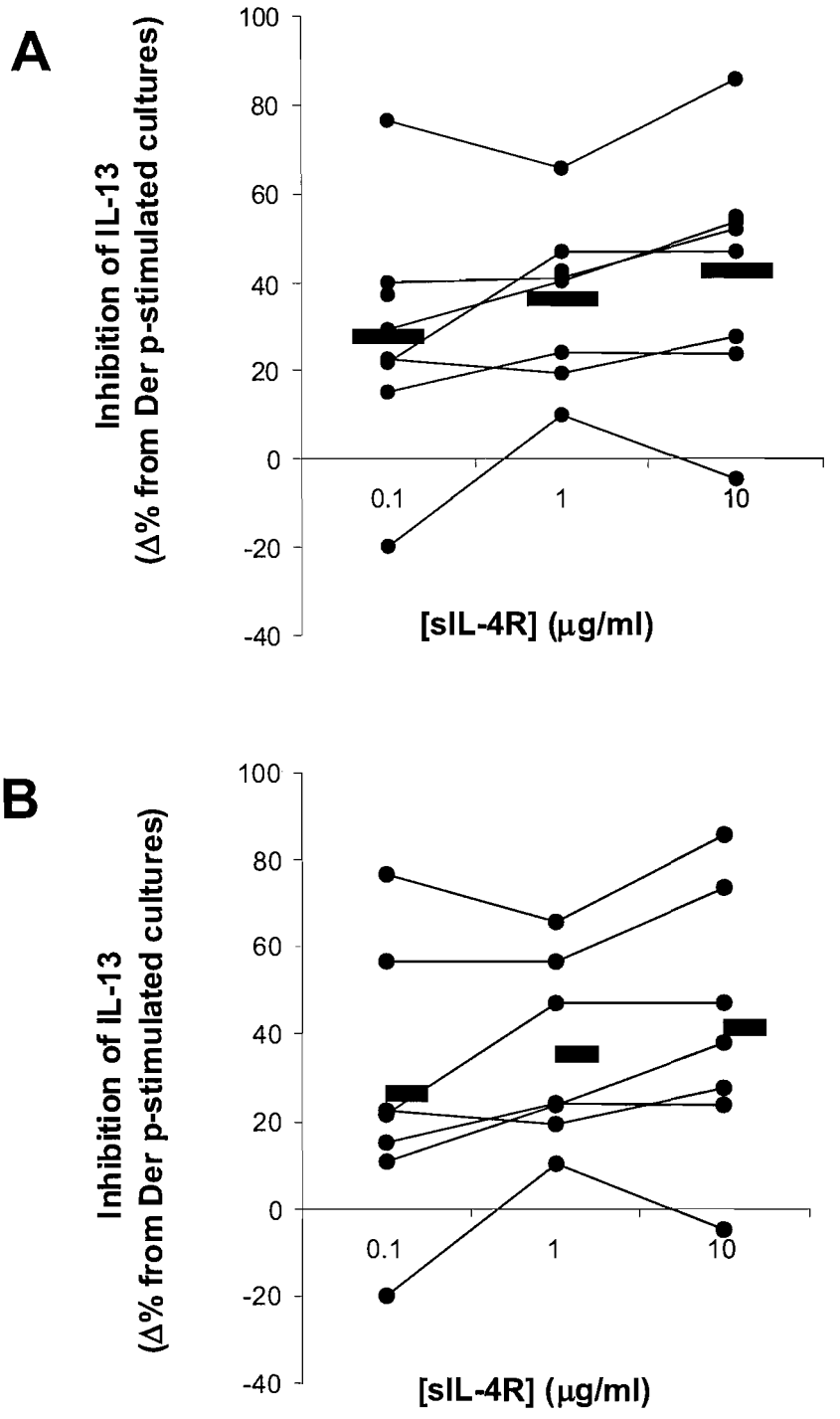


FIGURE 3.4-6: *Inhibition of IL-13 post treatment with sIL-4R (n=8). The Individual Δ% changes post-sIL-4R treatment at 3 days (A) and 7 days (B) are shown as filled circles. Means are shown as horizontal bars.*

3.4.5 The effects of sIL-4R on the production of IFN-γ

The modulatory effects of sIL-4R were further assessed by measuring IFN-γ production in seven-day cultures. Soluble IL-4R exerted a differential effect on Th2 and Th1 cytokines at low concentrations; whilst it caused significant ($p<0.05$) inhibition of *Der p*-induced release of IL-5 at 1 μg/ml, it did not alter IFN-γ production at the same concentration. Up-regulation of IFN-γ concentration was observed only when sIL-4R was used at 10 μg/ml (Figure 3.4-9); this caused a mean 2.6-fold increase in IFN-γ ($p<0.05$), (Figure 3.4-9). At this concentration sIL-4R caused a similar degree of inhibition of IL-5 and IL-13 (57.26% and 55.6%, respectively) (Figure 3.4-3, Figure 3.4-5, Figure 3.4-9).

3.4.6The effects of sIL-4R on IL-12 synthesis

Finally, I sought to elucidate whether the inhibition of IL-5 and IL-13 and the induction of IFN-γ were due to IL-12 synthesis. There was significant variability in the amounts of IL-12 produced, but overall there was no significant increase in IL-12 synthesis (Figure 3.4-9, Figure 3.4-10), suggesting that the neither the inhibition of Th2 cytokines, nor the induction of IFN-γ were due to induction of IL-12.

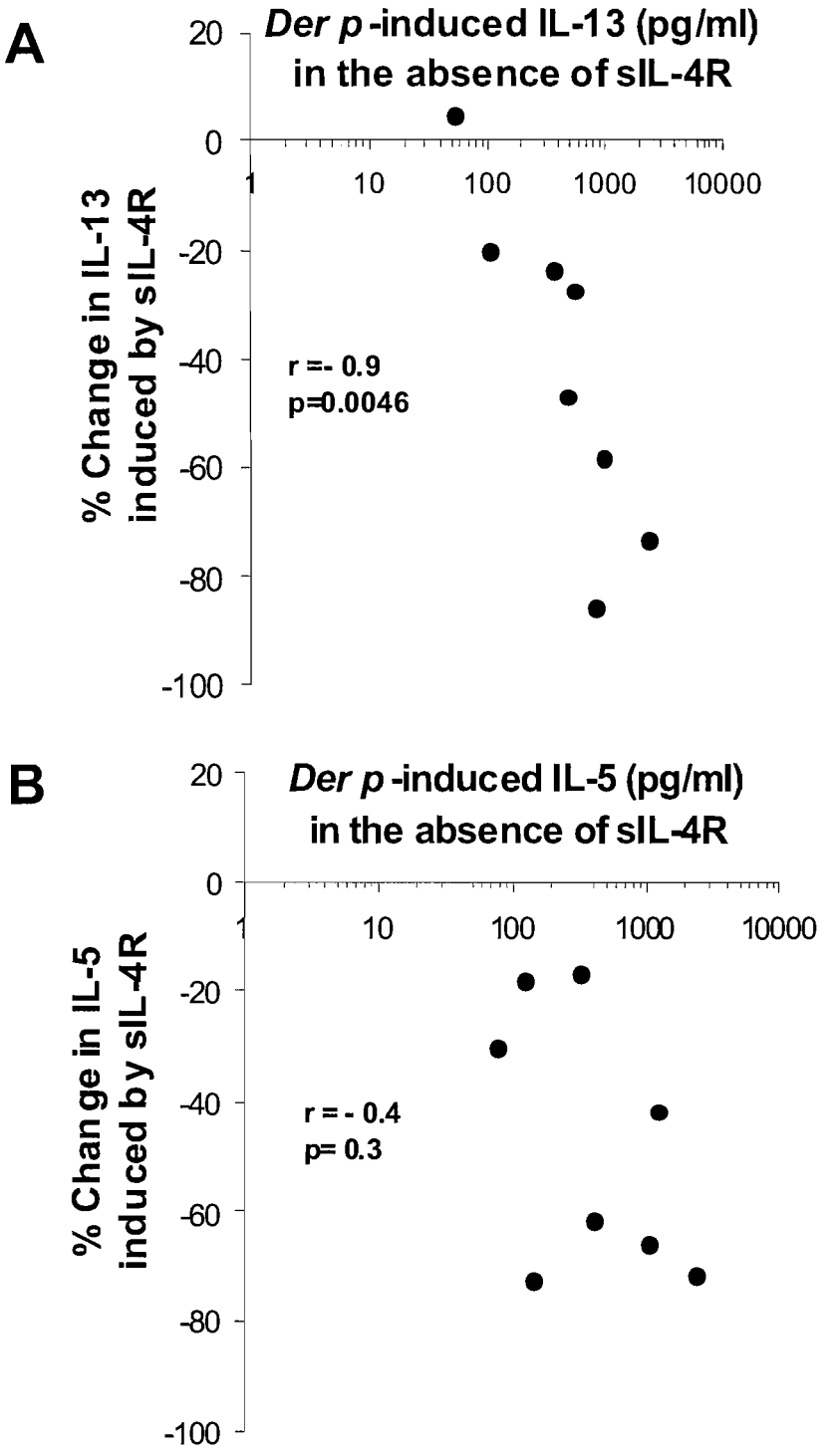


FIGURE 3.4-7: *Correlations between allergen-induced IL-13 (A) or IL-5 (B) and the degree of cytokine inhibition by sIL-4R (n=8).*

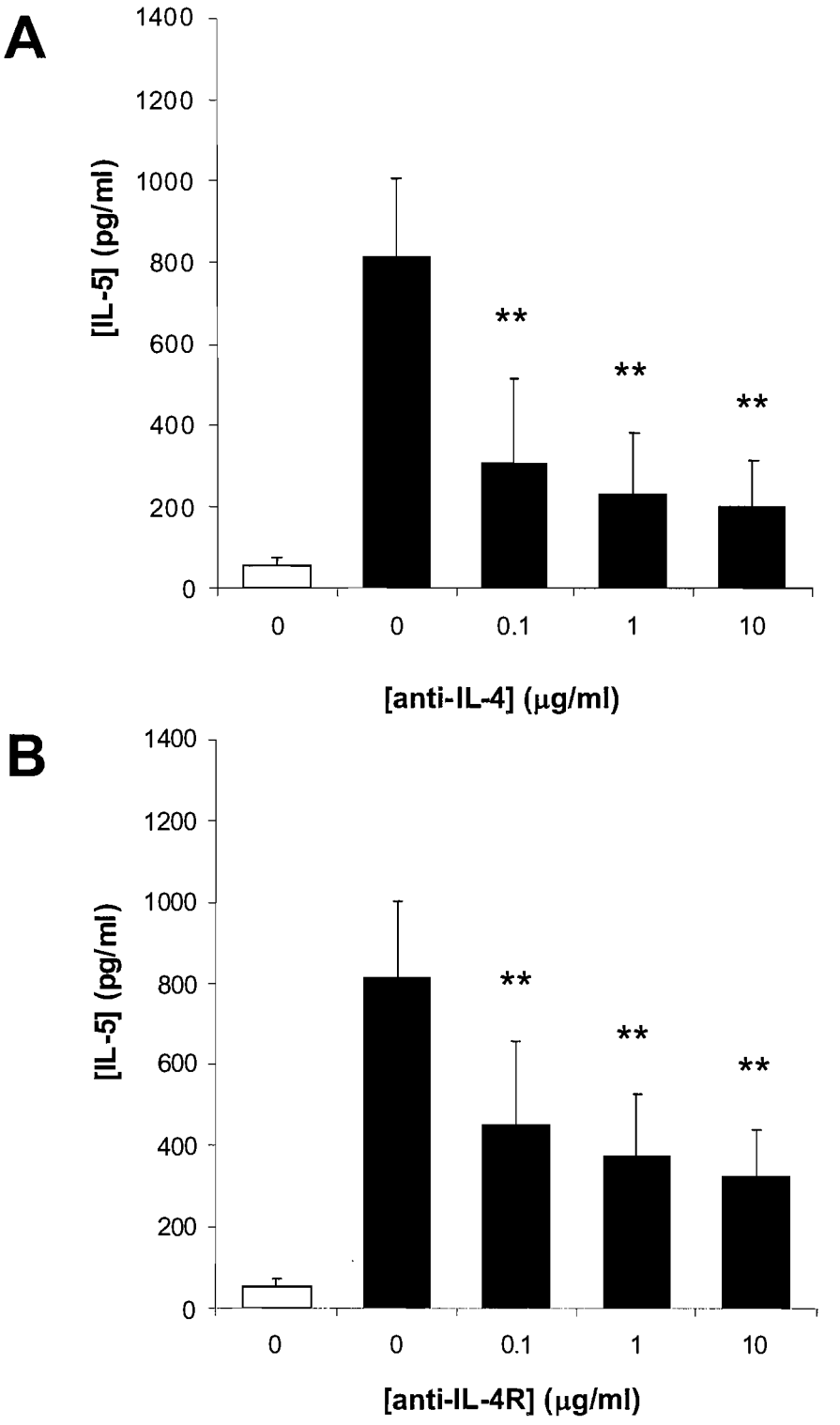


FIGURE 3.4-8: *The effect of anti-IL-4 (A) and anti-IL-4R (B) on the production of IL-5 (n=8).*

*PBMC were stimulated with 5,000 SQ U/ml Der p (closed bars) or cultured in medium alone (open bars) for seven days. The results are shown as mean ± SEM. The P values indicate levels of significance when compared with baseline Der p-stimulated culture (*P<0.05; **P<0.01).*

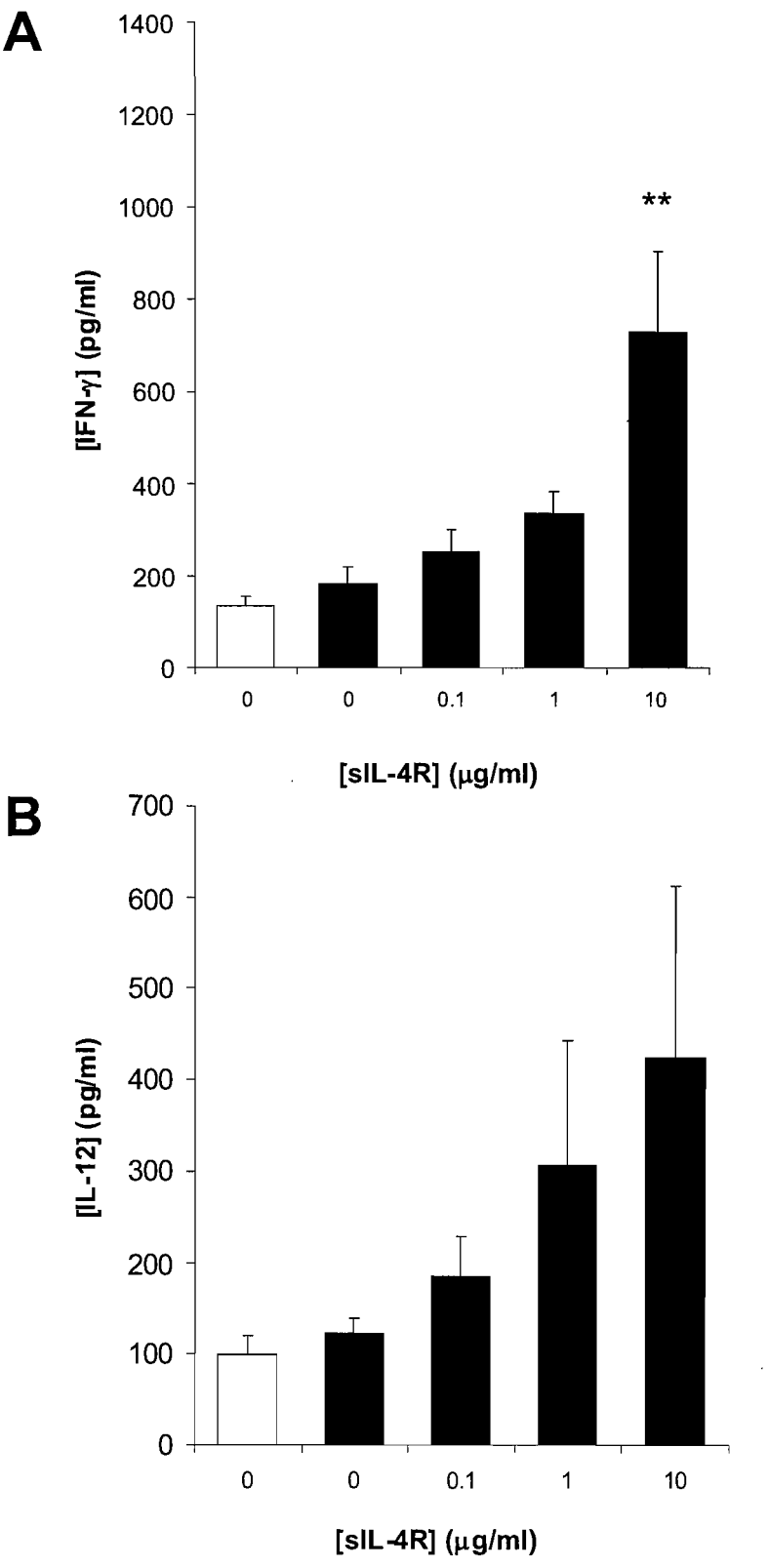


FIGURE 3.4-9: *The effect of sIL-4R on IFN-γ (A) and IL-12 (B) synthesis, (n=8). PBMC from eight subjects were stimulated for seven days with 5,000 SQ U/ml Der p (closed bars) or cultured in medium alone (open bars). The results are shown as mean ± SEM. The P values indicate levels of significance when comparing each condition with baseline (Der p-stimulated in the absence of sIL-4R) (**P<0.01).*

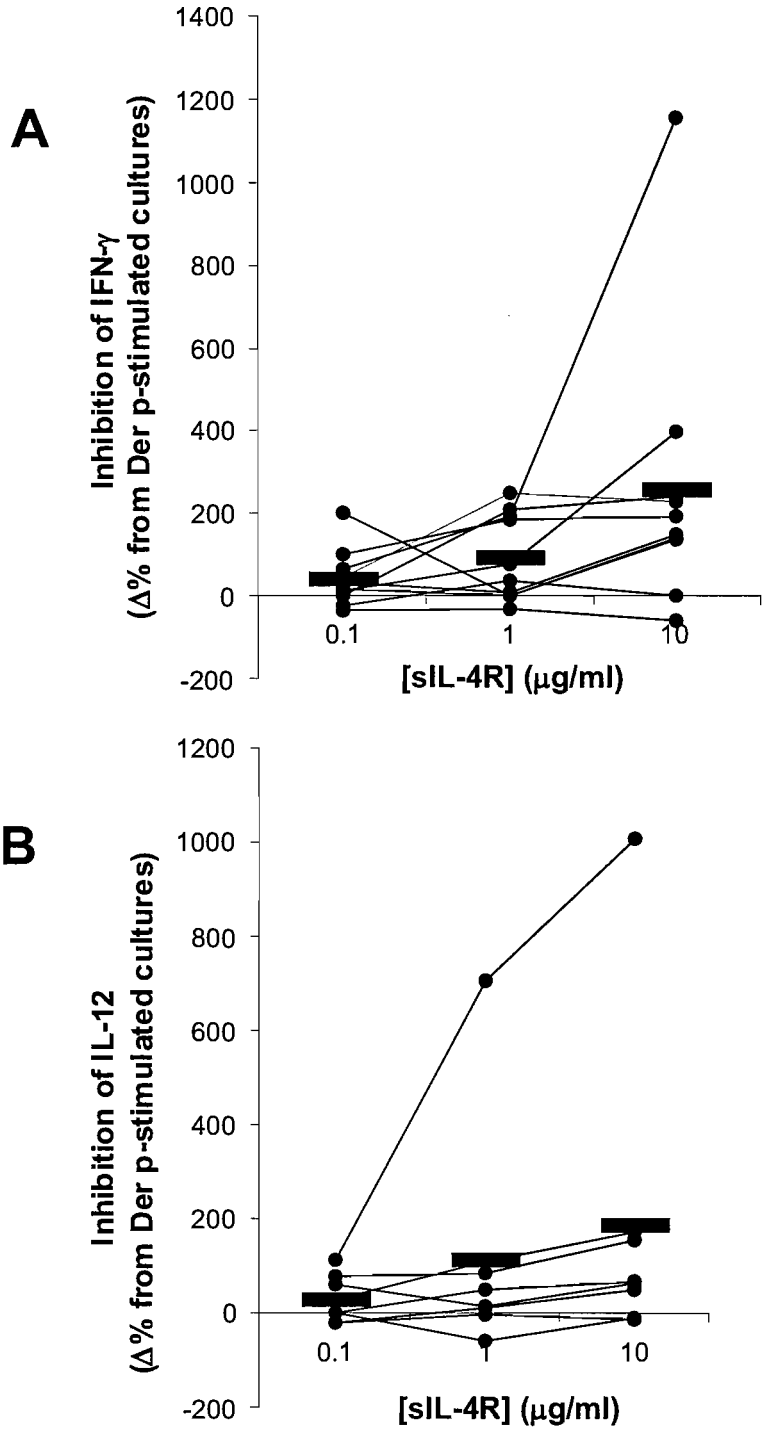


FIGURE 3.4-10: *Inhibition of IFN-γ (A) and IL-12 (B) synthesis by sIL-4R (n=8). The Individual Δ% changes post-sIL-4R treatment are shown as filled circles. Means are shown as horizontal bars.*

3.4.7 Kinetics of PBMC proliferation in response to allergen

Der p, in addition to stimulating cytokine production, is able to induce cell proliferation and clonal expansion. Proliferation occurs both antigen-specifically and as a consequence of a bystander effect, by which non-allergen-specific cells proliferate in response to the cytokine and growth factors released in the milieu.

To establish the extent and the time course of PBMC proliferation, PBMC from 18 subjects were cultured in the presence of *Der p* and then harvested after one, three, five and seven days. Allergen stimulated cells proliferated, reaching a peak at day five ($p<0.01$ compared to day one) (Figure 3.4-11). In contrast, no difference in proliferation was detected for medium stimulated cells. No difference was found between day five and day seven.

3.4.8Effects of sIL-4R on allergen-induced PBMC proliferation

IL-4 exerts powerful proliferative and antiapoptotic effects on target cells. One of the mechanisms by which sIL-4R could affect cytokine concentration *in vitro* is by inhibiting cell proliferation and promoting apoptosis. To test this hypothesis cells were stimulated with medium or *Der p* in the presence or absence of sIL-4R. I also compared the effects of sIL-4R on the *Der p*-induced proliferative response with those of the anti-IL-4 mAb. *Der p* at a concentration of 10.000 SQ units/ml was able to stimulate cell proliferation (Figure 3.4-11). A previous time-course study conducted on 24 asthmatic volunteers, showed that the optimal time point was day five or day seven (Figure 3.4-11). In this set of experiments, the addition of sIL-4 at the highest concentration (10 μg/ml) that was effective at blocking IL-5 and IL-13

synthesis had no inhibitory effect on the proliferative responses of PBMC stimulated by *Der p* in seven-day cultures (Figure 3.4-11).

The different proliferative response induced by Der p is likely to reflect the different number of subjects in the two set of experiments (eight vs twenty-four) and the overall more severe disease exhibited by the subjects included in the time-course study.

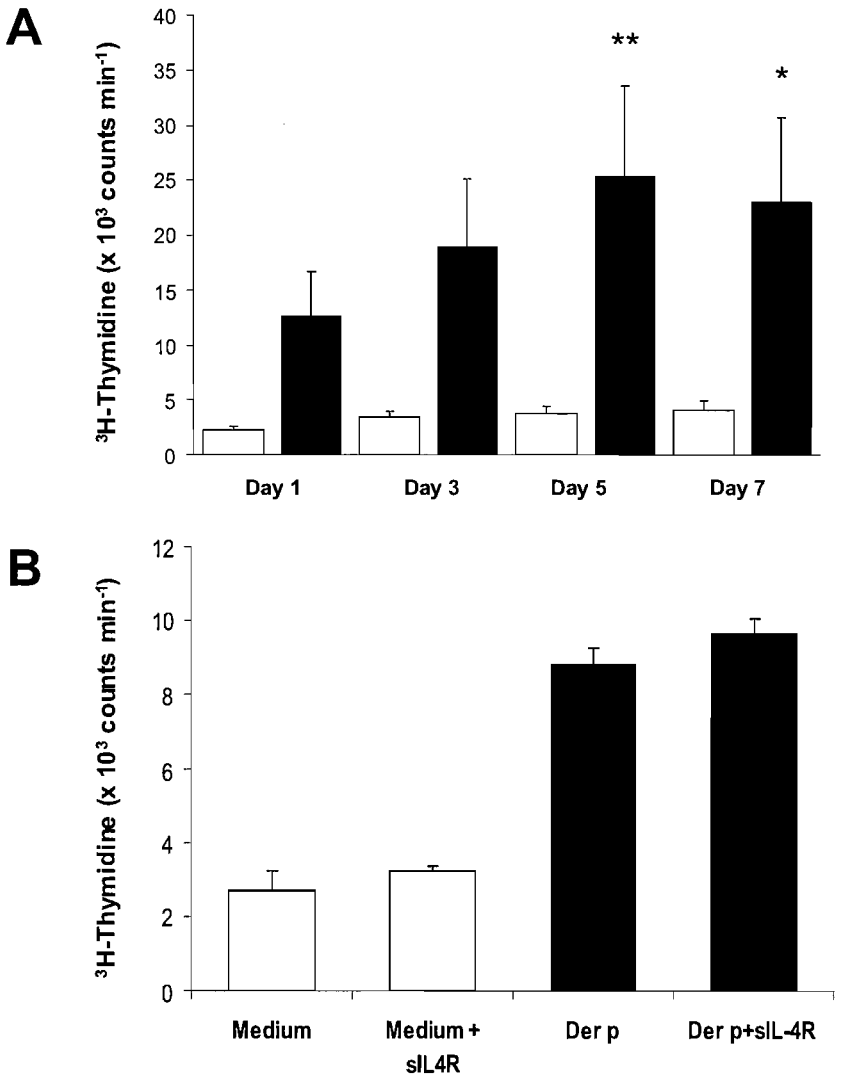


FIGURE 3.4-11 : *(A) The effect of allergen on proliferative responses of PBMC after one, three, five and seven days of culture (n=24). PBMC were cultured in the presence (closed bars) or absence of allergen (open bars). (B) The effect of allergen and sIL-4R on proliferative responses of PBMC in seven-day cultures (n=8). Allergen (closed bars) caused significant (P<0.01) stimulation of proliferation when compared to medium (open bars) but the addition of sIL-4R (10 μg/ml) had no effect on this proliferative response. The results are shown as mean ± SEM.*

3.5 DISCUSSION

Optimal development of Th2 cells requires polarising conditions that include neutralisation of the Th1 cytokine, IFN-γ, and the addition of the Th2 cytokine, IL-4 (Abehsira-Amar *et al.* 1992; Abbas *et al.* 1996). However, it has not been fully elucidated to what extent allergen-stimulated T-cells are dependent on IL-4 to generate other Th2-type cytokines that are relevant for allergic inflammation nor what the kinetics of any such involvement may be. In this study I have shown that IL-4 is centrally involved in the production of both IL-5 and IL-13. Whilst IL-4 is involved in the expansion of T lymphocytes, allergen-induced IL-13 and IL-5 synthesis is not entirely dependent on T-cell proliferation since the production of these cytokines was reduced by sIL-4R in the absence of any effect on PBMC proliferation. Significant inhibition of IL-5 and IL-13 could be achieved at concentrations of sIL-4R that did not result in significant IFN-γ production, which may be a desirable effect as it reduces the risk of inducing Th1-type inflammation. These studies further strengthen the case for IL-4 as a target for therapeutic inhibition, with the end result being not only inhibition of IL-4 but also reduction of the down-stream actions of this cytokine.

This study shows a slight difference in requirement for IL-4 for the production of IL-5 and IL-13. The effects of sIL-4R on IL-5 synthesis after three days of culture were negligible and reached significance only at day seven. This would suggest that during the early stages of stimulation there are sufficient numbers of polarised Th2 type T cells that do not require IL-4 to produce IL-5. This finds additional support in the fact that none of the IL-4 inhibitors, including anti-IL-4 and anti-IL-4R, could reduce IL-5 production to baseline levels that were seen in unstimulated cultures.

In contrast to IL-5, IL-13 production was significantly inhibited at both the three and seven-day time-points. Together with the finding that the concentration of IL-13 induced by *Der p* in

the absence of sIL-4R correlated strongly and significantly with the relative reduction in IL-13 in the presence sIL-4R, this suggests that IL-13 production by T cells is much more dependent on IL-4 than is the case with IL-5.

Because inhibition of IL-5 is seen only after several days of culture, it probably involves polarization of T cells towards the Th2 phenotype as they proliferate. The study also suggests that the inhibition of IL-5 or IL-13 by sIL-4R does not involve reduction of T-cell survival or inhibition of T-cell expansion as assessed by thymidine incorporation studies.

The observed differential effect of sIL-4R on cytokine synthesis and proliferation of *Der p* stimulated PBMC, could be due to various mechanisms. It is conceivable that sIL-4R had an important effect on STAT-6 pathway leading to cytokine suppression, whereas the proliferative cascade IRS-2/Atk/PKC was relatively unaffected. It is also possible that other IL-4 independent pathways of cell growth and proliferation remain unaffected or even potentiated following IL-4 inhibition by sIL-4R. In particular, IL-1 or STAT-5 dependent pathways could be involved in delivering an anti-apoptotoc signal. Therefore, although the overall effect of sIL-4R on proliferation was neutral, taking into consideration that the proliferative response reflected the whole population of PBMC rather than only Th2 cells, it is possible that analysis at single-cell level could reveal antiproliferative effects on IL-4-producing cells. Other studies on leukaemic cells have shown that proliferative effects of IL-4 can be uncoupled from cytokine secretion (Tuyt *et al.* 1995) although its molecular basis still remain to be fully elucidated. Whether the differential effects on cytokine secretion versus proliferation is a consequence of the mechanism of action of sIL-4R (binding IL-4 in solution rather than receptor antagonism of as in the case IL-4 muteins or anti-IL-4R antibodies), the type of cells targeted, the drug kinetic or the dose is not clear at present.

Inhibition of IL-5 and IL-13 synthesis was achieved at concentrations that were lower than those at which up-regulation of IFN-γ was seen, and although there was a trend for IL-12 synthesis to increase in the presence of sIL-4R, this never reached significance, suggesting that blockade of IL-4 does not involve IL-12 to any significant degree.

There have been several attempts to develop therapeutic strategies that would alter the Th1/Th2 imbalance that typifies allergic disease by enhancing Th1 or abrogating Th2 responses to allergen. The Th1-enhancing therapies, such as administration of IL-12 (Bryan *et al.* 2000) or IFN-γ, (Boguniewicz *et al.* 1993) have been unsuccessful so far, even though IL-12 caused a marked reduction in airway eosinophils, cells that have long been viewed as central in asthma (Bryan *et al.* 2000). Administration of anti-IL-5 antibodies has also been unsuccessful in clinical trials despite the fact that single-dose administration resulted in abolition of the eosinophil influx into the airways after allergen challenge (Leckie *et al.* 2000). Several approaches to IL-4 antagonism (Tournoy *et al.* 2002) have been considered, including (*a*) neutralisation with anti-IL-4 antibodies, (Finkelman *et al.* 1988; Renz *et al.* 1996), (*b*) use of antibodies directed against the IL-4 receptor α chain on cell surfaces, (*c*) IL-4 mutant proteins such as the double-mutein (Shanafelt *et al.* 1998; Srivannaboon *et al.* 2001) and (*d*) sIL-4R (Borish *et al.* 1999; Borish *et al.* 2001). In mice, anti-IL-4 monoclonal antibody treatment prior to allergic sensitisation markedly reduced IgE synthesis, but did not necessarily inhibit airway eosinophilia (Corry *et al.* 1996). The IL-4 double mutein was successful in murine (Tomkinson *et al.* 2001) and primate models of asthma (R. Gundel, W. Sebald *et al.*, unpublished observations) (Mueller *et al.* 2002) but, its development as a treatment of asthma has been abandoned. The approach which offered most hope was that using sIL-4R. In murine studies, sIL-4R, administered either intranasally or intraperitoneally before ovalbumin challenge in OVA-sensitised mice, significantly reduced allergen-specific

IgE responses, eosinophil accumulation and mucus hypersecretion (Henderson *et al.* 2000). In early clinical trials, involving 12 once-weekly nebulisations of sIL-4R, sudden and complete withdrawal of inhaled corticosteroids was possible without any deterioration in clinical activity of asthma (Borish *et al.* 1999; Borish *et al.* 2001).

The *in vitro* efficacy demonstrated in this study raises the question of why antagonism of IL-4 has shown mixed results in various allergic asthma clinical trial settings. In the case of sIL-4R, one possible explanation is insufficient availability of the molecule at sites where T cell polarization and expansion occurs. Studies have shown that these occur in regional (lung) lymph nodes in animal models of asthma, (Holt *et al.* 1991) and although the observations in animals cannot be directly extrapolated to human disease, it is possible that administration of sIL-4R via the inhaled route did not result in sufficient concentrations in lymph nodes.

Recombinant soluble IL-4 receptor (sIL-4R) has the same affinity for IL-4 as the cell-surface receptor; (Jacobs *et al.* 1993); thus, it has the potential to bind and sequestrate free IL-4 in competition with the cell surface receptor. However, this study has shown important differences when attempting to block IL-4: whilst sIL-4 had no effect on allergen-induced proliferation of T cells, the antibody against IL-4 itself was able to completely inhibit proliferation, and the degree of inhibition of IL-5 synthesis was similar for both. This observation is in keeping with what is known about the affinities of antibodies relative to soluble receptors and would suggest that inhibition by neutralising antibody may be therapeutically more effective that that with sIL-4R.

In this study I have tested the possibility of inhibiting allergen-induced Th2 cytokine response *in vitro* using a soluble form of IL-4 receptor which has a good safety profile in humans and been shown to have the same affinity for IL-4 as the membrane-bound receptor. Using this strategy I have demonstrated that sIL-4R is indeed a specific inhibitor of IL-4 and it is able to

inhibit IL-5 and IL-13 synthesis with similar efficacy as antibodies to IL-4 and surface-expressed IL-4R. In particular, sIL-4R at low dose caused significant inhibition of IL-5 and IL-13 without upregulating IFN-γ response, whereas at high concentrations, sIL-4R caused together with a further inhibition of IL-5 and IL-13 a significant increase in IFN-γ.

In contrast to the data observed at day seven, the effects of sIL-4R on IL-5 synthesis after three day culture were negligible, whereas IL-13 production showed significant inhibition. This would suggest that the allergen-inducible component of IL-5, although dependent on IL-4, is regulated by additional inflammatory mediators or alternatively, PBMC isolated from atopics contain a proportion of highly polarised T cells that no longer require IL-4 for IL-5 synthesis. This seems further confirmed by the studies using blocking antibodies for IL-4 and IL-4R, where, despite high concentrations of either antibodies, the levels of IL-5 (and also IL-13) failed to return to baseline (unstimulated levels).

However, when I analysed the proportion of IL-5 and IL-13 that was IL-4 dependent, I could demonstrate a strong correlation between allergen-induced IL-13 concentration and the extent of inhibition caused by sIL-4R, In contrast no correlation between the baseline concentration and the relative change in IL-5 post sIL-4R was found. Taken together these results suggest that IL-13 synthesis is more tightly controlled than IL-5 by the concentration of 'free' IL-4.

The mounting evidence for Th2 cell activation in allergic asthma suggests down-regulation or ablation of the Th2 response to be an appropriate aim in treating asthma and allergic disorders. However, such strategies can cause potentially harmful consequences such as those allowing a Th1 dominated immune activation in response to antigens leading to Th1-mediated autoimmune diseases. Furthermore, Th1 cells may contribute to asthmatic inflammation as shown by adoptive transfer studies in mice that demonstrate that the passive transfer of Th1

and Th2 cells enhances tissue eosinophilia in the airways of sensitised mice compared to when Th2 cells alone were administered (Hansen *et al.* 1999). Although other studies in mice did not confirm these results, (Huang *et al.* 2001) there is always the possibility that systemic inhibition of Th2 cells and increased concentration IFN-γ produced by Th1 cells may activate the production of pro-inflammatory cytokines and chemokines. It is not known whether sIL-4R down-regulates Th2 response to allergen simply by reducing the concentration of 'free' IL-4 available to activate the IL-4R, or if the reduction of Th2 cytokines is also caused by a secondary change in T-cell phenotype (or APC/T-cell interaction) induced by sIL-4R, which reflects the transition from a polarised Th2 type to a cell phenotype with regulatory properties.

The results of this study show the interesting differential effect that sIL-4R exerts on IFN-γ production. At low concentration sIL-4R did not alter IFN-γ production. Up-regulation of IFN-γ concentration was observed only when sIL-4R was used at the concentration 10-fold greater to the one able to induce a sub-maximal down-regulation of IL-5 and IL-13.

In conclusion, this study shows that IL-5 and IL-13 synthesis as well as T lymphocyte proliferation in response to stimulation with allergen involves IL-4. The efficacy of sIL-4 is similar to that of blocking antibodies against IL-4 or its cell-surface receptor in respect of cytokine production but is weaker than anti-IL-4 at inhibiting expansion of T cells. Targeting IL-4 has a greater therapeutic potential than targeting IL-5 or IL-13 alone because this treatment can inhibit not only IL-4 but also IL-5 and IL-13.

Other atopic disorders such as allergic rhinitis and atopic dermatitis are thought to be mediated by IL-4 and might also respond to IL-4 blockade with sIL-4R therapy. The present study shows that sIL-4R is able to down-regulate allergen-induced Th2 response *in vitro* without inducing a potentially harmful Th1 response.

CHAPTER FOUR

The effect of treatment with *Mycobacterium vaccae* (SRL172) in atopic asthma

4.1 INTRODUCTION

The non-pathogenic mycobacterium, *M. vaccae*, has been proposed for the prevention and therapy of allergic diseases (Rook *et al.* 1998). *Mycobacterium vaccae* is one of about 60 named species within the genus *Mycobacterium* and is one of a number of rapidly growing, yellow pigmented mycobacteria that normally occur as environmental saprophytes. Based on the 'hygiene hypothesis', bacterial products and non-pathogenic bacteria including *M.vaccae* have been employed as immunotherapeutic agents in studies aimed at preventing or treating atopic diseases such as asthma, rhinitis and dermatitis (Beasley *et al.* 2002; Walker *et al.* 2003).

In view of the accumulating evidence from animal studies and emerging evidence of the efficacy in the treatment of allergic dermatitis (Wang *et al.* 1998; Hopfenspirger *et al.* 2001; Hopfenspirger *et al.* 2002; Zuany-Amorim *et al.* 2002; Zuany-Amorim *et al.* 2002; Ozdemir *et al.* 2003), I have sought evidence that *M. vaccae* might be effective at altering the way the immune system of mild asthmatics responds to allergen.

I have tested the hypothesis that induction of an immune response to a non-pathogenic mycobacterial species, *Mycobacterium vaccae* can attenuate asthmatic reactions which occur following allergen challenge in patients with mild to moderate asthma. I have further hypothesised that *M. vaccae* may be able to reduce Th2-responses in established chronic adult asthma. In addition to testing the effect on the magnitude of the airways response, I have investigated the ability of *M. vaccae* to reduce inflammatory mediators in the induced sputum, serum IgE levels and to down-regulate allergen-induced *in vitro* synthesis of IL-5 by peripheral blood mononuclear cells (PBMC).

4.2 METHODS

4.2.1 Subjects

Twenty-four atopic asthmatics (mean age 34 years, range 20–53 years) with mild to moderate disease were recruited. All the subjects demonstrated an early airway response (EAR) and a late airway response (LAR). The subjects either had mild asthma (n=15), requiring only inhaled β_2-agonists, or moderately severe asthma (n=9), requiring treatment with low to moderate doses of inhaled corticosteroids (<600 μg of beclomethasone dipropionate or equivalent per day).

The study was approved by the Southampton University and Hospitals Ethics Committee and subjects gave their written informed consent.

4.2.1.1 Inclusion Criteria

- Study subjects were eligible for the study if they satisfied the following criteria:
- Male aged 18 to 55 years.
- History of mild to moderate, well-controlled asthma.
- Documented early airway response (EAR) to allergen challenge, defined as a decrease in FEV_1 of at least 25% compared to baseline within 15 minutes of allergen inhalation; and late airway response (LAR), defined as a decrease in FEV_1 of at least 15% compared to baseline occurring between three and eight hours after allergen inhalation.
- Presence of non-specific bronchial hyperresponsiveness indicated by a PC_{20} FEV_1 of not less than 0.5 mg/ml and not more than 16 mg/ml in a histamine challenge test.
- Blood pressure and heart rate considered to be clinically normal.

- Body weight within ± 15% of ideal body weight.
- Provision of written informed consent to participate as shown by the patient's signature on the Patient Consent Form.

4.2.1.2 Exclusion Criteria

- Women were excluded from the study. Patients were ineligible for the study if they met one or more of the following criteria:
- The use of systemic corticosteroids for asthma within the last six months.
- The use of high dose inhaled steroids (more than 600 μg beclomethasone by inhalation or equivalent) within the previous six months.
- FEV_1 less than 70% predicted.
- History of a cold or other respiratory tract infection within 14 days prior to screening.
- A need for prescription medication within 14 days of entry into study (screening), other than asthma medications.
- History of serious adverse reaction or hypersensitivity to any drug, or anaphylaxis to allergen.
- Administration of any investigational drug within three months prior to entry into the study.
- Existence of any surgical or medical condition which, in the judgement of the Investigator, might interfere with the absorption, distribution, metabolism or excretion of the vaccine or the performance of this study.
- Inability to communicate or cooperate with the investigator due to language problems, poor mental development or impaired cerebral function.

- Objection by the patient's General Practitioner to their patient's participation in the study.
- History of tobacco smoking during the previous 12 months.
- History of alcoholism or drug abuse.
- No clinically important abnormal physical findings other than those associated with mild to moderate asthma and allergic rhinitis.

4.2.1.3 Study design

This was a randomised, placebo-controlled, parallel group Phase I study. Twenty-four patients with mild and moderate asthma were randomised to receive a single dose of a *M. vaccae* preparation SRL172 (n=12) or a placebo (n=12). The study objective was to assess the effect of immunotherapy of SRL172 on allergen-induced bronchoconstriction and BHR to histamine. Three main aspects of allergic response were assessed: the early airway response (EAR), late airway response (LAR) and the bronchial hyperresponsiveness (BHR).

Furthermore, I assessed the effects of *M. vaccae* on sputum cellularity and Eosinophil cationic protein (ECP) and tryptase, serum IgE levels and *in vitro* allergen-induced IL-5 production and proliferation by PBMC of the study subjects. Subjects attended the department on nine occasions (Table 4.2-1). At the first screening visit, a medical history was taken, and a full physical examination, dental examination, routine haematological and biochemical tests, urinalysis and skin-prick tests were performed. In addition, baseline responsiveness to histamine was measured. After a run-in period of 28 days, during which disease activity was recorded in diary cards in order to assess stability, the first allergen challenge was performed at visit 2 using an incremental-dose challenge protocol. The following day (visit three) subjects returned for histamine challenge to assess any change in responsiveness secondary to

allergen challenge. Two weeks later (visit four) blood was drawn for the first in a series of three PBMC cultures and serum IgE measurements. Subjects then received an intradermal injection of 0.1 ml (1 mg) of SRL172 or placebo into the lateral aspect of the deltoid muscle region on the left arm. This dose was decided based on equivalence of dose obtained from animal studies and previous studies on tuberculosis (Prof. Stanford and Prof. Rook). After three days they returned to the department in order to record any local reaction (visit five). Eighteen days after injection (visit six), haematology and biochemistry blood tests, urinalysis and serum IgE measurement were repeated. At visit seven (three weeks after injection), a second blood sample was taken for PBMC culture and the allergen challenge was repeated. This was followed by a histamine challenge the next day (visit eight). Subjects were followed-up until 42 days post-injection (visit nine) when a full medical examination was conducted, a third blood sample was taken for serum IgE measurement, PBMC culture, haematology and biochemistry, and urinalysis was performed (Table 4.2-1). The timing of allergen challenge, sputum induction and immunological studies were arbitrarily established based on the supposed immunomodulatory mechanisms of *M. vaccae.*

In order to avoid observer bias the study medication was prepared by a pharmacist at Southampton General Hospital and injections were administered by a physician blinded to the study. Furthermore, the site of injection was concealed from other investigators with a bandage, and inspected by a physician external to the study. Adverse events and local complications were assessed and reported by the same physician.

Patients completed screening assessments within 28 days of admission to the study to establish their fitness to participate in the study and to provide written informed consent. The General Practitioner of each patient was informed in writing about the proposed participation of his/her patient in this study.

ASSESSMENTS	Screening phase			Treatment phase					
Visit	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Day	-28 to -15	-14	-13	0	+2	+18	+21	+22	+42
Patient written informed consent	✓								
Inclusion/exclusion criteria	✓	✓	✓	✓					
Patient demography	✓								
Medical history	✓								
Physical and clinical assessment	✓								✓
Dental examination	✓								✓
Blood - haematology/biochemistry	✓								✓
Urinalysis	✓								✓
PBMC culture + IgE levels				✓			✓		✓
Skin prick test	✓								
Allergen challenge		✓					✓		
Histamine challenge	✓		✓					✓	
Pulmonary function	✓	✓	✓	✓			✓	✓	✓
Blood - immunology/cytokines				✓		✓			✓
Sputum induction				✓		✓			✓
Randomisation				✓					
Injection of SRL 172				✓					
Injection site inspection					✓				
Concomitant medication / therapy	✓	✓	✓	✓	✓	✓	✓	✓	✓
Adverse experiences reporting		✓	✓	✓	✓	✓	✓	✓	✓

TABLE 4.2-1: *Study Checklist*

The following procedures were performed during screening visits and recorded in the Case Report Form:

4.2.1.3.1 Medical History

A full medical history and demographic data were recorded for each patient. This included the recording of a history of asthma and allergy, including onset, duration, known allergens (including medicines), other precipitating factors and family history. All patients had a dental examination for peridontal disease activity at Visit one. This was repeated at Visit nine to verify any change in peridontal disease activity.

4.2.1.3.2 Laboratory Investigations

Laboratory investigations included haematology, serum biochemistry and urinalysis. Biochemistry and haematology results are presented in Appendix 4.

4.2.1.3.3 Allergen Sensitivity (skin-prick test)

Allergen sensitivity was determined by skin-prick testing using several approved allergen extracts and following the labelled procedures as described in 2.2.5. Wheal size for each allergen in both groups is summarised in Table 1 of Appendix 1.

4.2.1.3.4 Lung Function

Forced expiratory volume in one second (FEV_1) was measured in triplicate and the largest value used for comparisons as described in 2.2.1.

4.2.1.3.5 Measurement of Bronchial Responsiveness to Histamine

Patients underwent a histamine challenge test in the morning to document the presence of acceptable ventilatory function and non-specific bronchial hyperresponsiveness as described in 2.2.2.

4.2.1.3.6 Allergen Inhalation Challenge

On a separate screening day at least two weeks before dosing, patients underwent an allergen inhalation challenge test to document the presence and magnitude of an EAR and LAR. Inhalation challenge was performed using an allergen dose that produced a wheal of at least 5 mm × 5 mm in the skin test and correlated with clinical history. After challenge, patients received nebulised salbutamol to achieve maximum bronchodilatation and abort a protracted LAR. Allergen challenge was performed as described in 2.2.3.

4.2.1.3.7 Repeat Histamine Challenge 24 hours Post-Allergen Challenge

The histamine challenge test was repeated at 24 hours ± 2 hours post allergen challenge in order to determine the presence and magnitude of any allergen-induced increase in bronchial hyperresponsiveness to histamine.

4.2.1.3.8 Concomitant Medications

Prescription medication, other than that required for asthma control, was not permitted for 14 days before the screening phase of the study and for 14 days before the treatment phase of the study (any concomitant medications taken during throughout the study were recorded).

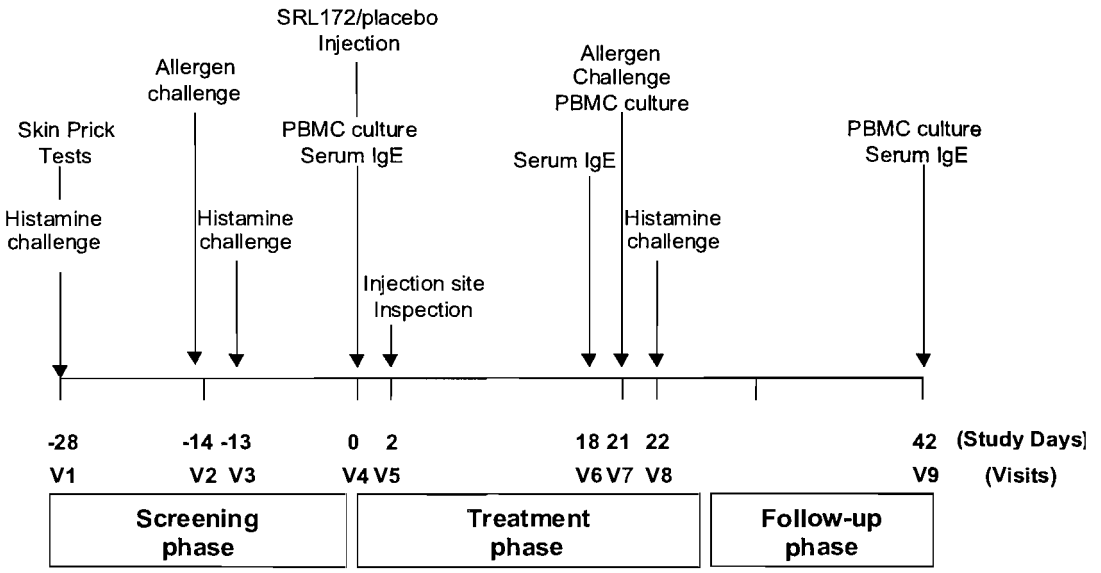


FIGURE 4.2-1: *M. Vaccae (SRL172): Study design*

In addition, over-the-counter medications were not permitted for a period of 72 hours prior to administration of the injection, except paracetamol (for minor analgesia), up to 24 hours before injection at the discretion of the investigator. If other medication was required, the name, strength, frequency of dosing and reason for use were documented.

Bronchodilators were not administered for the symptomatic relief of minor decreases in pulmonary function. In particular, bronchodilators were not administered within six hours of the start of the allergen or histamine challenges.

Patients were allowed to continue use of inhaled steroids if they had been on a stable low maintenance dose (specifically, not more than 600 µg beclomethasone or equivalent per day by inhalation) for at least one month, but use was discontinued for at least five days prior to each allergen challenge.

4.2.1.4 Allergen and histamine inhalation challenge

Allergen challenge was performed as described in 2.2.3.

4.2.1.5 *In vitro* IL-5 responses to allergen

Twenty millilitres of heparinised venous blood was collected from each study subject before and after *M.vaccae*/placebo administration respectively at visits two, seven and nine (Figure 4.2-1). PBMC were isolated by Ficoll-Hypaque gradient centrifugation as described in 2.2.6.1 and 2.2.6.2.

4.2.1.6 Serum IgE

Total serum IgE was measured by the Regional Immunology Laboratory, Southampton General Hospital by enzyme-linked immunosorbent assay (ELISA) using a monoclonal anti-IgE antibody developed in-house and a commercially available antibody (Dako, High Wycombe, UK).

4.2.1.7 Proliferation Assay

Proliferation of PBMC before and after treatment was performed as described in 2.2.6.4.

4.2.1.8 Sputum induction and processing

Sputum induction and processing was performed as described in 2.2.4.

4.2.1.9 Statistical analysis

The principal outcome variables were: maximum % falls in FEV_1 during the first 2 h following allergen challenge (EAR) and between three and eight h following allergen challenge (LAR), and changes in histamine responsiveness 24 h following allergen challenge. The changes in FEV_1 were also expressed as areas under the curve (AUC) for the EAR and LAR. The AUC was calculated using trapezoid integration as the area between the curve and the x-axis and divided by the duration (first two hours for the EAR and three to eight h for the LAR) over which the AUC was calculated. Comparisons of changes in maximum % falls in FEV_1, AUC for the EAR and LAR, and PC_{20} as a result of treatment with SRL172 or placebo were performed using formal analysis of variance models (ANOVA). Within-group changes in the same were made by paired Student's t test. The changes during the course of the study in IL-5 production *in vitro* and serum IgE levels relative to the first measurement were expressed as AUC by trapezoid integration of the percentage change at the three time-points. The AUC in the two treatment arms were then compared by Mann-Whitney U test, assuming a non-normal distribution. The changes in IgE levels and IL-5 synthesis in the two treatment groups were also compared using the Fisher's exact test.

Sputum and proliferation data were analysed for normality of distribution prior to statistical analysis. Differences between groups were analysed with unpaired t-test.

Correlation between the area of the wheal to *Der p* on skin-prick test and PBMC proliferation was analysed by Pearson's test.

4.3 RESULTS

4.3.1 Patient demographics

Twenty-four patients completed the study; Table 4.3-1 shows their baseline demographics and lung function data. Table 4.3-2 shows a summary of the past medical history of the two groups of subjects. The two groups of patients were well matched. Although more patients with asthma of moderate severity and therefore on inhaled corticosteroids were assigned to the placebo group, the baseline lung function and responsiveness to histamine was similar between the two groups.

	***M. vaccae* (n=12)**	**Placebo (n=12)**
Age (years), Mean [range]	33.7 [20–51]	35.8 [21–53]
Male gender	12 (100%)	12 (100%)
White ethnicity	12 (100%)	12 (100%)
Smoker previous year	0 (0%)	0 (0%)
Smokers prior to last year	2 (17%)	2 (17%)
Alcohol consumption (Units/week) [range]	9 [2-24]	11.1 [0-30]
Time since diagnosis, years Mean [Range]	21.6 [10–45]	22.5 [2–44]
Family history of asthma	9 (75%)	7 (58%)
Asthma severity - Mild - Moderate	 9 (75 %) 3 (25 %)	 6 (50%) 6 (50%)
Subjects on inhaled corticosteroids	3 (25%)	6 (50%)
Previous treatment for TB	0 (0%)	0 (0%)
Tuberculin Test	7 (58%)	9 (75%)
BCG scar	11 (92%)	11 (92%)
FEV_1 (% predicted) Mean ± SD	92% ± 10%	92% ± 12%
Baseline PC_{20} Histamine (mg/ml) Geometric mean [Range]	1.17 [0.65–2.69]	1.82 [0.35–13.63]

TABLE 4.3-1: *Baseline characteristics of the patients*

	M. vaccae (n=12)	Placebo (n=12)
Ears, Nose, Throat	1 (8%)	2 (17%)
Respiratory (other than asthma)	0 (0%)	2 (17%)
Cardiovascular	0 (0%)	0 (0%)
Gastrointestinal	1 (8%)	1 (8%)
Genitourinary	1 (8%)	1 (8%)
Musculoskeletal	0 (0%)	0 (0%)
Neurological	0 (0%)	1 (8%)
Endocrine-metabolic	0 (0%)	0 (0%)
Haematological	0 (0%)	0 (0%)
Dermatological	0 (0%)	1 (8%)
Dental Health	0 (0%)	1 (8%)
Psychological	0 (0%)	0 (0%)

TABLE 4.3-2: *Summary of past medical history*

4.3.2 Safety and side effects

Treatment was well tolerated by all subjects. No significant side effects were reported. Similarly, no significant changes were found in the laboratory data. Tables with reported adverse effects and laboratory data throughout the study are presented in Appendix 3.

4.3.3 Baseline airways responses

At baseline all randomised subjects developed both an EAR and LAR following allergen challenge.

4.2.3.1 Late asthmatic response (LAR)

During the LAR, the mean [range] maximum fall in FEV_1 was 35.8% [16.5–66.7%] in subjects receiving SRL172 and 29.5% [16.9–39.4%] in subjects receiving placebo (Figure 4.3-1, Figure 4.3-2). When taking into account the average FEV_1 between three and eight hours post-allergen challenge (AUC_{3-8}), the mean [range] AUC_{3-8} was 35.8 [16.5-66.7] for the SRL172 group and 29.5 [16.9-39.4] for the placebo group.

4.2.3.2 Early asthmatic response (EAR)

The mean [range] maximum fall in FEV_1 during the EAR were 33.0% [20.6–50.6%] and 33.0% [20.6–42.7] in the SRL172 and placebo groups, respectively.

Similarly, the mean [range] area under the curve in the first two hours (AUC_{0-2}), representing the EAR, was of 13.6 [3.4-25.4] and 12.6 [23.6-3.4] respectively for SRL172 and placebo.

4.2.3.3 Bronchial hyperresponsiveness to histamine

Baseline geometric mean [SD] PC_{20} histamine, although lower in the subjects treated with SRL172 (1.32 [0.7] mg/ml) when compared to those receiving placebo (3.34 [4.2] mg/ml) was not significantly different (p=0.23) (Figure 4.3-1, Figure 4.3-2).

4.3.4 Effects of treatment on airway responses to allergen challenge

4.3.4.1 Late asthmatic response (LAR)-All patients

Treatment with SRL172 caused a significant (p=0.005) reduction in the maximum fall in FEV1 during the LAR from a mean [range] of 35.8% [16.5–66.7%] before treatment to 26.7 % [3.6–60.6%] after injection of SRL172 (a mean relative reduction of 25.4%), (

Table 4.3-5, Figure 4.3-1, Figure 4.3-2). Similarly, the AUC of the % fall in FEV1 was reduced significantly (p=0.02) from 19.4 (5.8–41.4) to 12.8 [0.4–39.1] (a mean relative reduction of 34%), (

Table 4.3-5, Figure 4.3-1, Figure 4.3-2). In contrast, in the subjects receiving placebo there was no significant change in the maximum % fall in either FEV1 or AUC of the LAR. When comparing the magnitude of changes in the groups of subjects receiving SRL172 and those receiving placebo by ANOVA, the active group shows an average improvement of 6.6 % in AAUC after treatment compared to slightly worse (-1.03) in the placebo group. The estimated difference between treatment groups is 7.62% with the 95% confidence interval showing the difference between the active and placebo groups between –0.48 to 15.7%. The effect on the AUC during LAR failed to reach significance (p=0.06). Similarly, the estimated difference in maximum fall of FEV1 between the two treatment groups was 7.34% (95% CI, -2.2 to 12.9), which was statistically not significant (Figure 4.3-1, Figure 4.3-2,

Table 4.3-5). Of note, there was a greater inter-subject variability in the FEV1 during the second allergen challenge in both treatment groups. The mechanism is not clear, but could be due to the effects of the previous allergen challenge or could reflect the fact that for the second allergen challenge both groups had been off inhaled steroids for a longer period compared to the first allergen challenge. No distinguishable groups of responders and non-

responsers was identified. Allergen challenge curves for each patient are shown in Appendix 2.

	M. vaccae (SRL172)		Placebo	
Time	**Visit 2 FEV_1 Mean (SD) [range]**	**Visit 7 FEV_1 Mean (SD) [range]**	**Visit 2 FEV_1 Mean (SD) [range]**	**Visit 7 FEV_1 Mean (SD) [range]**
Baseline	3.6 (0.5) [2.8-4.4]	3.5 (0.5) [2.6-4.3]	3.5 (0.8) [2.3-4.7]	3.5 (0.9) [2.2-5.1]
20 min	2.4(0.5) [1.6-3.2]	2.8 (0.4) [1.8-3.3]	2.4 (0.6) [1.4-3.5]	2.7 (0.9) [1.6-4.2]
30 min	2.7(0.4) [1.7-3.2]	2.9 (0.5) [1.9-3.6]	2.6 (0.7) [1.4-3.5]	2.9 (0.9) [1.8-4.2]
45 min	2.9 (0.5) [1.9-3.6]	3.1 (0.5) [2.2-3.8]	2.9 (0.9) [1.5-4.3]	3.1 (1.0) [1.7-4.4]
60 min	3.1 (0.5) [2.0-3.6]	3.3 (0.5) [2.3-4.1]	3.06 (0.8) [1.6-4.5]	3.2 (0.9) [1.6-4.5]
90 min	3.4 (0.6) [2.4-4.2]	3.4 (0.5) [2.6-4.1]	3.3 (0.8) [1.9-4.4]	3.4 (1.0) [1.8-4.9]
2 h	3.5 (0.6) [2.4-4.4]	3.5 (0.6) [2.6-4.3]	3.4 (0.8) [2.1-4.5]	3.5 (1.0) [2.0-5.0]
3 h	3.5 (0.6) [2.3-4.3]	3.6 (0.6) [2.7-4.3]	3.4 (0.9) [2.1-4.5]	3.5 (1.0) [1.9-4.9]
3.5 h	3.3 (0.6) [2.6-4.2]	3.5 (0.6) [2.7-4.5]	3.3 (0.8) [2.0-4.7]	3.3 (1.0) [1.6-4.8]
4 h	3.2 (0.7) [2.3-4.1]	3.3 (0.7) [2.2-4.3]	3.3 (0.7) [2.1-4.4]	3.2 (0.9) [1.5-4.6]
4.5 h	3.1 (0.6) [2.2-3.9]	3.2 (0.7) [1.9-4.2]	3.2 (0.8) [1.8-4.5]	3.2 (1.0) [1.5-4.7]
5 h	3.0 (0.5) [2.0-3.8]	3.2 (0.7) [1.8-4.2]	3.1 (0.8) [2.1-4.7]	2.9 (0.8) [1.5-4.3]
5.5 h	2.9 (0.6) [1.9-3.7]	3.1 (0.8) [1.8-4.1]	2.9 (0.6) [1.8-4.1]	3.0 (0.9) [1.4-4.5]
6 h	2.8 (0.6) [1.8-3.8]	2.9 (0.8) [1.6-4.0]	3.0 (0.8) [1.8-4.4]	3.0 (1.0) [1.4-4.9]
6.5 h	2.8 (0.6) [1.9-4.0]	2.8 (0.7) [1.4-4.1]	2.9 (0.9) [1.6-4.6]	2.9 (1.0) [1.3-4.7]
7 h	2.6 (0.6) [1.8-3.6]	2.9 (0.7) [2.1-4.0]	2.8 (0.8) [1.8-4.7]	2.8 (1.0) [1.2-4.8]
7.5 h	2.6 (0.5) [1.6-3.6]	2.9 (0.7) [2.0-4.0]	2.7 (0.8) [1.5-4.6]	2.8 (1.0) [1.3-4.7]
8 h	2.5 (0.6) [1.7-3.5]	2.9 (0.8) [1.9-4.2]	2.7 (0.8) [1.4-4.6]	2.8 (1.0) [1.3-4.6]

TABLE 4.3-3: *Summary of Allergen challenge data after 25% fall in FEV_1.*

	M. vaccae (SRL172)		Placebo	
Time	Visit 2 FEV_1 Mean (SD) [range]	Visit 7 FEV_1 Mean (SD) [range]	Visit 2 FEV_1Mean (SD) [range]	Visit 7 FEV_1 Mean (SD) [range]
Baseline	3.6 (0.5) [2.8-4.4]	3.5 (0.5) [2.6-4.3]	3.5 (0.8) [2.3-4.7]	3.5 (0.9) [2.2-5.1]
20 min	-23.9(10.0) [-19.1 to -50.6]	-21.8 (8.8) [-6.7 to -38.4]	-32.2 (8.2) [-16.5 to -42.7]	-24.1 (9.5) [-4.33 to -39.4]
30 min	-24.8(9.0) [-13.3 to – 39.6]	-17.2 (9.2) [-3.7 to – 34.1]	-25.2 (8.7) [-9.3 to – 39.6]	-17.5 (8.6) [-5.1 to – 32.9]
45 min	-19.7 (10.0) [-3.2 to -36.3]	-12.4 (9.0) [0 to – 33.5]	-17.9 (11.2) [-5.5 to – 36.3]	-13.1 (7.7) [0.91 to – 27.7]
60 min	-14.9 (6.8) [-4.6 to -29.6]	-7.04 (8.1) [4.0 to -23.5]	-12.8 (9.6) [-2.2 to – 27.6]	-9.8 (7.0) [0.3 to – 25.8]
90 min	-7.0 (5.1) [4.3 to -14.6]	-3.3 (5.8) [5.0 to -16.9]	-6.1 (6.53) [3.6 to – 17.8]	-2.3 (7.8) [11.3 to -19.5]
2 h	-4.7 (5.4) [5.7 to -15.7]	-1.2 (5.9) [7.5 to -14.6]	-3.8 (7.3) [13.4 to -11.7]	-2.1(6.8) [7.6 to -16.6]
3 h	-3.1 (7.3) [11.7 to – 19.3]	1.23 (4.5) [9.2 to -9.5]	-2.28 (8.1) [15.9 to -15.8]	-0.35 (9.8) [19.3 to -15.4]
3.5 h	-8.5 (10.2) [5.7 to -29.3]	-2.1 (8.5) [8.0 to -16.2]	-6.0 (8.1) [8.2 to -18.6]	-5.2 (11.0) [13.6 to -27.2]
4 h	-13.1 (10.4) [0.3 to -36.6]	-5.8 (13.7) [12.9 to -33.6]	-5.7 (7.9) [13.1 to -16.7]	-9.0 (10.2) [5.4 to -31.2]
4.5 h	-15.5 (10.8) [-0.3 to -40.1]	-9.8 (13.9) [3.8 to -44.9]	-8.2 (8.7) [7.7 to -19.2]	-10.3 (12.5) [3.5 to -33.9]
5 h	-16.7 (11.9) [0.8 to -47.2]	-8.7 (15.8) [18.6 to -47.0]	-11.1 (10.4) [5.7 to -33.6]	-15.6 (12.1) [1.9 to -33.1]
5.5 h	-20.3 (11.5) [-6.0 to -47.7]	-13.4 (16.2) [7.9 to -48.4]	-15.3 (10.2) [6.9 to -31.6]	-14.0 (13.0) [1.4 to -38.0]
6 h	-22.1 (14.1) [4.3 to -51.0]	-17.6 (15.7) [-2.2 to -52.5]	-14.2 (10.2) [3.1 to -31.9]	-15.5 (11.2) [-1.8 to -35.8]
6.5 h	-23.0 (12.6) [-5.0 to -49.3]	-21.8 (17.2) [-0.27 to -60.6]	-17.4 (11.5) [1.8 to -37.8]	-17.9 (12.6) [3.9 to -41.6]
7 h	-27.8 (11.0) [-12.9 to -52.6]	-17.6 (10.5) [-1.9 to -34.7]	-19.3 (13.2) [4.8 to -30.8]	-20.7 (13.7) [2.9 to -43.9]
7.5 h	-28.8 (8.1) [-13.9 to -44.5]	-19.6 (10.7) [-3.6 to -37.0]	-21.3 (13.8) [3.8 to -35.0]	-21.2 (13.0) [-1.6 to -44.8]
8 h	-30.2 (11.9) [-15.7 to -48.8]	-18.4 (12.2) [0.7 to -38.6]	-21.3 (15.3) [2.4 to -39.4]	-21.0 (12.2) [-0.3 to -39.5]

TABLE 4.3-4: *Summary of changes from baseline in allergen challenge data after 25% fall in FEV_1.*

		M. vaccae (SRL172) (n=12)	Placebo (n=12)	p
AAUC FEV_1, mean % change from baseline (SD) [range]	Pre-intervention (Visit 2)	-19.4 (9.4) [-5.8 to – 41.4]	-13.0 (7.0) [-2.0 to -22.2]	
	Post-intervention (Visit 7)	-12.8 (12.0) [-0.4 to – 39.1]	-14.0 (9.5) [0.0 to – 35.5]	
	Change (visit 7 – Visit 2)	p=0.02	p>0.05	
	Treatment difference Mean (95% CI)	7.6 (-0.5 to 15.7)		p=0.06
Maximum fall FEV_1, mean % change from baseline (SD) [range]	Pre-intervention (Visit 2)	-35.8 (14.1) [-16.5 to – 66.7]	-29.5 (8.2) [-16.9 to –39.4]	
	Post-intervention (Visit 7)	-26.7 (14.8) [-3.6 to -60.6]	-27.6 (11.4) [-28.9 to -44.8]	
	Change (visit 7 – Visit 2)	p=0.005	p>0.05	
	Treatment difference Mean (95% CI)	7.3 (-2.2 to 16.8)		p>0.05

TABLE 4.3-5: *Effects of M. vaccae (SRL172) on allergen induced late asthmatic response (LAR).*

4.3.4.1.1 Patients with mild asthma

In the subgroup of patients with mild asthma, *M.vaccae* caused a mean 47.2% relative reduction in the AUC of the LAR (p=0.026) (Table 4.3-6). However, as in the analysis including all patients, the estimated absolute difference between treatment groups did not achieve statistical significance (7.1%; 95% CI -2.54 to 16.7%), (Table 4.3-6).

Similarly, the *M. vaccae* group showed a significant 31% relative reduction in maximum % fall after treatment (visit seven *Vs* visit two) (p=0.024). However, the estimated absolute difference between treatment groups was statistically not significant (8.2%; 95% CI –3.92 to 20.34%), (Table 4.3-6).

		M. vaccae (SRL172) (n=9)	Placebo (n=6)	p
AAUC FEV_1, mean % change from baseline [95% CI]	Change (Visit 7 – Visit 2)	7.9 [1.2 to 14.7]	0.84 (7.9) [-8.4 to 12.3]	
		p=0.026	p>0.05	
	Treatment difference Mean (95% CI)	7.1 (-2.54 to 16.7)		p=0.06
Maximum fall FEV_1, mean % change from baseline [95% CI]	Change (visit 7 – Visit 2)	9.84 [1.81 to 17.9]	1.6 (10.6) [-14.0 to 13.7]	
		p=0.024	p>0.05	
	Treatment difference Mean (95% CI)	8.2 (-3.9 to 20.3)		p>0.05

TABLE 4.3-6: *Changes from visit 2 in LAR – patients with mild asthma*

4.3.4.2 Early asthmatic response (EAR)

Treatment with placebo or SRL172 had no significant effect on the EAR, expressed as maximum % fall in either FEV_1 or AUC (Table 4.3-7).

		M. vaccae (SRL172) (n=12)	Placebo (n=12)	p
AAUC FEV_1, mean % change from baseline (SD) [range]	Pre-intervention (Visit 2)	-13.9 (5.9) [-3.4 to -25.4]	-12.6 (7.0) [-3.4 to -23.6]	
	Post-intervention (Visit 7)	-7.8 (6.6) [-2.4 to -22.5]	-8.6 (6.2) [0.8 to – 20.4]	
	Change (visit 7 – Visit 2)	p>0.05	p>0.05	
	Treatment difference Mean (95% CI)	2.0 (-4.2 to 8.2)		p>0.05
Maximum fall FEV_1, mean % change from baseline (SD) [range]	Pre-intervention (Visit 2)	-33.0 (9.8) [-20.6 to -50.6]	-29.5 (8.2) [-16.9 to –39.4]	
	Post-intervention (Visit 7)	-22.6 (8.5) [-7.7 to -38.4]	-24.4 (8.8) [-8.0 to -39.4]	
	Change (visit 7 – Visit 2)	p>0.05	p>0.05	
	Treatment difference Mean (95% CI)	1.89 (-7.76 to 11.5)		p>0.05

TABLE 4.3-7 :*Effects of M. vaccae (SRL172) on the allergen induced early asthmatic response (EAR).*

4.3.4.2.1 EAR – mild asthmatics

The treatment effect for each group is shown in (Table 4.3-8). The active group shows an average improvement of 7.56% in AAUC after treatment compared to 1.70% in the placebo group. The estimated difference between treatment groups was statistically not significant (5.86%; 95% CI –2.22 to 13.93%; p>0.05). When calculating the maximum % fall in FEV1, the active group shows on average a 13.48% lower maximum % fall after treatment compared to 8.13% in the placebo group. The estimated difference between treatment groups was statistically not significant (5.35%;95%CI –7.76 to 18.5%; p>0.05) (Table 4.3-8).

		***M. vaccae* (SRL172) (n=12)**	**Placebo (n=12)**	**p**
AAUC FEV_1, mean % change from baseline (SD) [range]	Pre-intervention (Visit 2)	-13.9 (5.9) [-3.4 to -25.4]	-12.6 (7.0) [-3.4 to -23.6]	
	Post-intervention (Visit 7)	-7.8 (6.6) [-2.4 to -22.5]	-8.6 (6.2) [0.8 to – 20.4]	
	Change (visit 7 – Visit 2)	p>0.05	p>0.05	
	Treatment difference Mean (95% CI)	2.0 (-4.2 to 8.2)		p>0.05
Maximum fall FEV_1, mean % change from baseline (SD) [range]	Pre-intervention (Visit 2)	-33.0 (9.8) [-20.6 to -50.6]	-29.5 (8.2) [-16.9 to –39.4]	
	Post-intervention (Visit 7)	-22.6 (8.5) [-7.7 to -38.4]	-24.4 (8.8) [-8.0 to -39.4]	
	Change (visit 7 – Visit 2)	p>0.05	p>0.05	
	Treatment difference Mean (95% CI)	1.89 (-7.76 to 11.5)		p>0.05

TABLE 4.3-8 : *Effects of M. vaccae (SRL172) on allergen induced early asthmatic response (EAR) in mild asthmatics.*

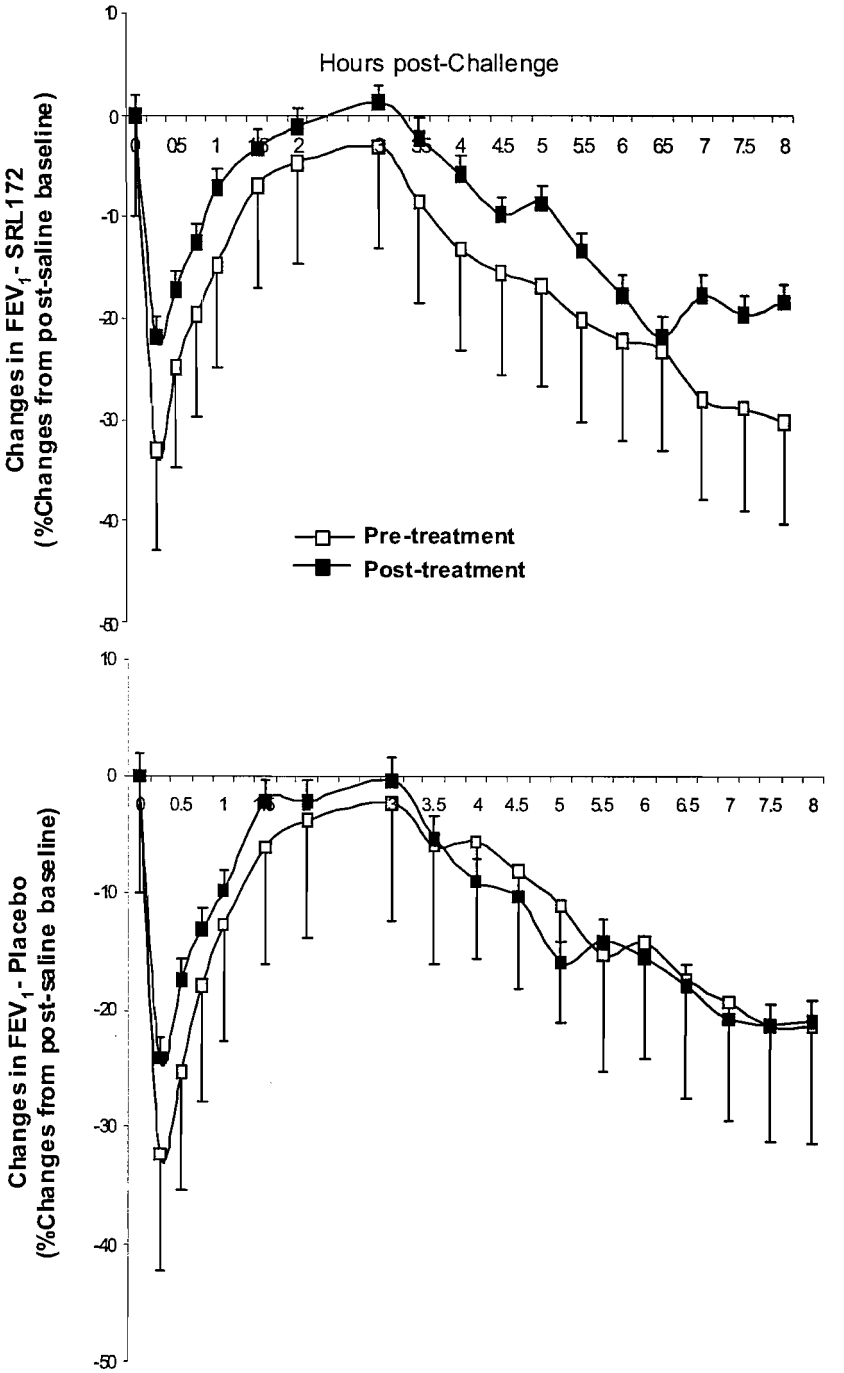


FIGURE 4.3-1: *Effects of SRL172 (top panel) and placebo (bottom panel) on allergen-induced late phase asthmatic responses.*

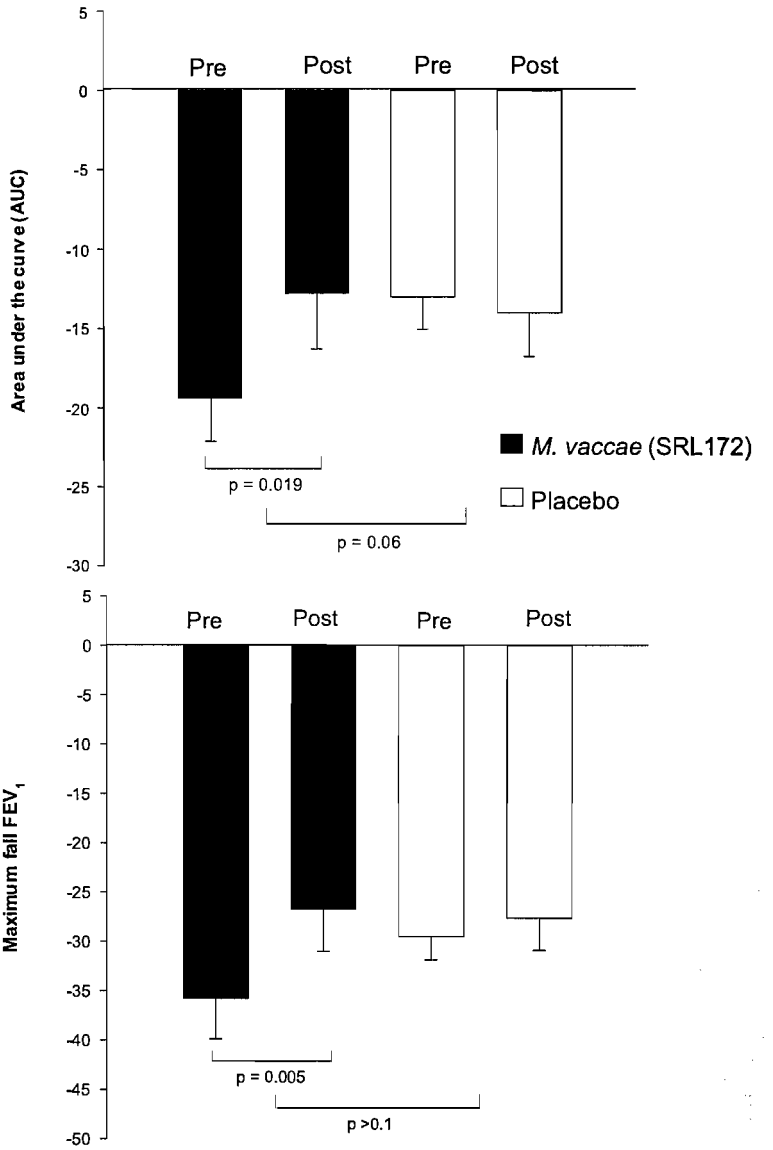


FIGURE 4.3-2: *Effects of SRL172 on area under the curve (AUC) and maximum fall in FEV_1 during allergen-induced late phase asthmatic responses (LAR). The p values of 0.06 and 0.1 denote levels of significance when comparing the two groups with respect to the changes (Δ) in AUC and maximum fall in FEV_1, respectively.*

4.3.4 Effect of treatment on airway responsiveness

The two groups of subjects were not different in respect of baseline $PC_{20}FEV_1$ histamine. Allergen challenge performed before any treatment caused a significant (p=0.007) change in $PC_{20}FEV_1$ from a geometric mean [95% CI] of 1.17 mg/ml [1.0-1.3 mg/ml] to 0.55 mg/ml [0.3-0.8 mg/ml] in the subjects that subsequently received SRL172 and a significant change (p=0.02) from 1.82 mg/ml [1.5–2.1 mg/ml] to 0.78 mg/ml [0.5–1.1 mg/ml] in the subjects that received placebo. The changes in $PC_{20}FEV_1$ between the two treatment groups were not significantly different (p=0.98). Comparisons of the geometric means for post-allergen challenge $PC_{20}FEV_1$ recorded before and after treatment with placebo or SRL172 using ANOVA showed no difference between the two treatment arms, with the mean value changing from 0.55 mg/ml to 0.76 mg/ml in the actively treated subjects and from 0.77 to 1.41 mg/ml in the subjects receiving placebo.

		***M. vaccae* (SRL172) (n=12)**	**Placebo (n=12)**	**p**
$PC_{20}FEV_{1(histamine)}$ Arithmetic mean (mg/ml) (SD) [range]	Pre-intervention (Visit 1)	1.32 (0.7) [0.6-2.7]	3.3 (4.2) [0.35-13.63]	
	Pre-intervention (Visit 3)	0.7 (0.5) [0.1-1.68]	1.76 (2.7) [0.1 -8.8]	
	Post-intervention (Visit 8)	1.1 (1.1) [0.2-4.2]	2.6 (2.9) [0.2 – 8.3]	
$PC_{20}FEV_{1(histamine)}$ (mg/ml) Geometric mean [95% CI]	Pre-intervention (Visit 1)	1.17 [1.0 – 1.3]	1.82 [1.5-2.1]	
	Pre-intervention (Visit 3)	0.55 [0.3-0.8]	0.78 [0.5-1.1]	
	Post-intervention (Visit 8)	0.77 [0.5-1.0]	1.41 [1.1-1.7]	
	Change post allergen challenge (Visit 3-Visit 1)	p=0.007	p=0.02	
	Treatment difference (visit 8 – Visit 3) Mean (95% CI)	-0.27 (-2.56 to 2.01)		p=0.98

TABLE 4.3-9: *Effects of SRL172/placebo on $PC_{20}FEV_1$ histamine.*

4.3.5 Effects of *M. vaccae* on sputum cellularity

Sputum supernatant was collected at visit four (prior to treatment) and visits six. Not all patients were able to produce sputum on all occasions, therefore eight patients in the placebo group and seven in the active group had complete data for analysis.

		***M. vaccae* (SRL172) (n=7)**	**Placebo (n=8)**	**p**
Neutrophils mean% (SD) [range]	Pre-intervention (Visit 4)	17.9 % (11.2) [1.3-40.9]	31.1% (16.9) [9.2–57.5%]	p>0.05
	Post-intervention (Visit 6)	16.2% (9.7) [0.21 – 30.7]	32.2% (22.8) [8.4–74.7%]	p>0.05
		p>0.05	p>0.05	
Eosinophils mean% (SD) [range]	Pre-intervention (Visit 4)	6.97% (5.9) [0.8–17.4%]	5.04% (4.6) [2.24–14.2%]	p>0.05
	Post-intervention (Visit 6)	8.03% (6.0) [2.3–21.8%]	3.93% (3.1) [0.64–8.64%]	p>0.05
		p>0.05	p>0.05	
Mo/Ma mean% (SD) [range]	Pre-intervention (Visit 4)	68.2% (17.1) [36.5–95.8%]	56.2% (15.5) [29.6–79.8%]	p>0.05
	Post-intervention (Visit 6)	73.0% (10.2) [57.6–89.7%]	60.7 % (21.4) [22.4–85.3%]	p>0.05
		p>0.05	p>0.05	
Lymphocytes mean% (SD) [range]	Pre-intervention (Visit 4)	3.2% (1.6) [0.9–5.94%]	3.45% (1.4) [1.7–4.9%]	p>0.05
	Post-intervention (Visit 6)	0.96% (1.2) [0–3.7%]	0.85% (0.8) [0–2.24%]	p>0.05
		p<0.001	p<0.001	

TABLE 4.3-10: *Effect of treatment on sputum cellularity*

No statistically significant difference was found in the number of neutrophils, monocyte/macrophages and lymphocytes pre- and post-treatment within each group and between treatment groups (Table 4.3-10)

4.3.6 Effects of *M. vaccae* on sputum mediators

Eosinophil cationic protein (ECP) can be detected in the sputum of patients with asthma (Fujimoto *et al.* 1997), in nasal and bronchoalveolar lavage fluids after the experimental inhalation of antigens, in saliva (Schmekel *et al.* 2001), serum (Niimi *et al.* 1998) and within involved tissues. In allergic diseases, eosinophil granule proteins may cause damage and desquamation of airway epithelial cells, alter airway hyperreactivity and cilial function, and elicit local oedema (Giembycz *et al.* 1999). ECP levels decrese in bronchial wash following steroid treatment (Robinson *et al.* 1995). Eosinophilia and ECP correlate with lung function and the clinical severity of the disease (Bousquet *et al.* 1990; Fujimoto *et al.* 1997). Serum or local levels of ECP are frequently measured in clinical and research settings as a parameter of eosinophil involvement or therapeutic efficacy.

Tryptase is the major enzyme stored in the cytoplasmic granules of human mast cells, and this neutral protease occurs in most, if not all, human mast-cell populations (Oskeritzian *et al.* 2000). Because this enzyme appears to be highly characteristic of, if not unique to, the human mast cell, measurements of mast-cell tryptase in biologic fluids such as plasma, serum, and inflammatory exudates have been used to assess mast-cell activation in these settings (Fahy *et al.* 1994; Oskeritzian *et al.* 2000).

In this study, the median [range] of ECP in the *M. vaccae* group was of 131.0 μg/ml [21.6–647 μg/ml] at visit four, compared to a median [range] of 173.3 μg/ml [10–799.2 μg/ml] at visit seven ($p>0.05$) (Figure 4.3-3). Similarly, the median [range] of ECP in the placebo group was of 48.47 μg/ml [10.7–243.8 μg/ml] at visit four, compared to a median [range] of 65.4 μg/ml [19.6–1,185 μg/ml] at visit seven ($p>0.05$). No difference was found between the two treatment groups (Figure 4.3-3).

When looking at the tryptase levels, the median [range] of tryptase in the *M. vaccae* group was of 5.9 μg/ml [0.04–29.6 μg/ml] at visit four, compared to a median [range] of 12.1 μg/ml [8.2–25.3 μg/ml] at visit seven ($p>0.05$) (Figure 4.3-3). Similarly, the median [range] of tryptase in the placebo group was of 14.3 μg/ml [2.8–53.5 μg/ml] at visit four, compared to a median [range] of 8.7 μg/ml [6.7-55.8 μg/ml] at visit seven ($p>0.05$). No difference was found between the two treatment groups (Figure 4.3-3).

4.3.7 Effects of *M. vaccae* on proliferation of peripheral blood mononuclear cells (PBMC)

Administration of *M. vaccae* may cause immunomodulation to allergen by affecting T cell phenotype in response to allergen with or without changes in proliferative response. To assess the change in allergen-induced proliferative response in the two treatment groups, PBMC from subjects belonging to the two study groups were stimulated with *Der p* at visit four (before the administration on *M. vaccae*/placebo) and at visit seven and eight (Figure 4.3-4).

No significant change was found within each group between visits, nor was there significant difference in proliferation between the two groups at sequential time points.

No correlation was found between the area of the wheal to *Der p* following the skin-prick test and the PBMC proliferation to allergen.

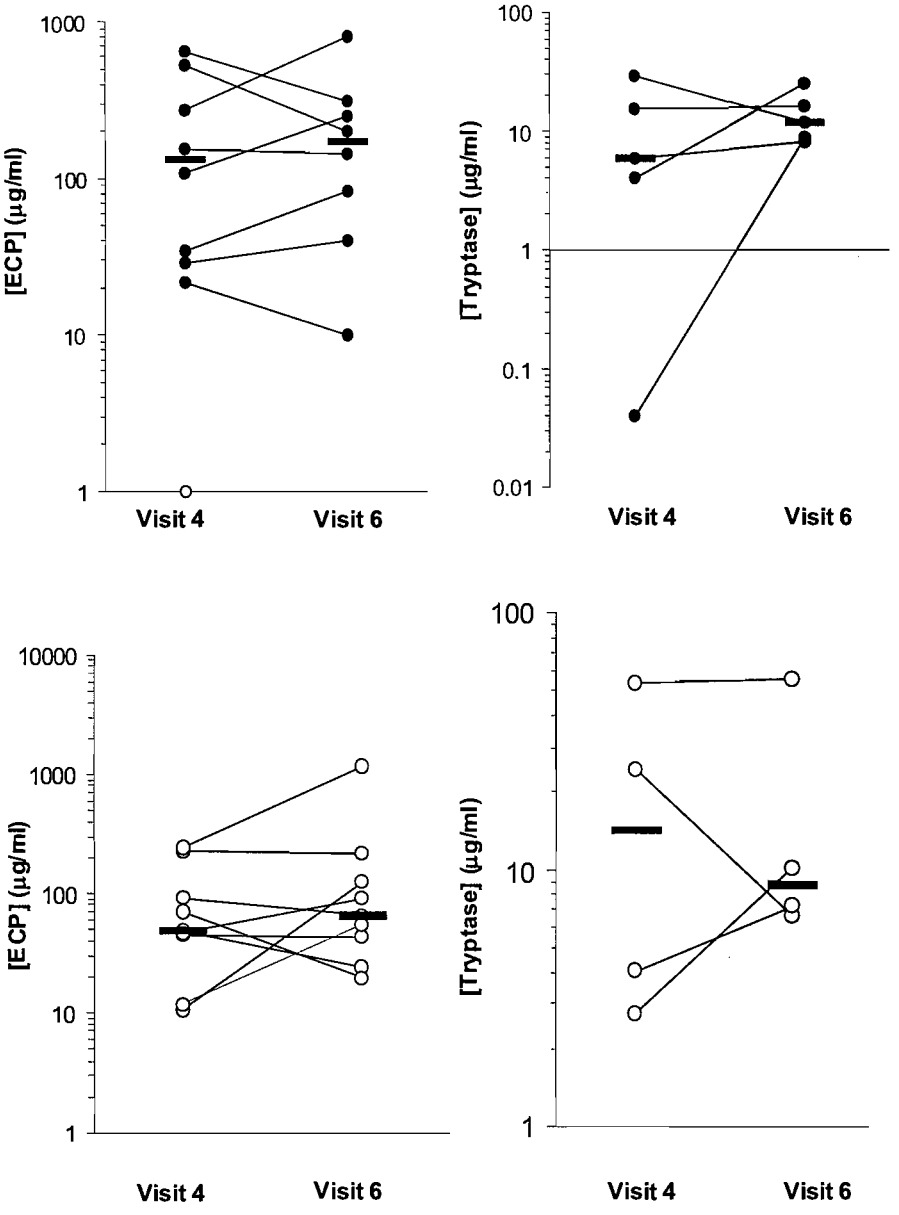


FIGURE 4.3-3: *Effects of M.vaccae (solid circles) or placebo (open circles) on the levels of eosinophil cationic protein (ECP) (upper panel) and tryptase (lower panel) in the sputum supernatant. Horizontal bars represent median values.*

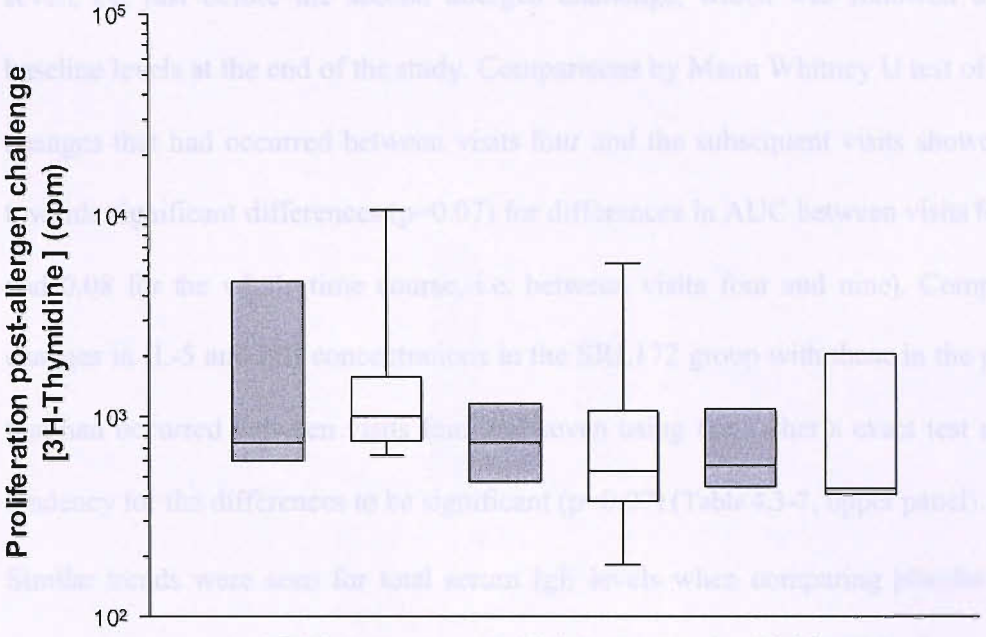


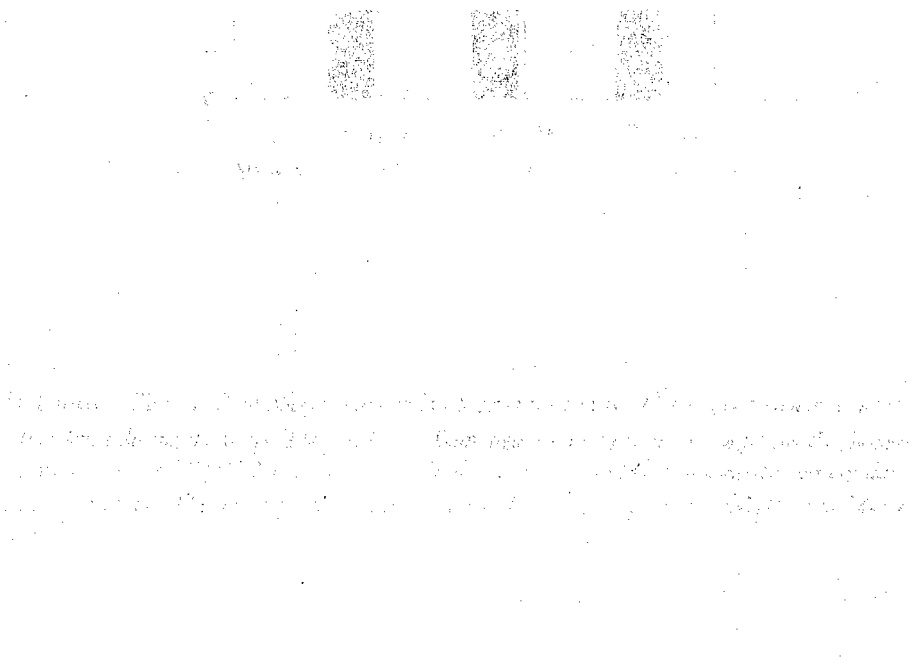
FIGURE 4.3-4: *Effects of M.vaccae (grey boxes) or placebo (open boxes) on allergen-induced PBMC proliferation.*

4.3.8 Effect of treatment on IL-5 synthesis *in vitro* and serum IgE levels

Cultures of PBMC were possible on all three occasions in 19 out of 24 subjects (ten treated with SRL172 and nine subjects receiving placebo). Subjects who were randomised to receive active treatment had a tendency to exhibit higher IL-5 responses to allergen *in vitro* but these were not significantly different from subjects receiving placebo (Table 4.3-7, upper panel). In seven out of ten subjects treated with SRL172 there was a gradual decline in IL-5 synthesis over the course of the study. In contrast, in only two subjects receiving placebo was there a decrease in IL-5. In the latter group there was a tendency towards a transient increase at visit

seven, i.e. just before the second allergen challenge, which was followed by a return to baseline levels at the end of the study. Comparisons by Mann Whitney U test of AUC of IL-5 changes that had occurred between visits four and the subsequent visits showed a tendency towards significant differences (p=0.07) for differences in AUC between visits four and seven and 0.08 for the whole time course, i.e. between visits four and nine). Comparison of the changes in IL-5 and IgE concentrations in the SRL172 group with those in the placebo group that had occurred between visits four and seven using the Fisher's exact test also showed a tendency for the differences to be significant (p=0.07) (Table 4.3-7, upper panel).

Similar trends were seen for total serum IgE levels when comparing placebo and SRL172 treatment groups (p=0.07 when comparing visits four and seven by Fisher's exact test) (Table 4.3-7, lower panel). Comparison of AUC showed no difference between the two treatment groups.



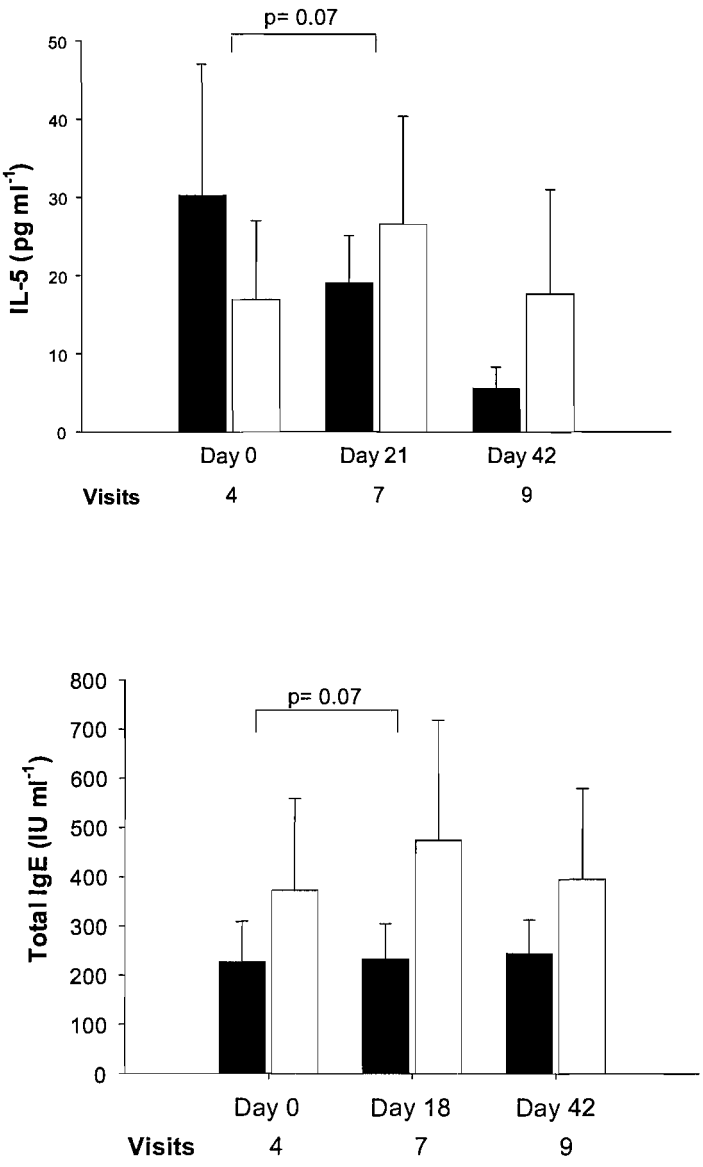


FIGURE 4.3-5: *Effects of SRL-172 on allergen-induced IL-5 generation by peripheral blood mononuclear cells (PBMC) and total serum IgE levels during the study. The p values indicate significance levels when comparing the changes (Δ) in IL-5 and IgE concentrations in the SRL172 group with those in the placebo group that had occurred between day 0 and day 21 using the Fisher's exact test. The bars represent mean ± standard error of the mean (SEM).Placebo (oper bars) M. vaccae (Black bars).*

4.4 DISCUSSION

This study has shown that a single intradermal injection of SRL172 in allergic asthmatics reduces the allergen-induced LAR, and is associated with a tendency for reduced IL-5 synthesis by T cells stimulated with allergen *in vitro*.

Although the difference between active treatment and placebo failed to reach conventionally accepted significance levels for comparisons of AUC of the LAR, and was not statistically significant for maximum falls in FEV_1, these findings suggest that SRL172 may have the potential to modulate both the airways reaction to specific allergens and the associated upregulation of Th2-type cytokines that is typical of atopic asthma. These observations and conclusions are supported by a recent study showing that intradermal administration of *M. vaccae* can reduce the requirement for bronchodilators in mild pollen-induced seasonal asthma (Hopkin *et al.* 1998) and a study in which SRL172 reduced the intensity of atopic dermatitis in children (Arkwright *et al.* 2001)

Allergen challenge has been widely used in asthma research. It has to be recognised that comparison of the effects on airway responses of SRL172, specifically the AUC of the LAR, with those of placebo just fails to reach the 5% significance level ($p=0.06$) that is usually sought in clinical trials. However, the high significance of the comparisons of both the maximum fall in FEV_1 and the AUC of the LAR within the group of subjects treated with SRL172 strongly suggests that *M. vaccae* may be able to attenuate allergen-induced airway responses.

One difficulty with the design of this study has been the lack of sufficient knowledge about the time-course of action of SRL172. Studies of inter-subject variability and reproducibility of late phase responses have shown that in order to demonstrate a 30% reduction in the LAR, with 95% power and probability of less than 5% of the difference occurring by chance, 12

subjects are needed (Inman *et al.* 1995). However, it is likely that the imunomodulatory capacity of SRL172 varies considerably from mildly to highly allergic individuals and it is, therefore, possible that a greater degree of confidence in the biological effect might have been seen in the present study if more subjects had been included to take into account this variability. The dose of SRL172 was selected as the largest dose used in man giving a Th1 pattern of response to the mycobacterial antigen that did not cause unacceptable local inflammation. Although no dose-response studies have been performed in humans, in the mouse model the dose of *M. vaccae* does not seem to be critical and a wide range (100-fold) of doses have similar effects. It is also possible that the timing of the challenge in relation to the injection of the vaccine was not optimal. I based this timimg on a study in the murine ovalbumin-sensitised mouse showing the attenuated allergic response seen three weeks after administration of *M. vaccae* (Wang *et al.* 1998). Finally, it is also possible that a greater effect may have been observed with multiple injections of SRL172. Although, there are no published data on the effects of multiple injections of *M. vaccae* in adults with moderate asthma asthma, a multi-centred phase II, randomized, placebo-controlled study on 178 patients, funded by by Sakai has shown in an exploratory analysis a trend towards improvement among patients receiving two high doses of *M.vaccae* showed a significant reduction in asthma symptoms score and exacerbations (SRPharma). In a trial using repeated, weekly injections of killed *M. bovis* BCG for four weeks, showed no difference in asthma symptoms or any other markers of asthma severity. Importantly, the trial was stopped early because of unacceptably high local reactions to BCG injection (Shirtcliffe *et al.* 2004). It would be interesting to see whether a similar schedule with the non-pathogeic *M. vaccae* would give similar results in terms of asthma symptoms or local side effects.

There is no data on whether *M. vaccae* is immunogenic *in vivo*, and no studies have been published in humans on the safety and efficacy of multiple doses of *M. vaccae* in allergic asthma (Dr L. R. Brunet, SRpharma, personal communication). However, as discussed in Chapter one, preliminary unpublished data report an improvement in asthma symptoms in patient who received 2 doses of M. vaccae (SRpharma).

The trend towards an effect of a single injection of SRL172 to reduce the capacity of peripheral blood T cells from allergic individuals to generate IL-5 in response to specific allergen adds support for its potential to inhibit relevant allergic mechanisms. In contrast to the transient rise in allergen-induced IL-5 synthesis *in vitro* that could be seen in subjects receiving placebo, in seven out of ten subjects treated with SRL172 there was a steady decline in IL-5 synthesis. This would suggest a protective effect against allergen exposure in sensitised individuals.

In the absence of PBMC culture at the beginning of the study, i.e. before the first allergen challenge, I have no definitive explanation for the time-course of IL-5 synthesis, and speculate that the transient rise in placebo-treated subjects was a consequence of the first allergen challenge, with the potential for IL-5 generation returning to baseline at the end of the study. By way of its effects on the maturation of eosinophils from bone marrow-derived precursors, and their activation and prolonged survival in the affected organ, IL-5 is one of the key cytokines in allergic diseases. It is, however, becoming apparent that IL-5 is not the only cytokine involved. Indeed a recent clinical trial using a humanised anti-IL-5 antibody has showed no significant reduction in the LAR (Leckie *et al.* 2000). It is therefore important to appreciate that, unlike agents which target single mediators, *M. vaccae* has a potentially wider effect through its ability to upregulate IFN-γ and reduce both IL-4 and IL-5 (Wang *et al.* 1998).

Contrary to animal studies (Erb *et al.* 1998), no change in sputum eosinophilia, ECP and tryptase was found following *M. vaccae* treatment. This observation could be might be explained by an already low baseline eosinophil count, the mild disease severity in both groups and possibly a too small sample size. Other factors that could explain the apparent lack of effect on sputum cellularity include the timing of the sputum induction (time between allergen challenge-intervention-sputum induction), the dose of *M.vaccae* and single versus multiple injections, and the route of administration. Previous studies using *M. bovis* BCG have shown that the intranasal route, but not systemic route, was able to reduce pulmonary eosinophila and IL-5 synthesis (Erb *et al.* 1998). The intranasal administration of *M. bovis* BCG raises concerns over unacceptable nasal inflammation, colonisation of the oro-pharyngeal epithelium or development of granulomatous disease, and therefore has not been trialed in humans. In a recent study, however, *M. vaccae* has been administered intragastically in a mouse model of asthma (Hunt *et al.* 2005). The authors have demonstrated that a single administration of *M. vaccae* by gastric route was able to reduce eosinophilic infiltrate following allergen challenge. Furthermore, there was an increase in the synthesis of IL-10, implying an expansion of cells with regulatory properties (Hunt *et al.* 2005). These cells include T regulatory cells and regulatory APC $CD11c^{+}$, which in turn could down-regulate the Th2 response independently from the reduction in eosinophils or mast cells (Zuany-Amorim *et al.* 2002; Zuany-Amorim *et al.* 2002; Adams *et al.* 2004).

In demonstrating a benefit from stimulating the immune system with *Mycobacteria*, the results of this study lend support to the 'hygiene hypothesis'. As discussed more in detail in Chapter three of this thesis, the strong association between delayed hypersensitivity to *Mycobacterium tuberculosis* and atopic disease seen in Japanese children, (Shirakawa *et al.* 1997) and the increase in IgE levels following successful treatment of pulmonary TB in South

Africa (Adams *et al.* 1999) suggest that immune responses to mycobacteria are an important means of inhibiting allergic responses. It should, however, be appreciated that *M. vaccae* is unique in its ability to modulate immune responses. Its Th1 adjuvant property is unaffected by killing, whereas other mycobacteria, such as BCG, have little Th1 adjuvant effect when dead (Abou-Zeid *et al.* 1997). *M. vaccae* also down-regulates pre-existing Th2 responses in a manner that appears to be independent of its ability to enhance Th1 responses (Wang *et al.* 1998). Thus, a recombinant strain expressing the dominant T cell epitope of ovalbumin or organisms to which ovalbumin had been covalently bonded are both able to induce a Th1 response to ovalbumin, but this does not result in greater suppression of the pre-existing Th2 response than is achieved with heat-killed organisms alone (Wang *et al.* 1998). A further unique property is the priming of $CD8^+$ T cells in mice (Skinner *et al.* 1997). When mixed with ovalbumin and injected intraperitoneally, killed *M. vaccae* drives the formation of $CD8^+$ cytotoxic T cells that lyse recombinant EL-4 thymoma cells expressing ovalbumin (Watson 1998). In view of the suggested role of $CD8^+IFN\text{-}\gamma^+$ T cells in the control of Th2 responses, (MacAry *et al.* 1997) this property may be of relevance in interpreting the present study. However, as discussed in Chapters four and six, it seems now increasingly clear that the immunomodulatory properties of *M. vaccae* are distinct from pathogenic bacteria (such as *M. tuberculosis*) and also BCG. There is now evidence that the bacteria implicated in the 'hygiene hypothesis' are not those that cause infectious diseases, but are the harmless commensal species present in the environment throughout human evolution. These include Lactobacilli (Kalliomaki *et al.* 2003) and saprophytic environmental mycobacteria (Rook *et al.* 1998; Rook *et al.* 2003). These 'old friends' are recognised by the innate immune system and are able to stimulate regulatory T (Treg) cells (Rook *et al.* 2003). Treg cells can then

reduce the effector response and, by maintaining a constant antigen source provide the immune stimulus necessary for immunity to re-infection (Belkaid *et al.* 2002).

In conclusion, the results of this study provide the first evidence that SRL172 mayo reduce the magnitude of the late phase response to allergen. This may be associated with an inhibitory effect on IL-5. Whilst the findings of my study are not definitive, I believe that they justify the need for a larger clinical trial, involving multiple dosing, to study the potential of SRL172 as a treatment for chronic asthma.

CHAPTER FIVE

Effects of killed *Mycobacterium vaccae* on *in vitro* allergic inflammation

5.1 INTRODUCTION

Bronchial asthma is characterised by chronic airway inflammation orchestrated by activated Th2 cells secreting the cytokines interleukin (IL)-4 and -5 which promote airway eosinophilia and IgE synthesis and ultimately lead to airway remodelling (Romagnani 1994).

Epidemiological studies have suggested a link between the worldwide rise in atopic disorders and a change in exposure and immunity to microbial agents (Holt 1996; Hopkin 1997; Strachan 2000; Braun-Fahrlander *et al.* 2002), forming the basis for the 'hygiene hypothesis' of allergic diseases. Many bacteria and their components have the potential to modulate the function of immune cells, which could, in principle, protect against the development of Th2 diseases (Shaheen *et al.* 1996; Strachan *et al.* 1996). Mycobacteria have attracted particular attention as epidemiological evidence suggests that immunity to mycobacteria is associated with lower risk of atopy (Shirakawa *et al.* 1997; Rook *et al.* 1998). However, there is now evidence that the bacteria involved in the 'hygiene hypothesis' are not those that cause infectious diseases but the harmless commensal species present in the environment throughout human evolution. These include Lactobacilli (Kalliomaki *et al.* 2003), saprophytic environmental mycobacteria (Rook *et al.* 1998; Rook *et al.* 2003), the helminths (reviewed in (Yazdanbakhsh *et al.* 2002; Rook *et al.* 2003)) and bacterial DNA.

How mycobacteria might exert their immunomodulatory effects in humans are unclear. Most of the studies to date have been conducted with BCG which elicits particularly strong protective Th1 immune responses. As shown in Chapter four, my previous *in vivo* study in human volunteers (Camporota *et al.* 2003) showed trends in reduction of *ex vivo* IL-5 synthesis by peripheral blood mononuclear cells (PBMC) and a tendency towards reduced serum IgE following treatment with a single intradermal injection of *M. vaccae*. In this Chapter, I report the results of a study in which the *in vitro* effects of *M. vaccae* (SRP299) on

cytokine production by PBMC from mild atopic asthmatics were investigated. I show that SRP299 causes a potent inhibition of allergen-induced IL-5 and IL-13 synthesis. I also show that this effect does not involve IL-12 induction but involves IL-10, providing further evidence that suggests regulatory T cell induction by *M. vaccae*. Furthermore, I show that the inhibitory effect of *M. vaccae* involves generation of prostaglandins.

5.2 METHODS

5.2.1 Subjects

Eight non-smoker atopic asthmatics with mild asthma requiring only inhaled β_2-agonists, were recruited. None of the subjects had reported a respiratory infection within 14 days of the study nor had ever used oral or inhaled corticosteroids. The study was approved by the Southampton University and Hospitals Ethics Committee and subjects gave their written informed consent.

5.2.2 *In vitro* cytokine responses to allergen

PBMC were isolated from 40 ml of heparinised venous blood by Ficoll-Hypaque gradient centrifugation as described in 2.2.6.1 and 2.2.6.3.

It has been previously proposed that cyclo-oxygenase-2 (COX-2) plays an important role in limiting inflammatory processes. In order to study the contribution of prostaglandins to the inhibitory effects of *M. vaccae*, PBMC were cultured in the presence of indomethacin (a COX-1 and COX-2 inhibitor) at a concentration of 0.2mM. To further determine whether the two main prostanoids prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) exerted a differential effect on PBMC functional phenotype, PBMC were stimulated with either PGE_2 (Iloprost,

Cayman chemical, Ann Arbor, MI, USA) or PGI_2 (Schering Health Care Limited, Burgess Hill, West Sussex, UK) at a concentration of 1 μM.

5.2.3 Statistical analysis

For statistical analyses, the Graph Pad InStat® package (Graph Pad Software, San Diego, USA) program was used. Data were analysed for distribution with the Kolmogorov-Smirnov test. Repeated measure ANOVA was used for the comparisons among the groups. When differences were statistically significant, Dunnett's post test for multiple comparisons test was used for between-group comparisons (groups versus *Der p* stimulated cultures). For between group analysis the Bonferroni's test for multiple comparisons was also used. When multiple comparisons were not required t-test and Wilcoxon test was employed. A $p<0.05$ was considered statistically significant.

5.3 Results

5.3.1 Effects of M. vaccae on type 2 and type 1 cytokine production

Stimulation of PBMC with *Der p* caused a significant increase in IL-5 ($p<0.01$) and IL-13 ($p<0.001$) after seven days of culture (Figure 5.3-1a and b). The addition of *M. vaccae* to the allergen-stimulated culture resulted in a significant ($p<0.01$) mean 65.2 % inhibition in *Der p*-induced IL-5 and a smaller but still highly significant ($p<0.01$) mean 36.7 % inhibition in IL-13 synthesis (Figure 5.3-1a and b). Stimulation of PBMC with *Der p* alone did not cause any significant increase in IFN-γ synthesis (First three bars Fig 1c), but in the presence of *M.*

vaccae there was a significant ($p<0.05$) 48.6 % mean increase in IFN-γ production (Figure 5.3-1c).

5.3.2 Effects of *M. vaccae* and IL-12 on cytokine responses

The ability of *M. vaccae* to stimulate Th1 immune response was demonstrated by time-course/dose-response experiments in which *M. vaccae* stimulated both IL-12 and IFN-γ synthesis in a dose-dependent fashion (Figure 5.3-2a, b). The production of IL-12 and IFN-γ peaked at day five. The relative role of IL-12 in the inhibition of Th2 cytokine synthesis caused by *M. vaccae* was further studied.

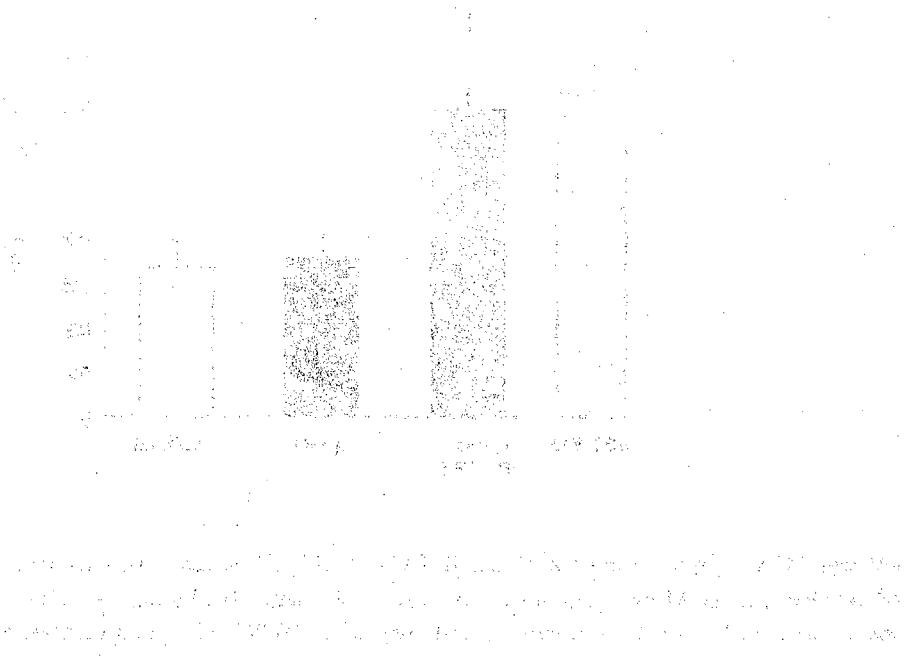
M. vaccae and IL-12 were effective in down-regulating allergen-induced IL-5 and IL-13 responses (Figure 5.3-2a,b), although *M. vaccae* was marginally, but not statistically significantly, more effective at inhibiting IL-5 (Figure 5.3-2), while IL-12 was able to cause a significantly greater inhibition of IL-13 ($p<0.05$) (Figure 5.3-2). In the presence of *Der p, M. vaccae* and IL-12 were both able to inhibit the polarisation of T cells induced by allergen. Neutralisation of IL-12 with blocking antibody did not significantly reverse the inhibition of *Der p*-induced IL-5 and IL-13 production caused by *M. vaccae* (Figure 5.3-2). In contrast, neutralisation of IL-12 was effective at inhibiting *M. vaccae*-induced IFN-γ generation ($p<0.05$) (Figure 5.3-2).

5.3.3 The role of IL-10 in Th2 and Th1 cytokine generation

Another mechanism by which *M. vaccae* is thought to down-regulate allergic response is by inducing IL-10 synthesis to expand the population of allergen-specific regulatory T cells. I therefore carried out a time-course and dose-response experiment in which a dose-dependent

increase in IL-10 synthesis by PBMC in response to *M. vaccae* (Figure 5.3-3) was observed. IL-10 production peaked at day three, i.e. before the peak of the IL-12 and IFN-γ responses. To further test the role of IL-10, I added anti-IL-10 to cultures in which PBMC were stimulated by allergen in the presence of *M. vaccae* and neutralising antibody to IL-12 in order to see whether there was any additional inhibition. The levels of IL-5 in cultures of *M. vaccae/Der p*-stimulated PBMC were significantly increased in the presence of antibodies blocking IL-10 ($p<0.05$) (Figure 5.3-4), showing restoration of IL-5 production. However, IL-13 levels were not significantly affected by additionally blocking IL-10 (Figure 5.3-4).

When neutralising antibodies for IL-10 were added to PBMC stimulated by allergen in the presence of *M. vaccae* and anti-IL-12 antibodies, the production of IFN-γ was increased significantly (Figure 5.3-4), in keeping with the notion that IL-10 regulates both Th1 and Th2 responses. Isotype control antibodies (IgG2a) had no effect on the allergen-stimulated cytokine levels.



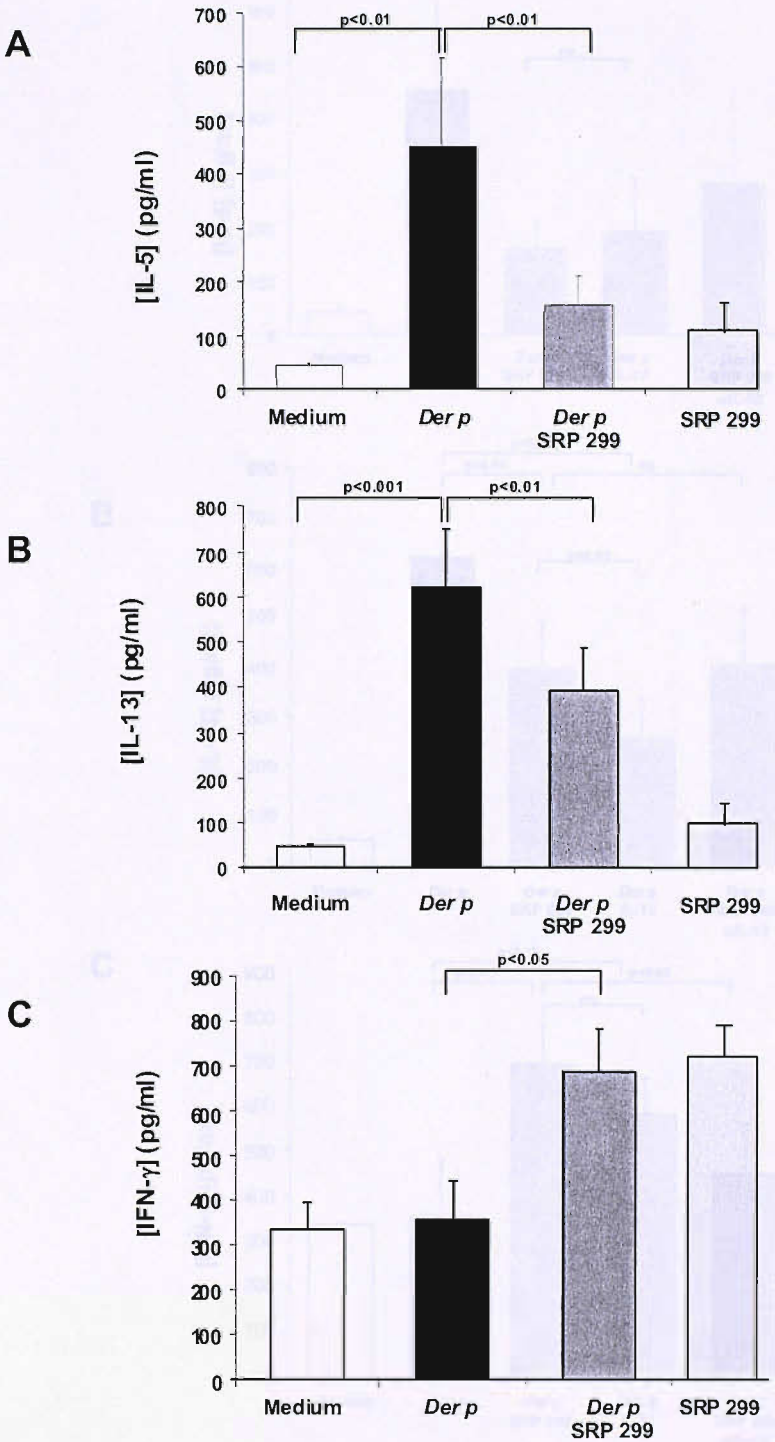


FIGURE 5.3-1: *Effects of M. vaccae on IL- 5 (A), IL-13 (B) and IFN-γ production (C). PBMC were stimulated with 5,000 SQ units/ml Der p (closed bars), cultured in medium alone (open bars), with M. vaccae (SRP299) 10 μg/ml light grey bars) Der p and M. vaccae (SRP299) 10 mg/ml (grey bars) for seven days . The results are shown as mean ± SEM.*

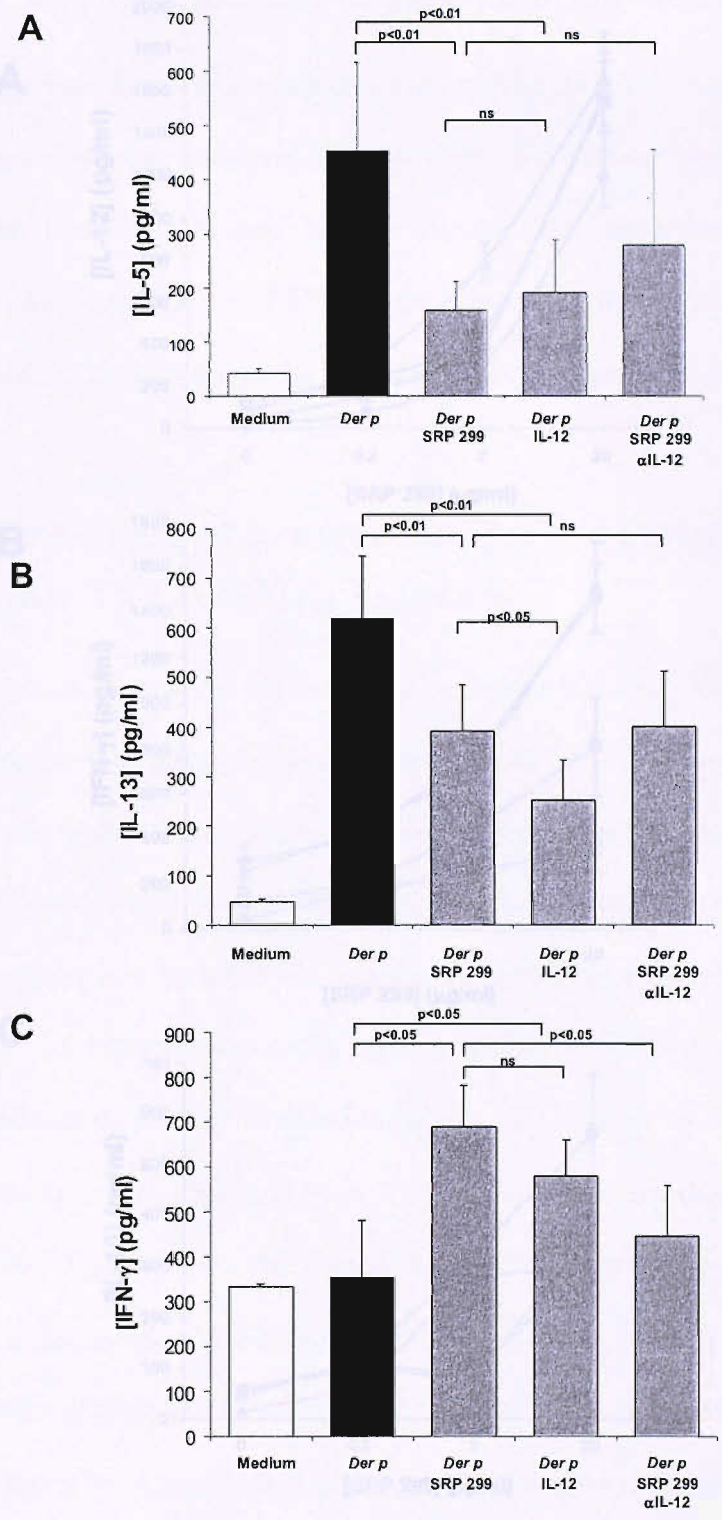


FIGURE 5.3-2: *Effects of M. Vaccae (SRP299) and IL-12 on IL-5 (A), IL-13 (B) and IFN-γ production (C). PBMC were stimulated with 5,000 SQ units/ml Der p (closed bars), cultured in medium alone (open bars) or with Der p and SRP299; Der p and IL-12; Der p, SRP299 and αIL-12mAb (grey bars) for seven days. The results are shown as mean ± SEM.*

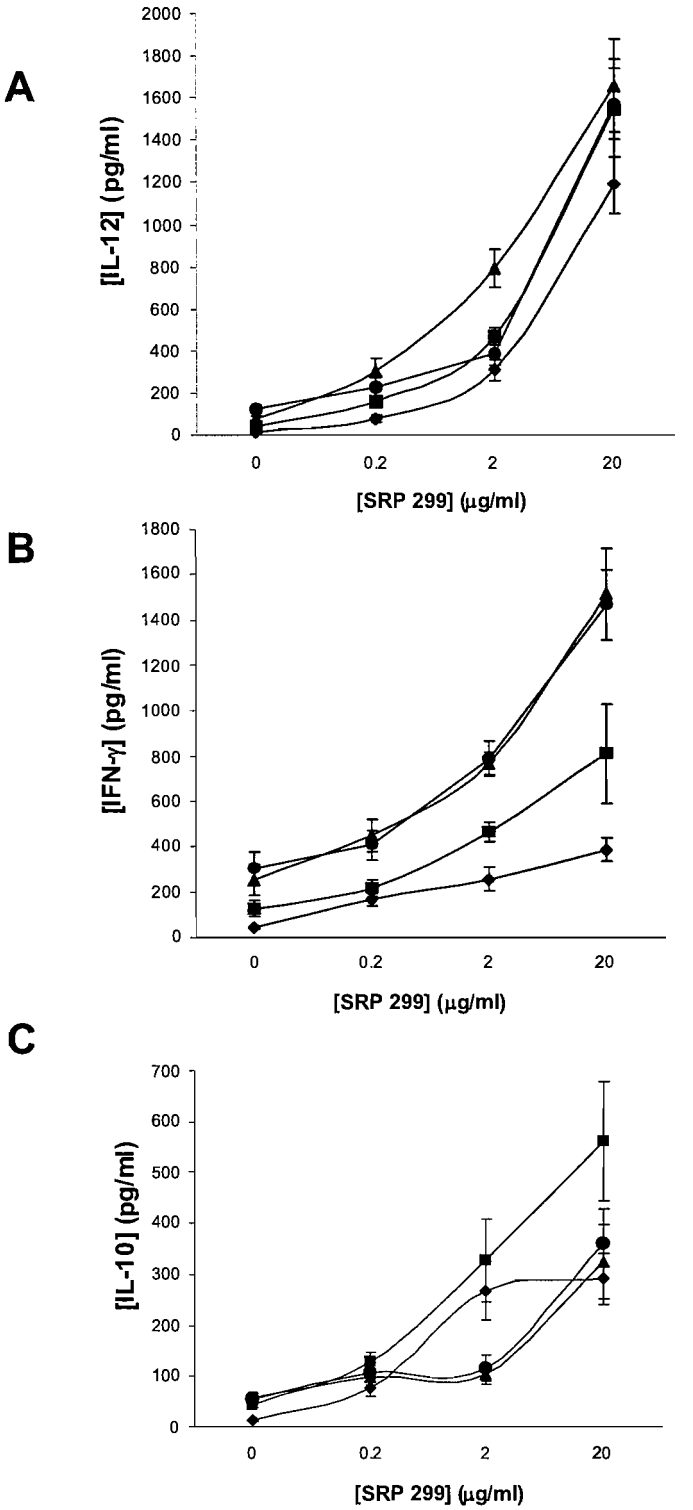


FIGURE 5.3-3: *Time-course and dose-response of IL-12 (A), IFN-γ (B) and IL-10 (C). PBMC were simulated with medium alone, M. vaccare (SRP299) 0.2 µg/ml, SRP299 2 µg/ml, SRP299 20 µg/ml for one (♦), three (■), five (▲) and seven days (●).The results are shown as mean ± SEM.*

5.3.4 The role of IL-18 in association with IL-12

IL-12 has been shown to act in association with IL-18 (El-Mezayen *et al.* 2004). Therefore, I investigated whether additional blocking of IL-18 would contribute to the reversal of the Th2 cytokine inhibition induced by *M. vaccae.* The restoration of IL-5 production in *M.vaccae/Der p*-stimulated PBMC in the presence of the combination of antibodies against IL-12 and IL-18 was only partial and statistically not significant (Figure 5.3-5).

5.3.5 The role of prostanoids on the ability of *M. vaccae* to inhibit Th2 cytokine response

Given that blocking IL-10 did not have an effect on IL-13 synthesis, I hypothesized that other mediators such as prostaglandins may be induced by *M. vaccae* and that these would play a role in the inhibition of cytokine responses. To test this hypothesis I assessed the effects of indomethacin, which irreversibly inactivates both cyclo-oxygenase (COX)-1 and COX-2. Indomethacin had no effect on allergen-induced cytokine production. When given in the presence of blocking antibodies against IL-10, IL-12/IL-18, treatment of PBMC with indomethacin completely inhibited the ability of *M. vaccae* to down-regulate both IL-5 and IL-13 in response to allergen (Figure 5.3-5). However, no significant effect was seen on IFN-γ production (Figure 5.3-5). The presence of blocking antibodies against IL-12, IL-18 and IL-10 did not impair the ability of PBMC to respond to non specific stimuli such as Phytohaemagglutinin (PHA) when added to the culture supernatant at day six (24 hours before harvesting) (Figure 5.3-6). This indirectly shows that the modulation of cytokine responses by *M. vaccae* and the observed contribution of IL-10, IL-12 and IL-18 are not due to a non-specific reduction in cell responsiveness or cell death/apoptosis. Isotype control antibodies (IgG2a) had no effect on the allergen-stimulated cytokine levels.

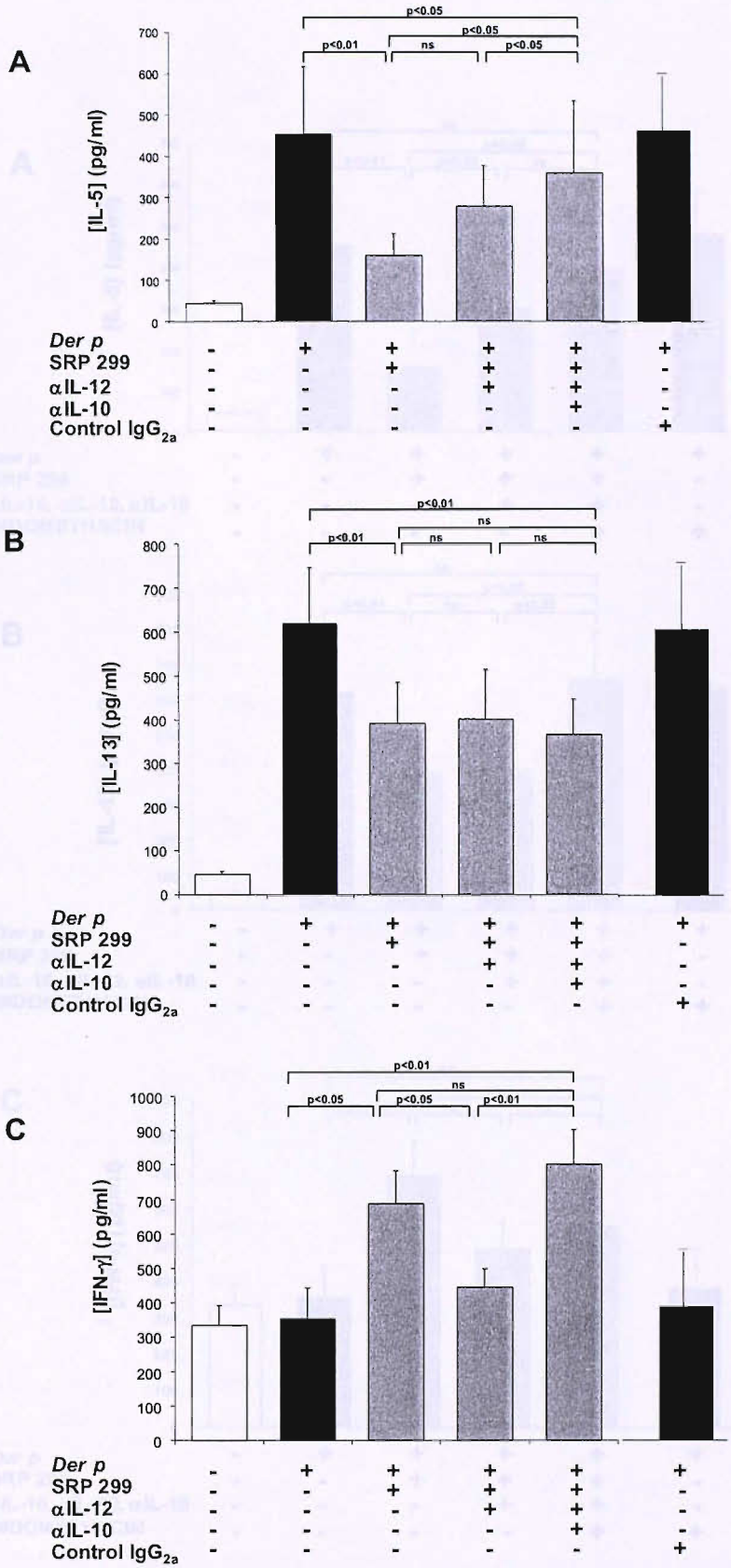


FIGURE 5.3-4: *Effects of M. Vaccae (SRP299) and IL-12 on IL-5 (A), IL-13 (B) and IFN-γ production (C). PBMC were stimulated with 5,000 SQ units/ml Der p (closed bars), cultured in medium alone (open bars) or with Der p and SRP299; Der p, SRP299 and αIL-12mAb, Der p, SRP299 and αIL-10mAb (grey bars) for seven days. The results are shown as mean ± SEM.*

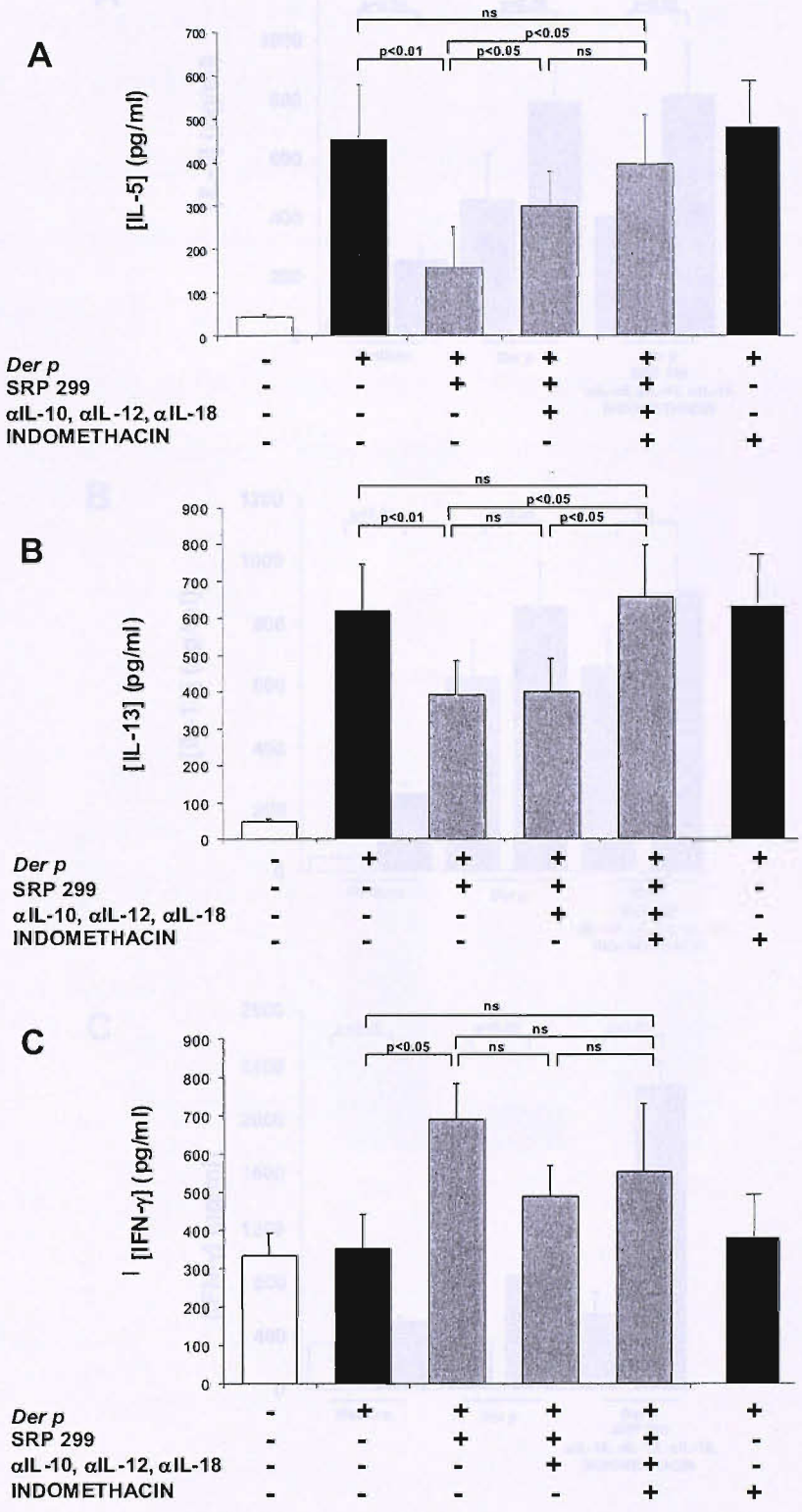


FIGURE 5.3-5: *Effects of M. vaccae (SRP299) and IL-12 on IL- 5 (A), IL-13 (B) and IFN-γ production (C). PBMC were stimulated with 5,000 SQ units/ml Der p (closed bars), cultured in medium alone (open bars) or with Der p and SRP299; Der p , SRP299 and αIL-12mAb/ αIL-18mAb/ αIL-10mAb , ± Indomethacin (grey bars) for seven days . The results are shown as mean ± SEM.*

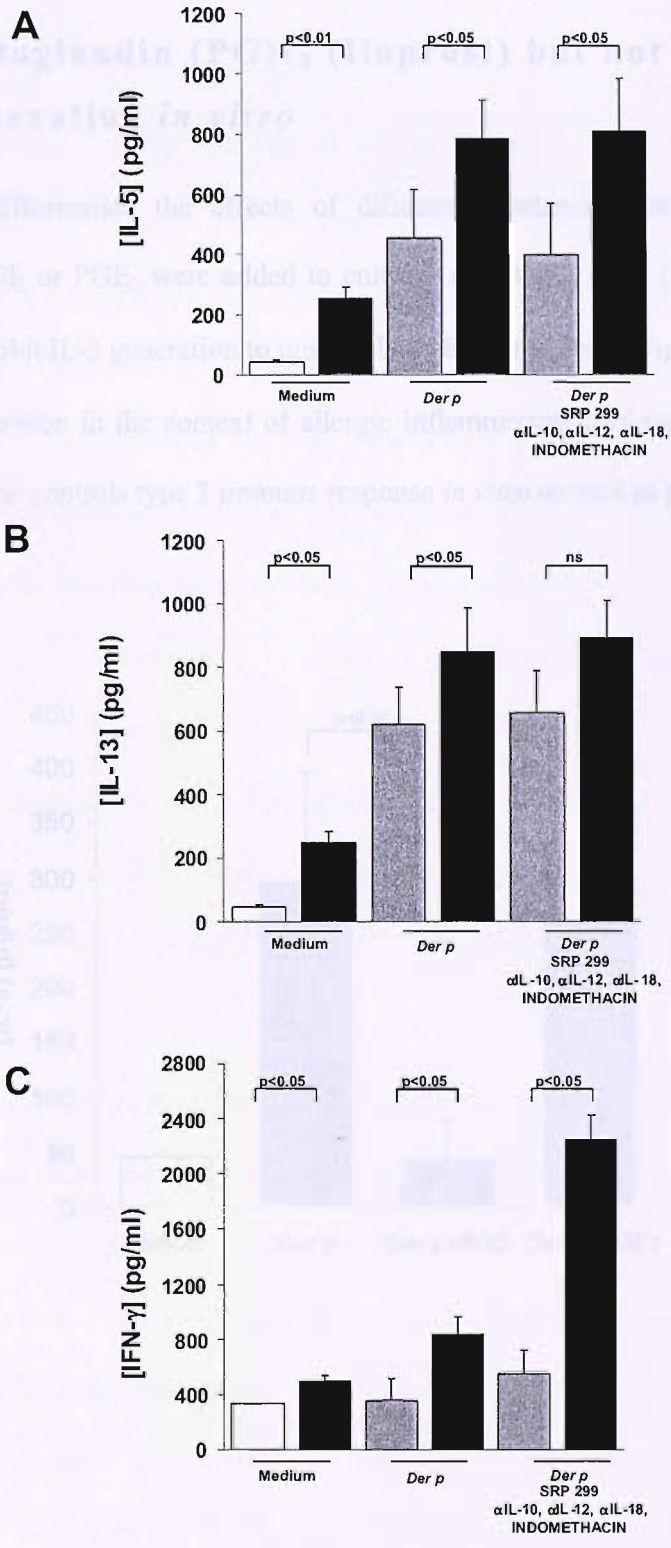


FIGURE 5.3-6: *Blocking antibodies did not impair PBMC responsiveness to non-specific stimuli (Phytohaemagglutinin , PHA). Effects of M. Vaccae (SRP299) on and IL-12 on IL- 5 (A), IL-13 (B) and IFN-γ production (C). PBMC were stimulated with 5,000 SQ units/ml Der p), cultured in medium alone (open bars) or with Der p and SRP299; Der p , SRP299 and αIL-12mAb/ αIL-18mAb/ αIL-10mAb , ± Indomethacin in the presence (filled bars) or absence of PHA(grey bars) for seven days. The results are shown as mean ± SEM.*

5.3.6Prostaglandin (PG)I_2 (Iloprost) but not PGE2 inhibits IL-5 generation *in vitro*

In order to differentiate the effects of different prostanoids on allergen-induced IL-5 production, PGI_2 or PGE_2 were added to cultures of PBMC. PGI_2 (Iloprost), but not PGE_2, was able to inhibit IL-5 generation to unstimulated baseline levels (Figure 5.3-7). This suggests that PGI_2 generation in the context of allergic inflammation may represent a mechanism by which *M. vaccae* controls type 2 immune response *in vitro* as well as possibly *in vivo.*

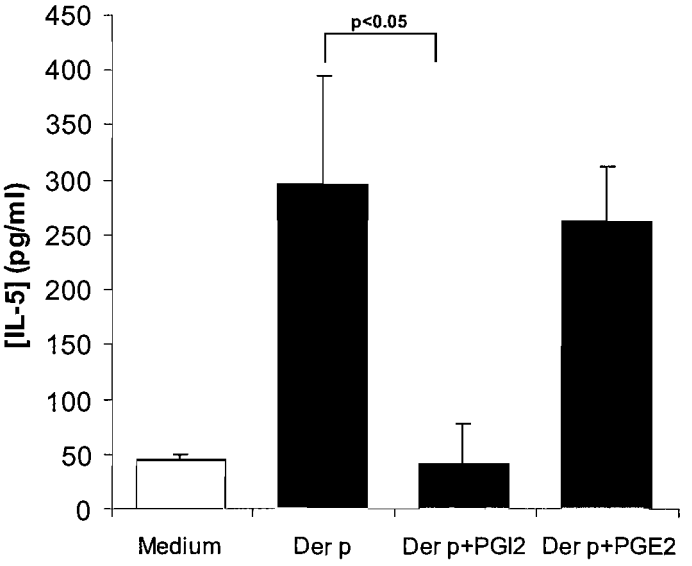


FIGURE 5.3-7: *Effects of Prostaglandins on IL-5 synthesis. The results are shown as mean ± SEM.*

5.4 DISCUSSION

This study shows for the first time that *M. vaccae* is able to down-regulate allergen-induced IL-5 and IL-13 synthesis by human T cells. Although *M. vaccae* caused an increase in IFN-γ synthesis, it was the generation of IL-10 and PGI_2, and not IL-12/IFN-γ, that were crucial for the inhibitory effects of *M. vaccae* on allergen-induced Th2 cytokines synthesis. Neutralisation of IL-10 reversed the inhibitory effects of *M. vaccae* on IL-5 but not IL-13 production, whilst prostaglandins had an additional effect on IL-13 production. It is not entirely clear why blocking IL-10 reverses the effects of *M. vaccae* on IL-5 but not IL-13 synthesis. It is possible that other cytokines (e.g. TGF-β), not investigated in the current study, play a more important role in regulating IL-13 synthesis. Our study showing that indomethacin completely reverses the inhibitory effects of *M. vaccae* but only minimally adds to the inhibitory effects of IL-10 my study suggests that *M. vaccae* regulates IL-5 synthesis largely via IL-10, whereas the regulation of IL-13 involves IL-10 and prostanoids.

Heat-killed *M. vaccae* has been shown to suppress allergic airway inflammation in a mouse model of asthma when administered either prior to sensitisation or during an established allergic response. Treatment with *M. vaccae* results in reduction in airway hyperresponsiveness, airway eosinophilia and IL-5 production induced by OVA challenge (Wang *et al.* 1998; Hopfenspirger *et al.* 2002; Smit *et al.* 2003). The down-regulation of the Th2 response has been attributed to the capacity of mycobacteria to strongly induce an upregulation of Th1 cytokines (Wang *et al.* 1998; Jouanguy *et al.* 1999; Janssen *et al.* 2001) or decrease in the IL-4/IFN-γ ratio (Xie *et al.* 2002). This would, in turn, dampen the Th2 response to allergen, theoretically by virtue of the cross-regulatory properties of the two T cell phenotypes. However, contrary to BCG, which stimulates a Th1 response, *M.vaccae* is an

environmental organism with different immunomodulatory properties, and reduced propensity to stimulate an exaggerated effector immune response (Rook *et al.* 2003). Indeed, recent animal studies have failed to demonstrate an increase in IFN-γ or the Th1-related IgG2a antibodies in serum following treatment with *M. vaccae* (Zuany-Amorim *et al.* 2002) and more recently, studies in mice have demonstrated that the effects of *M. vaccae* on Th2 cytokine generation and airways responsiveness might not be secondary to an increase of Th1-type cytokines, as previously thought, but to the expansion of a population of IL-10 producing regulatory T cells and $CD11c^{+}$ antigen presenting cells with regulatory properties (Zuany-Amorim *et al.* 2002; Adams *et al.* 2004). The inhibition of the Th2 response in this model is mediated through IL-10 and transforming growth factor-β (TGF-β). This is demonstrated by the ability of antibodies against IL-10 and TGF-β to completely reverse the inhibitory effect of Treg cells transferred from animals treated with *M. vaccae* to recipient animals prior to sensitisation with allergen (Zuany-Amorim *et al.* 2002).

Human studies demonstrate that human Treg cells can suppress responses to recall antigens and allergens (Taams *et al.* 2002; Bellinghausen *et al.* 2003; Jutel *et al.* 2003). Dendritic cells exposed to antigen produce IL-10 and stimulate the development of IL-10-producing Treg cells (Akbari *et al.* 2001; Akbari *et al.* 2002) and T cells expanded with IL-10 both *in vivo* and *in vitro* show suppressive activity (Akdis *et al.* 2004). Synthesis of IL-10 is believed to be important in the induction of tolerance that may provide an effective means of reducing T cell-driven airway inflammation in asthma.

A recent study of PBMC of healthy and allergic individuals has shown that Treg cells represent the dominant subset specific for common environmental allergens in healthy individuals; in contrast there is a high frequency of allergen-specific Th2 cells in allergic individuals, indicating that a change in the dominant T-cell subset – the frequency of allergen-

specific Treg cells over Th2 -may lead to allergy development or *vice versa* in recovery (Akdis *et al.* 2004). Treg cells are able to exert both an allergen-specific and bystander suppression of T cell proliferation in response to allergen stimulation *in vitro* (Akdis *et al.* 2004).

Synthesis of IL-10 by regulatory T cells has been shown to be important in the pathogenesis of atopic disorders. Indeed, children who outgrow cow's milk allergy have higher frequencies of circulating Treg cells and decreased *in vitro* proliferative responses to bovine ß-lactoglobulin. Depletion of Treg cells from PBMCs of tolerant children leads to an increase in allergen-specific proliferation, suggesting that Treg cells are capable of suppressing the effector Th2 cells (Karlsson *et al.* 2004). Similarly, successful treatment with grass-pollen immunotherapy results in increased frequency of IL-10 mRNA-positive cells in the nasal mucosa (Nouri-Aria *et al.* 2004) and increased IL-10 synthesis by allergen-stimulated Treg cells isolated peripheral T cells (Francis *et al.* 2003; Nouri-Aria *et al.* 2004)

In the current study I have shown that *M. vaccae* increases IL-10 synthesis and the inhibition of IL-10 by blocking antibodies significantly reverses the inhibitory effects of *M. vaccae* on allergen-induced cytokine production (both IL-5 and IFN-γ). This demonstrate that *M. vaccae*-induced IL-10 has the capacity to down-regulate both Th2 and Th1 responses induced by specific allergen.

Although *M. vaccae* is also able to stimulate IFN-γ and IL-12 synthesis by human PBMC, the fact that the inhibition by blocking antibodies to IL-12 and IL-18 is not able to restore IL-5 and IL-13 production, would suggest that the IL-12/IFN-γ system is not the main mechanism involved in the immunomodulatory activity of *M. vaccae.* The importance of IL-10 over IFN-

γ generation is in accordance with the animal studies utilising *M. vaccae* (Zuany-Amorim *et al.* 2002; Zuany-Amorim *et al.* 2002; Adams *et al.* 2004).

As discussed above, other factors may play an important role in the observed inhibition of the allergic response induced by the administration of *M. vaccae* such as the synthesis of Transforming Growth Factor-beta (TGF-β) and the induction of the gene Forkhead/winged helix transcription factor-3 (*Foxp3*) (Sakaguchi 2003).The expression of TGF-β and the transcription-repressor gene *Foxp3,* as opposed to GATA-3, uniquely identifies Treg cells. In atopic subjects, *Foxp3* expression has been shown to be greater in cells with a Treg cells phenotype (Ling *et al.* 2004). Regulatory T cells can be generated peripherally through costimulation with T cell receptors (TCRs) and TGF-β by a *Foxp3*-dependent mechanism (Chen *et al.* 2003). They exert their inhibitory effects on T cell proliferation through a variety of mechanisms that includes the generation of IL-10 and TGF-β or the engagement of inhibitory co-stimulatory molecules like Cytotoxic T-lymphocyte antigen-4 (CTLA-4) and PD-1 (Akdis *et al.* 2004). In a murine asthma model, co-administration of TGF-β-induced regulatory T cells prevented house dust mite-induced allergic pathogenesis in lungs (Chen *et al.* 2003) and *M. vaccae* has been shown to induce TGF-β and suppress proliferation via a IL-10 and TGF-β-dependent nechanism (Zuany-Amorim *et al.* 2002).

In the current study I have also demonstrated a role for prostanoids in Th2 cytokine generation induced by allergen. When added to culture, PGI_2, but not PGE_2, completely inhibited the production of IL-5, to a greater extent than either *M. vaccae* or IL-12. Indomethacin reversed the inhibitory effects of *M. vaccae* on IL-13 synthesis but had a marginal effect on IL-5 synthesis. Importantly, IFN-γ production was not affected. My study has not completely elucidated which of the prostanoids is involved but strongly suggests a

role for PGI_2. The spectrum of prostanoids elicited by the COX-2 pathway of the arachidonic acid metabolism differs from that synthesised by COX-1 (Wickens *et al.* 2002), and prostaglandins have diverse effects on the regulation of cytokine production by T cells. PGE_2 has been implicated in the enhancement of Th2-type responses by inhibiting IL-12 production. Acting on B cells, PGE_2 stimulates isotype-class switching to induce the production of IgG1 and IgE. PGE_2 acts on antigen-presenting cells, such as macrophages and dendritic cells, to induce the expression of IL-10 and inhibit the expression of IL-12 receptor (IL-12R) (Wickens *et al.* 2002). On the contrary PGI_2 has been shown to down-regulate Th2 response in an animal model of allergic asthma (Jaffar *et al.* 2002). My findings are in agreement with recent animal data which demonstrated that high levels of PGI_2 are produced in the airways following OVA inhalation, that PGI_2 induces IL-10 production, and that inhibition of COX-2 *in vivo* specifically reduces PGI2 synthesis and results in a marked increase in Th2-mediated, but not Th1-mediated, lung inflammation (Jaffar *et al.* 2002).

In conclusion, I have shown that *M. vaccae* down-regulates allergen-induced Th2 allergic responses through the generation of IL-10 and prostanoids. Further insight into the mechanism of action of *M. vaccae* is needed, particularly in respect to the mechanisms leading to regulatory T cells generation and the characterisation of receptors/ligand interaction influencing the effects of *M. vaccae* treatment on responses of APC and T cells.

CHAPTER SIX

Effects of Bacterial Lipopolysaccharide

in an *in vitro* model of allergic inflammation

6.1 INTRODUCTION

6.1.1 Endotoxin, atopy and asthma: epidemiological considerations

Endotoxin or lipopolysaccharide (LPS) is a structural component of the cell wall of Gram-negative bacteria and a potent proinflammatory agent. Exposure to endotoxin can cause acute and chronic occupational inflammatory airway diseases (Haglind *et al.* 1987; Schwartz 1996). It may worsen the annual decline in FEV_1 (Vogelzang *et al.* 1998) and can increase BHR in asthmatic patients (Michel *et al.* 1991; Michel *et al.* 1992; Michel *et al.* 1996). Studies (Jagielo *et al.* 1996; O'Grady *et al.* 2001) have shown that a single exposure to aerosolised LPS can induce airflow obstruction in healthy subjects (Malmberg *et al.* 1993; Kline *et al.* 1999) and long-term exposure to endotoxin-containing dust in the workplace can induce airway inflammation leading to progressive airflow impairment and chronic lung disease.

Contrary to its historical 'roguish' reputation, endotoxin has recently emerged as a model of how microbial exposure might protect individuals against the development of allergy and asthma. Changes in environmental factors and a reduction in childhood infections and exposure to microbes have been considered as part of the 'hygiene hypothesis', which, as discussed in Chapter one, postulates that reduced exposure to bacterial pathogens during early childhood results in the abnormal expansion of Th2 cells and predisposition to allergy. The 'hygiene hypothesis' has originated from the epidemiological observation of an inverse relationship between the prevalence of infectious diseases and allergic sensitisation. This has been shown by inverse association between atopic sensitisation and the number of siblings (Strachan 1989; von Mutius *et al.* 1994; Strachan 1997; Strachan 2000), day-care attendance early in life (Kramer *et al.* 1999; Ball *et al.* 2000) and exposure to orofaecal pathogens, which may be regarded as a generic marker of poor hygiene. However, as discussed in Chapter one,

this theory has evolved such that harmful infections *per se* may not be as critical as the exposure to microbial burden and, even in the absence of infections, as bacteria and their components may be found in varying concentrations in many indoor and outdoor environments.

6.1.1.1 Farming and rural environment protects from atopy and asthma

In the last decade, a series of epidemiological studies have consistently shown a reduced risk of atopic diseases and allergic sensitisation in children from farming families when compared with their peers from non-farming families (Braun-Fahrlander *et al.* 1999; Ernst *et al.* 2000; Kilpelainen *et al.* 2000; Klintberg *et al.* 2001) (reviewed in (Braun-Fahrlander 2003)). In particular, exposure to livestock and stables was shown to confer protection from allergy.

A higher exposure to endotoxin has been hypothesised as contributing to the observed lower prevalence of allergic diseases in children growing up on a farm. Indeed, endotoxin levels tend to be highest in environments where there are farm animals because the faecal flora of larger mammals is a major source of endotoxin. Elevated levels of endotoxin have also been found in the homes of farmers' children and children with regular contact with livestock as compared with non-farming children without animal contacts (von Mutius *et al.* 2000; Gehring *et al.* 2002; Braun-Fahrlander 2003).

A cross-sectional study looking at endotoxin levels in mattress dust from farming and non-farming households in rural areas of central Europe found a relationship between higher levels of endotoxin in the dust and a decreased frequency of hay fever, allergic asthma, eczema (Phipatanakul *et al.* 2004) and allergic sensitisation in children from the households (Braun-Fahrlander *et al.* 2002; Gehring *et al.* 2002; Braun-Fahrlander 2003; van Strien *et al.* 2004).

The European Community Respiratory Health Survey showed that the generational increase in atopy and allergic rhinitis was not observed in individuals who were exposed to a farming environment in childhood (Leynaert *et al.* 2001). A follow-up study in primary school children demonstrated that the new occurrence of skin-prick test positivity over three years was reduced in children from part-time and full-time farmers as compared with non-farm children (Horak *et al.* 2002; Braun-Fahrlander 2003). Similarly, having lived in the country – a proxy for a farm environment– was negatively associated with a positive skin-prick test, particularly in subjects with childhood exposure (Filipiak *et al.* 2001; Kauffmann *et al.* 2002) (reviewed in (Braun-Fahrlander 2003).

A comparison of endotoxin levels in house dust from a country with a low (Estonia) and a high (Sweden) prevalence of allergy showed higher LPS levels in Estonian than in Swedish house dust and an inverse relationship between this parameter and the development of atopic disease and sensitisation in the children during the first two years of life (Bottcher *et al.* 2003). These findings support the assertion that high levels of endotoxin, or other bacterial products with Th1-stimulating properties, might protect children from developing atopic disease, and is also confirmed by a cross-sectional surveys carried out in a rural population in Austria, Bavaria (Germany) and Switzerland by the collaborative Allergy and Endotoxin (ALEX) research group (Braun-Fahrlander *et al.* 2002). Similarly, in a study in urban homes on infants with a high risk of developing asthma, allergen-sensitised infants had significantly lower house dust endotoxin levels than non-sensitised infants (Gereda *et al.* 2000).

In addition, in a subset of these infants it was shown that endotoxin levels correlated with IFN-γ-producing T cells (Th1) but not with IL-4, IL-5 or IL-13-producing cell proportions (Th2) (Gereda *et al.* 2000).Taken together, these studies of infants and children from non-

farming environments support the notion of a protective effect of endotoxin exposures on atopic sensitisation or atopic eczema during the early year of life.

6.1.2 Endotoxin, its receptors and the innate immune system

6.1.2.1 Endotoxin and Toll-like Receptors

Bacterial lipopolysaccharide is a product of Gram-negative bacteria and a potent stimulator of innate immune responses. LPS consists of a highly conserved hydrophobic lipid domain known as lipid A, a nonrepeating 'core' oligosaccharide and a distal polysaccharide (or O-antigen). The polysaccharide group can be highly variable, functions as an antigen and is recognised by the adaptive immune system. The lipid moiety, by contrast, is highly conserved and constitutes a molecular pattern recognised by the receptors of the innate immune system (Raetz *et al.* 2002) (Figure 6.1-1). LPS is found in the dust in houses and outdoors in dirt. It is continuously shed into the environment and is abundant in occupational organic dust, house dust and human oral and nasal cavities (Di Luzio *et al.* 1973; Hasday *et al.* 1999). It is also found in domestic water, which can be inhaled in bathrooms and from air humidification systems.

Given their conserved architecture, most types of lipid A molecules are detected at picomolar levels by an ancient receptor of the innate immune system (Aderem *et al.* 2000; Medzhitov *et al.* 2000). Mechanisms relating to the recognition of these microbial compounds by the innate immune system and the regulation of the resulting inflammatory responses through adaptive immunity are likely to be of key importance for the development of atopic diseases.

Environmental cofactors may also be important. Endotoxin can attach itself to heat-shock protein (hsp) and β-1,3 glucan (an immunostimulatory cell-wall component of fungi, yeast and plants), and particularly to bacterial DNA, all of which might potentiate the immune effects of endotoxin (Roy *et al.* 2003). Recent studies show that bacterial DNA in dust correlates with endotoxin. The highest bacterial DNA levels are present in farm barns, followed by rural homes, farm homes and urban homes, leading to the speculation that some of the effects of environmental LPS allergy and asthma prevention could be attributable to bacterial DNA (Roy *et al.* 2003).

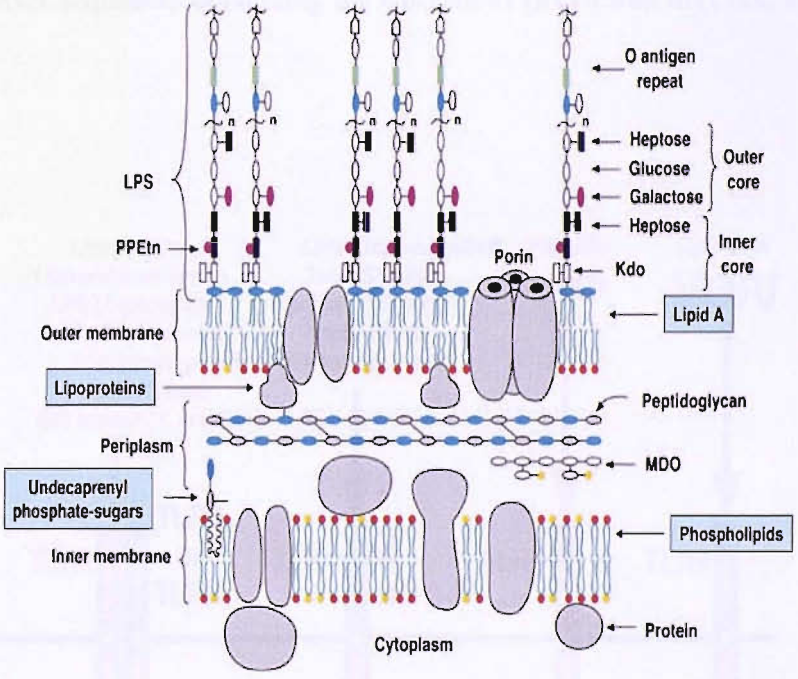


FIGURE 6.1-1: *Model of the inner and outer membranes of E. coli. (from (Raetz et al. 2002)).*

Endotoxin acts as a potent immunostimulatory molecule through its lipid A moiety, which signals, through the LPS binding protein (LBP)/CD14 complex, toll-like receptor 4 (TLR4) and, to a lesser extent, other molecules (MyD88 and TLR9) of the innate immunity pathway. Toll-like receptors – highly conserved, germ-line receptors – represent the main receptors of

the innate immune system recognising conserved microbial patterns and are expressed ubiquitously in most tissues in healthy adults (Zarember *et al.* 2002). Among the members of the TLR family, TLR4 (Poltorak *et al.* 1998; Hoshino *et al.* 1999), a membrane-spanning protein related to the IL-1 receptor, together with CD14, serves as receptor for LPS, while TLR2 appears to be less specific than TLR4 in that it is activated by diverse ligands, including bacterial lipoproteins and peptidoglycan fragments. TLR6 may function together with TLR2 to recognize a subset of bacterial membrane proteins and lipopeptides. TLR3 is activated by double-stranded RNA, and TLR5 is activated by bacterial flagellin. TLR9 responds to bacterial DNA sequences containing the CpG motif (Reviewed in (Cook *et al.* 2003)) (Figure 6.1-2).

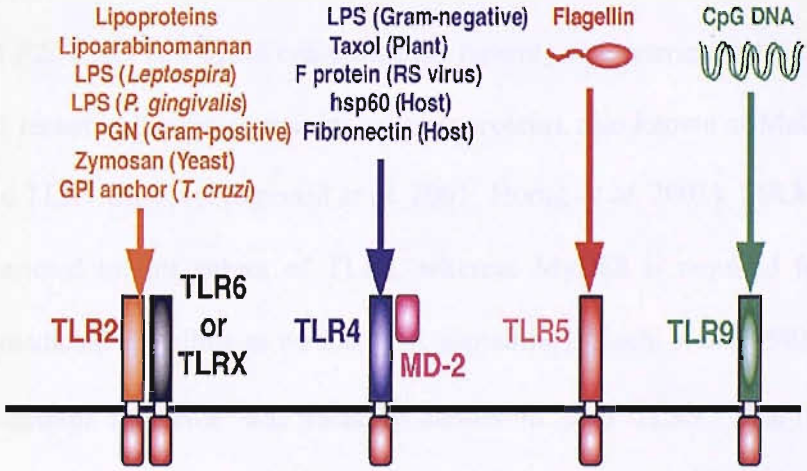


FIGURE 6.1-2: *Toll-like receptors and their ligands.*

Recognition of LPS by TLRs requires interactions between TLRs and adaptor molecules that link the receptor complex to downstream signalling molecules. These protein-protein interactions are mediated by modular homophilic domains present in both TLRs and IL-1 receptors. The best-characterized Toll/IL-1 receptor domain-containing adaptor molecule is

MyD88, which is utilised by all known TLRs. MyD88 links TLRs to members of the IL receptor-associated kinase (IRAK) family, including IRAK1 and IRAK4, which activate tumour necrosis factor receptor-associated factor-6 (TRAF-6). TRAF-6 is also required for mitogen-activated protein kinase (MAPK) activation, leading, via nuclear factor-kappaB (NF-κB), to activation of transcription of multiple pro-inflammatory genes, including TNF-α, IL-1 and IL-6 (Figure 6.1-3) (Cook *et al.* 2003). In addition to this MyD88-dependent pathway, analysis of MyD88-deficient mice revealed that TLR4 can also signal through a MyD88-independent pathway that results in the activation of NF-κB and MAPK cascades and can induce type I interferons and interferon-inducible genes through interferon regulatory factor-3 (IRF3) activation (Kawai *et al.* 1999; Horng *et al.* 2002; Yamamoto *et al.* 2002; Oshiumi *et al.* 2003). Emerging data suggest that at least part of the difference between responses conferred by individual TLRs results from their use of adaptor molecules other than MyD88. TLR1, TLR2, TLR4 and TLR6 can utilize the recently characterized adaptor molecule TIRAP (Toll/IL-1 receptor domain-containing adaptor protein), also known as Mal, but TLR3, TLR5, TLR7 and TLR9 cannot (Fitzgerald *et al.* 2001; Horng *et al.* 2001). TIRAP function appears to be restricted to this subset of TLRs, whereas MyD88 is required for IL-1 and IL-18 receptor-mediated signalling as well as TLR signalling (Adachi *et al.* 1998).

A third adaptor molecule was recently shown to bind TLR3. This Toll/IL-1 receptor-containing adaptor molecule (TICAM)-1, also known as TIR domain-containing adaptor inducing IFN-β (TRIF), functions in concert with TLR3, and possibly TLR4, to induce interferon-β production by activating interferon regulatory factor-3 (Yamamoto *et al.* 2002; Oshiumi *et al.* 2003) (reviewed in (Cook *et al.* 2003)) (Figure 6.1-3). TLR signalling is modulated by IRAK-M, which inhibits cell signalling by preventing dissociation of phosphorylated IRAK1 and IRAK4 from MyD88. The suppressor of cytokine signalling

(SOCS)-1 protein also inhibits the response to endotoxin (Kinjyo *et al.* 2002; Kobayashi *et al.* 2002; Nakagawa *et al.* 2002).

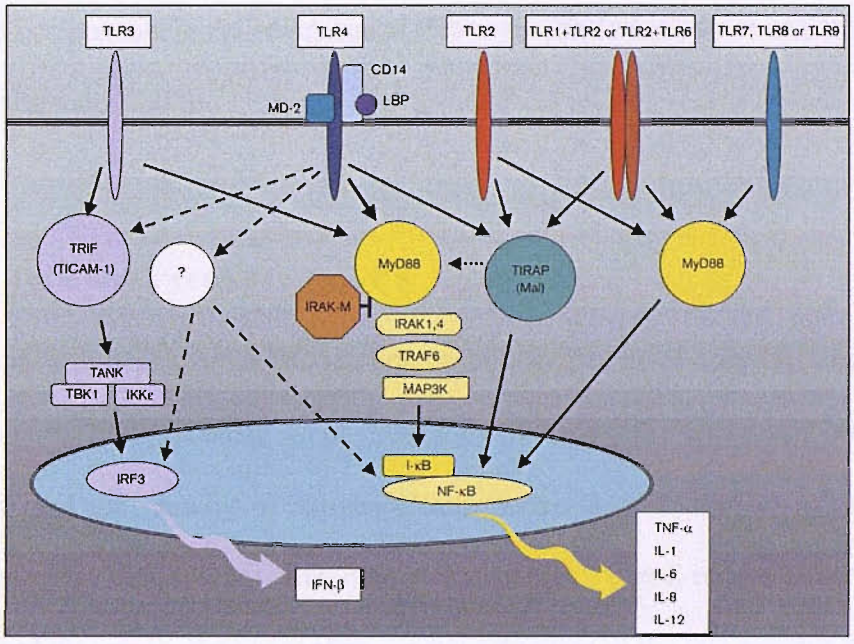


FIGURE 6.1-3: *Toll-like receptor signalling pathways. Toll-like receptors (TLRs) bind different ligands, but each TLR signals through the adaptor molecule, MyD88, and leads to nuclear factor-κB (NF-κB) activation (shown in yellow). Differential gene expression might arise through the selective use of alternative adaptor molecules (shown as circles), including TICAM-1 (toll/IL-1 receptor-containing adaptor molecule-1), TIRAP (toll/IL-1 receptor domain-containing adaptor protein) or possibly other, unidentified proteins (white circle). The TICAM-1 pathway (shown in purple) results in IFN-γ gene expression, and is MyD88-independent. MyD88-dependent responses are negatively regulated by IL receptor-associated kinase (IRAK)-M (red octagon) and suppressor of cytokine signaling (SOCS)-1 (not shown). IKKε, I-κB kinase; IRF3, interferon regulatory factor 3; LBP, lipid binding protein; TANK, TRAF-associated NF-κB activator; TBK1, TANK-binding kinase; TLR, Toll-like receptor; TRAF, tumour necrosis factor receptor associated factor; TNF-α. (From (Cook et al. 2003)).*

Differential expression of lipopolysaccharide receptors in children from farming and non-farming households has recently been reported, suggesting that the innate immune system responds to the high microbial burden of the farming environment. In a subset of the ALEX Study population it was shown that the blood cells of farmers' children express higher amounts of CD14 and TLR-2, which are innate immune receptors for microbial compounds

that include endotoxin (Flo *et al.* 2001; Lauener *et al.* 2002). An increased expression of CD14 as well as TLR-2 was also observed in human leukocytes after treatment with lipopolysaccharide *in vitro*, which suggested that the differences found *in vivo* between farmers' and non-farmers' children mirror different degrees of exposure to such microbes or microbial components in the environment (Flo *et al.* 2001; Lauener *et al.* 2002) (reviewed in (Braun-Fahrlander 2003)).

6.1.2.2 Immune response to LPS

TLRs have a critical role in DC maturation and induction of adaptive immune responses (Kawai *et al.* 1999; Schnare *et al.* 2001) by inducing the expression of costimulatory molecules and the priming of antigen-specific naïve T cells, leading to the activation of antigen-specific Th1 but not Th2 immune responses. These results suggest that distinct pathways of the innate immune system control activation of the two effector arms of adaptive immunity (Schnare *et al.* 2001). In addition, MyD88-deficient mice also fail to produce IFN-γ and ovalbumin-specific IgG_{2a} antibodies (Schnare *et al.* 2001). These defects are due at least in part to the inability of these mice to synthesise IL-12. However, MyD88-deficient mice are able to produce normal amounts of antigen-specific IgG_1 and IgE and higher levels of IL-13 than control wild-type mice (Schnare *et al.* 2001). These mice appear to have a selective defect for mounting Th1 but not Th2 responses, and TLRs seem to control induction of only Th1-type inflammation (Schnare *et al.* 2001). Indeed, all the known pathogen-asssociated molecular pattern (PAMPs) derive from pathogens which elicit a Th1 response.

The interaction of PAMPs with TLRs results in the release of several cytokines, such as TNF-α, IFN-γ, IL-1, IL-6, IL-10 and IL-12. In particular, LPS is a potent inducer of IL-12 and IFN-

γ (Gereda *et al.* 2000), which are cytokines that stimulate Th1-mediated immunity, and suppressors of Th2 inflammatory cytokines such as IL-4, IL-5 and IL-13.

The above immunological mechanisms elicited by endotoxin give a framework to hypothesise how natural microbial exposures might have an immune modulatory and atopy-protective effect without the harm of infections.

6.1.3 Apparent paradox of endotoxin

The multitude of endotoxin exposure studies lead to conflicting results as to whether the LPS protects against the development of asthma and allergic sensitisation or it is a proinflammatory factor, leading to asthma exacerbations, airway inflammation and chronic airway diseases. At first glance, the ability of endotoxin to have both an exacerbating as well as ameliorating effects seems to suggest a paradox; however two important factors, timing or age of exposure and dose of LPS, may play an important role in governing the augmentation or amelioration of atopic diseases.

6.1.3.1 Timing of endotoxin exposure

The timing of the exposure to LPS seems to be important in inducing a protective effect. In both animal models and studies in humans, exposure to endotoxin early in life, during the development of the immune system, seems to be most important in providing protection against the development of allergic disease. Clinical studies have shown that children who are placed in daycare within the first six months of life are less likely to have asthma and recurrent wheezing in later childhood (Ball *et al.* 2000). In a rural study, exposure to stables before the first birthday is associated with protective effects (Riedler *et al.* 2001) and early

endotoxin exposure is positively associated with the proportion of Th1 cells at the age of two years (Bolte *et al.* 2002). The ALEX Study Group carried out a cross-sectional study that showed that the risk of asthma or atopic sensitisation was reduced to approximately one third if the child had first been exposed to stables during the first year of life compared with the first exposure to stables during school age or with no exposure (Riedler *et al.* 2001). Pet exposure before one year of age was associated with less allergic sensitisation and later episodes of wheezing, but wheezing increased when exposure occurred after 3 years of age (Celedon *et al.* 2002; Ownby *et al.* 2002). In contrast, mite-sensitized volunteers with allergic rhinitis who were challenged with subthreshold doses of mite allergen with simultaneous low-level lipopolysaccharide exposure (1 μg) showed greatly augmented eosinophilic inflammation in the nasal lavage (Eldridge *et al.* 2000).

Although murine experiments are limited in the extent to which the results may be relevant to humans, they have given valuable insight into the role of the timing and dosage of lipopolysaccharide in the process of allergic sensitisation. In BALB/c mice – known to be genetically determined to have a high Th2 response – systemic LPS administration before allergen sensitisation reduced total and specific IgE serum levels, Th2 cytokine production and the extent of airway eosinophilia. However, if endotoxin exposure was delayed until several days after allergen sensitisation endotoxin promoted a more robust IgE response (Gerhold *et al.* 2002). In another rodent model, a single dose of nebulized LPS was administered at different time-points relative to the induction of allergen sensitisation that leads to allergic inflammation in these animals. If LPS was administered before or shortly after allergen sensitisation, lung inflammation and airways hyperresponsiveness were abrogated. When lipopolysaccharide was administered at later time-points after allergen sensitisation however, lung inflammation was markedly increased (Tulic *et al.* 2000).

6.1.3.2 Dose of LPS

The dose of endotoxin can also play a decisive role in modifying the risk of atopy. The notion of a dose-dependent effect has been supported by a number of recent studies. The Childhood Allergy Study showed a dose-response relationship of lowering allergic sensitisation with increasing number of pets (Ownby *et al.* 2002). Two or more pets were needed before a fourfold benefit was demonstrated (Ownby *et al.* 2002). A strong Th2 response was generated when ovalbumin was combined with a low dose of endotoxin. By contrast, a neutrophilic Th1 response and protection against BHR resulted when the ovalbumin allergen was combined with higher doses of endotoxin. Thus, the dose of endotoxin present during allergen sensitisation determined Th2-pro-asthmatic versus Th1-asthma-protective response. Low level inhaled LPS signalling through TLR4 induces Th2 responses to inhaled antigens in a mouse model of allergic sensitisation (Eisenbarth *et al.* 2002). The mechanism by which LPS signalling results in Th2 sensitisation involves the activation of dendritic cells. In contrast to low levels, inhalation of high levels of LPS with antigen results in Th1 responses. These studies suggest that the level of LPS exposure can determine the type of inflammatory response generated and provide a potential mechanistic explanation of epidemiological data on endotoxin exposure and asthma prevalence (Eisenbarth *et al.* 2002). Again, a high dose of LPS during intranasal ovalbumin priming resulted in a Th1-associated response, with the production of IFN-γ, neutrophilic airway inflammation and the production of ovalbumin-specific Th1 isotype antibodies (Eisenbarth *et al.* 2002).

No study to date has defined the precise levels or nature of endotoxin where the switches occur, and it may be co-dependent on other environmental exposures.

Besides the epidemiological role of LPS in preventing allergic diseases, as postulated in the 'hygiene hypothesis', the importance of studying the interaction between LPS and allergens is

of relevant for the treatment of allergic diseases. Indeed, a non-toxic LPS analogue, Monophosphoryl lipid A, MPL® (Corixa Corporation, Seattle, WA, USA) has been developed and is being tested in clinical trials as an adjuvant in immunotherapy.

Monophosphoryl lipid A (MPL®), can induce increases in IL-12, IFN-γ, and decreases in IL-5 consistent with immune deviation in favour of a Th1-phenotype.

Immunization strategies utilizing MPL® alone or in combination with other adjuvants have been successfully employed to enhance responses to a variety of antigens (Schneerson *et al.* 1991; Baldridge *et al.* 1999; Baldrick *et al.* 2001; Baldrick *et al.* 2002; Evans *et al.* 2003; Baldrick *et al.* 2004; Puggioni *et al.* 2005). Recently, MPL® has been used as an adjuvant in a successful vaccination preparation used for grass and tree-pollen immunotherapy (Wheeler *et al.* 2001; Drachenberg *et al.* 2003; Puggioni *et al.* 2005; Thompson *et al.* 2005). MPL®, in conjugation with modified whole grass pollen (allergoid), reduced allergic symptoms in patients suffering from hay fever (Wheeler *et al.* 2001). In the murine system, MPL® can promote activation of antigen-presenting cells (APC) *in vitro*. Pretreatment of APC with MPL® increased their immunostimulatory properties and resulted in the increased production of IFN-γ, IL-4 and IL-5 by B-cells and macrophages. The MPL also has the ability to up-regulate expression of co-stimulatory molecules on APC. Similarly to LPS, the effects of MPL on allergen-induced responses occurs through the binding to the TLR-2 and TLR-4, with generation of IL-12 production by monocytes which induces allergen-stimulated T cells to preferentially produce IFN-γ.

6.1.4 Aims and hypothesis

Although several epidemiological studies suggest that LPS is able to either exacerbate or prevent allergic asthma, based on timing and dose of exposure, there is little direct evidence linking dose and timing of LPS exposure to responses to allergen challenge in humans either *in vivo* or *in vitro*.

The aim of this study was to assess the effect of LPS on allergen-induced cytokine response by PBMC from patients with mild atopic asthma. In particular, three aspects were evaluated:

1. The effect of increasing concentrations of LPS on allergen-stimulated PBMC

2. The effect of timing of LPS exposure in relation to allergen challege *in vitro*

3. The importance of the generation of a Th1 response in preventing Th2 cytokine generation in response to allergen.

LPS in known to be a potent inducer of IL-12 *in vitro*. As IL-12 is also a potent inducer of Th1 responses, I hypothesised that IL-12 production by LPS/allergen-stimulated PBMC varies with the timing of LPS stimulation in relation to allergen challenge and the dose of LPS. The production of IL-12 - with higher levels obtained at high doses of LPS and when PBMC are pre-exposed to LPS - would down-regulate the magnitude of Th2 cytokines generated in response to allergen stimulation.

6.2 METHODS

6.2.1 Study Design

Experiments were carried out according to the following protocol.

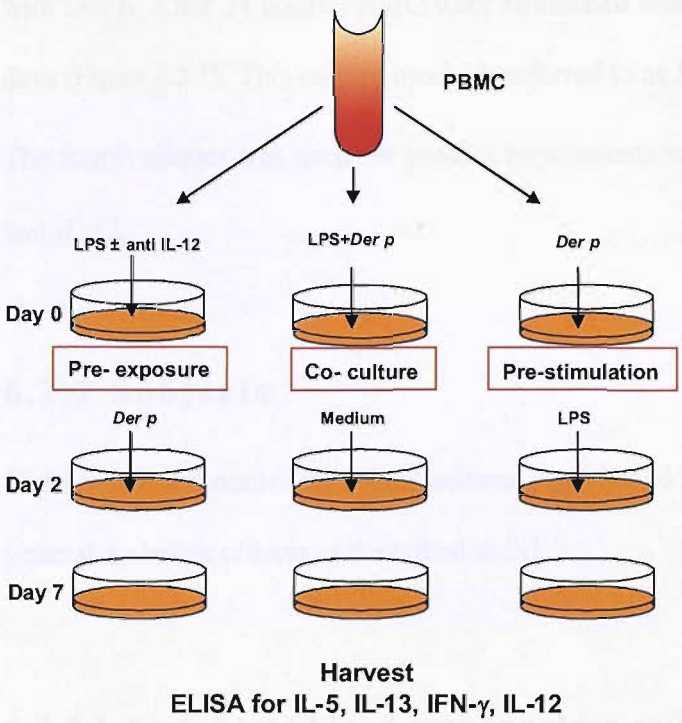


FIGURE 6.2-1: *Experimental Design*

PBMC from each patient were separated in four aliquots. The first aliquot was used for the pre-exposure experiments where cells were stimulated with LPS in the presence or absence of anti-IL-12 monoclonal antibodies. After 24 hours PBMC, were stimulated with *Der p* and cultured for a total of seven days (Figure 6.2-1). This culture model is referred to as LPS→ *Der p* in this Chapter.

The second aliquot was used for the co-culture experiments where cells were stimulated with LPS and *Der p* and cultured for a total of seven days (Figure 6.2-1). This culture model is referred to as LPS+*Der p* in this Chapter.

The third aliquot was used for the pre-stimulation experiments where cells were stimulated with *Der p.* After 24 hours PBMC were stimulated with LPS and cultured for a total of seven days (Figure 6.2-1). This culture model is referred to as *Der p* →LPS in this Chapter.

The fourth aliquot was used for parallel experiments where cells were stimulated with *Der p* and IL-12.

6.2.2 Subjects

Eight atopic asthmatics with mild asthma participated in the study. The subjects satisfied the general inclusion criteria as described in 2.1.2.

6.2.2.1 Peripheral blood mononuclear cells (PBMC)

Blood was drawn into heparinised tubes. PBMC were isolated as described in 2.3.6.1 and cultured as described in 2.2.6.3.

6.2.2.2 Solid phase sandwich Enzyme Linked-Immunosorbance Assay (ELISA)

ELISA was performed as described in 2.2.7.

6.2.2.3 Statistics

All statistical analyses were performed using InStat® version 3 for Windows (GraphPad™ Software Inc.). Data were analysed for distribution with the Kolmogorov-Smirnov test.

Multiple comparison analysis was performed by one-way ANOVA. Time-course and dose-response of experiments run in parallel were analysed using repeated measures ANOVA. When indicated, *post hoc* analysis was performed by Bonferroni multiple comparison test. Repeated measure Friedman's Test was used for non-parametric multiple comparison analysis with Dunn's test for *post hoc* between-group analysis. For all tests $p<0.05$ was considered statistically significant.

6.3 RESULTS

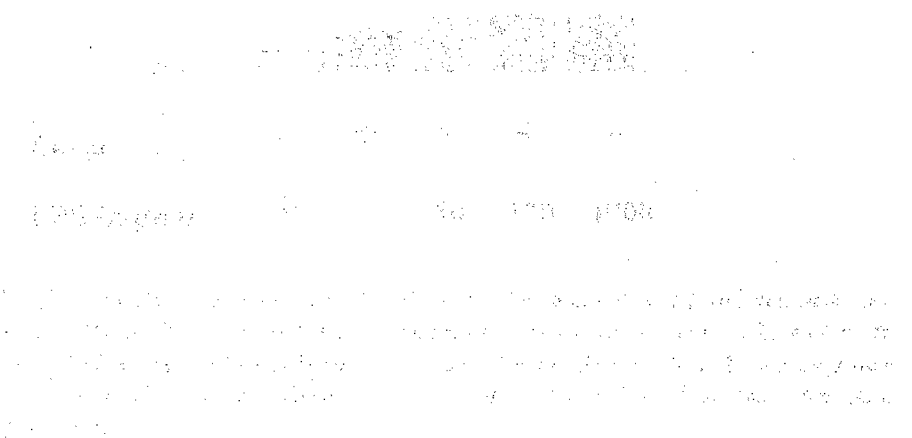
LPS in known to be a potent inducer of IL-12 *in vitro*. As IL-12 is also a potent inducer of Th1 responses, I hypothesised that IL-12 production by LPS/allergen-stimulated PBMC varies with the timing of LPS stimulation in relation to allergen challenge and the dose of LPS. The highest production of IL-12, being generated with high doses of LPS and when PBMC are pre-exposed to LPS, would down-regulate the magnitude of Th2 cytokines generated in response to allergen stimulation.

6.3.1 Effects of pre-exposure to LPS on allergen-induced IL-12 and IL-5 synthesis

PBMC secreted high concentrations of IL-12 in response to LPS→*Der p* compared to *Der p* alone (mean±SD 4,874±820 pg/ml vs 478±103 pg/ml; $p<0.001$). To test the hypothesis that high dose of LPS has a greater down-regulating effect on Th2 response by inducing greater

production of IL-12, I carried out concentration-response experiments where PBMC were pre-incubated with increasing concentrations of LPS (10 ng/ml, 100 ng/ml and 1 μg/ml) and, following 24 hours of culture, they were stimulated with allergen at doubling concentrations of 5,000, 10,000 and 20,000 SQ units/ml, (Figure 6.3-1). There was no difference in the concentration of IL-12 measured in the culture supernatants when using the low or high dose of LPS/low dose allergen (4,771±1,446 pg/ml vs 4,874±820 pg/ml; $p>0.05$) (Figure 6.3-1). Similarly, there was no difference in the concentration of IL-12 in supernatants of cells stimulated with low or high dose of LPS/high dose allergen (5,517±249 pg/ml vs 5,742±296 pg/ml; $p>0.05$), (Figure 6.3-1). The difference between high and low dose of allergen was also not significant.

When looking at the effects of LPS in pre-exposure experiments on IL-5 response, stimulation with allergen caused a significant increase in IL-5 production, as also seen in Chapter three. Pre-exposure to LPS caused a significant inhibition of IL-5 both at low dose (104±64 pg/ml vs 343±239 pg/ml; $p<0.05$) and at high dose of LPS (74±35 pg/ml vs 343±239 pg/ml; $p<0.01$) (Figure 6.3-2).



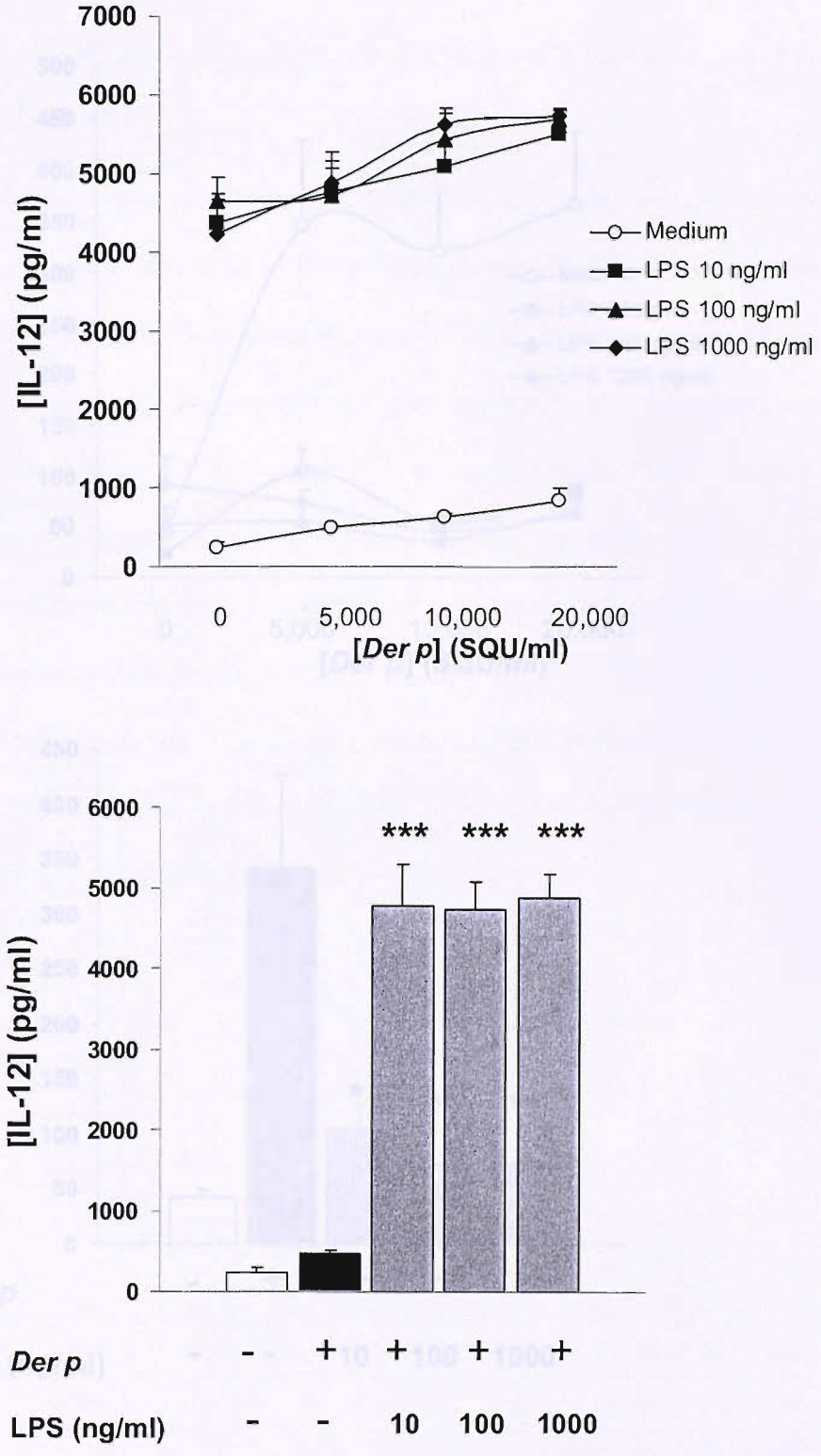


FIGURE 6.3-1: *Effects of LPS pre-exposure on IL-12 synthesis in Der p-stimulated PBMC from atopic asthmatic donors (n=8),(Model LPS → Der p). Upper panel shows a concentration-related effect of LPS 10 nm/ml (■), 100 ng/ml (▲), 1000 ng/ml (♦) given 24 hours before challenge with doubling doses of Der p. Lower panel shows the concentration dependence of LPS's effect on subsequent allergen challenge (Der p, 5000 SQU/ml). Bars depict mean ± SD, (n=8). *** p<0.001.*

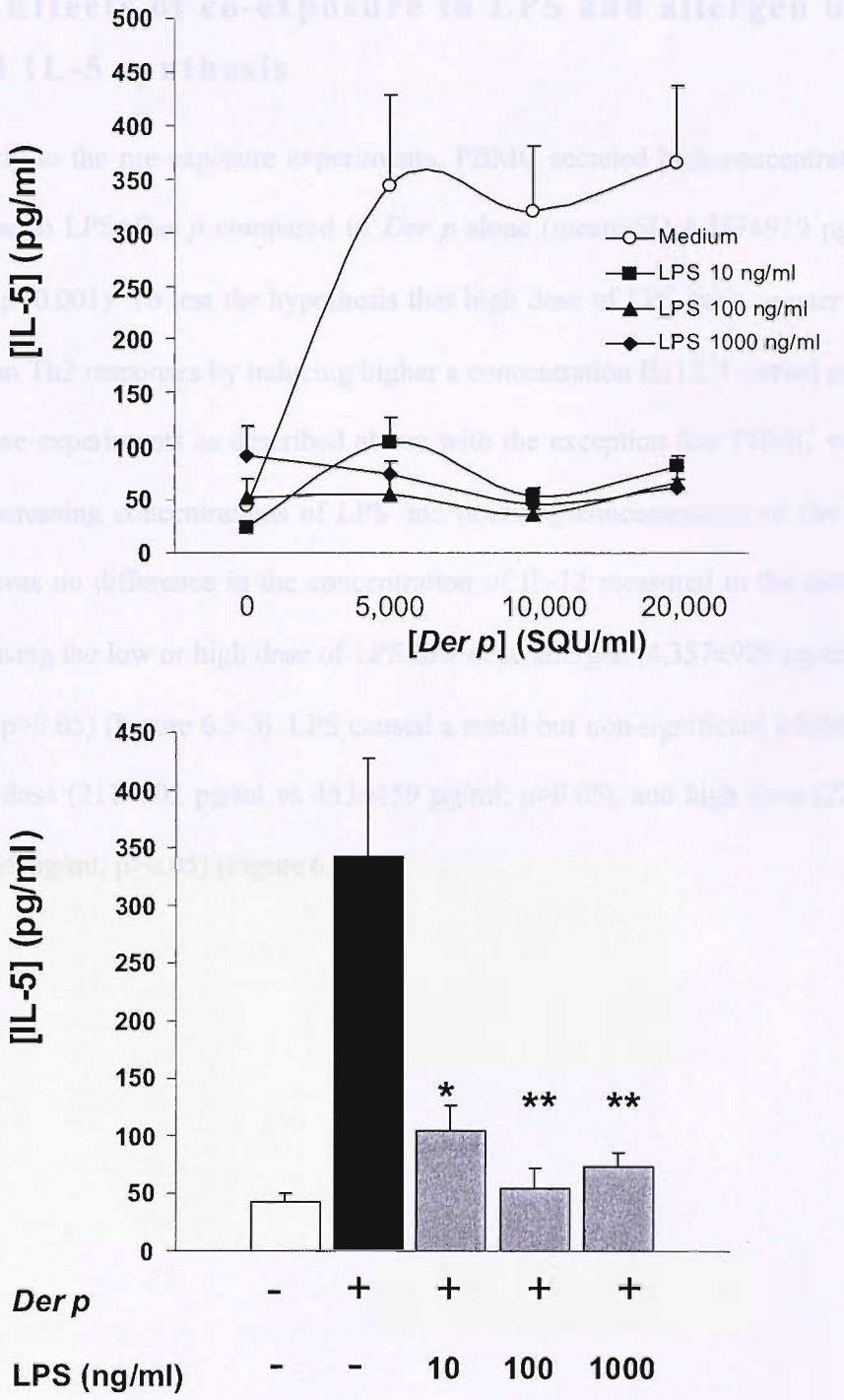


FIGURE 6.3-2. *Effects of LPS pre-exposure on IL-5 synthesis in Der p-stimulated PBMC from atopic asthmatic donors (n=8) (Model LPS → Der p). Upper panel shows a concentration-related effect of LPS 10 nm/ml (■), 100 ng/ml (▲), 1000 ng/ml (♦), given 24 hours before challenge, on responses to doubling doses of Der p. Lower panel shows to the concentration dependence of LPS's effect on subsequent allergen challenge (Der p, 5000 SQU/ml) . Bars depict mean ± SEM, (n=8). *p<0.05; ** p<0.01*

6.3.2Effects of co-exposure to LPS and allergen on IL-12 and IL-5 synthesis

Similarly to the pre-exposure experiments, PBMC secreted high concentrations of IL-12 in response to LPS+*Der p* compared to *Der p* alone (mean±SD 4,357±929 pg/ml vs 482±122 pg/ml; $p<0.001$). To test the hypothesis that high dose of LPS has a greater down-regulating effect on Th2 responses by inducing higher a concentration IL-12, I carried out concentration--response experiments as described above with the exception that PBMC were co-incubated with increasing concentrations of LPS and doubling concentrations of *Der p* (Figure 6.3-3). There was no difference in the concentration of IL-12 measured in the culture supernatants when using the low or high dose of LPS/low dose allergen (4,357±929 pg/ml vs 3,788±1,152 pg/ml; $p>0.05$) (Figure 6.3-3). LPS caused a small but non-significant inhibition of IL-5 both at low dose (211±102 pg/ml vs 453±459 pg/ml; $p>0.05$), and high dose (220±180 pg/ml vs 453±459 pg/ml; $p>0.05$) (Figure 6.3-4).

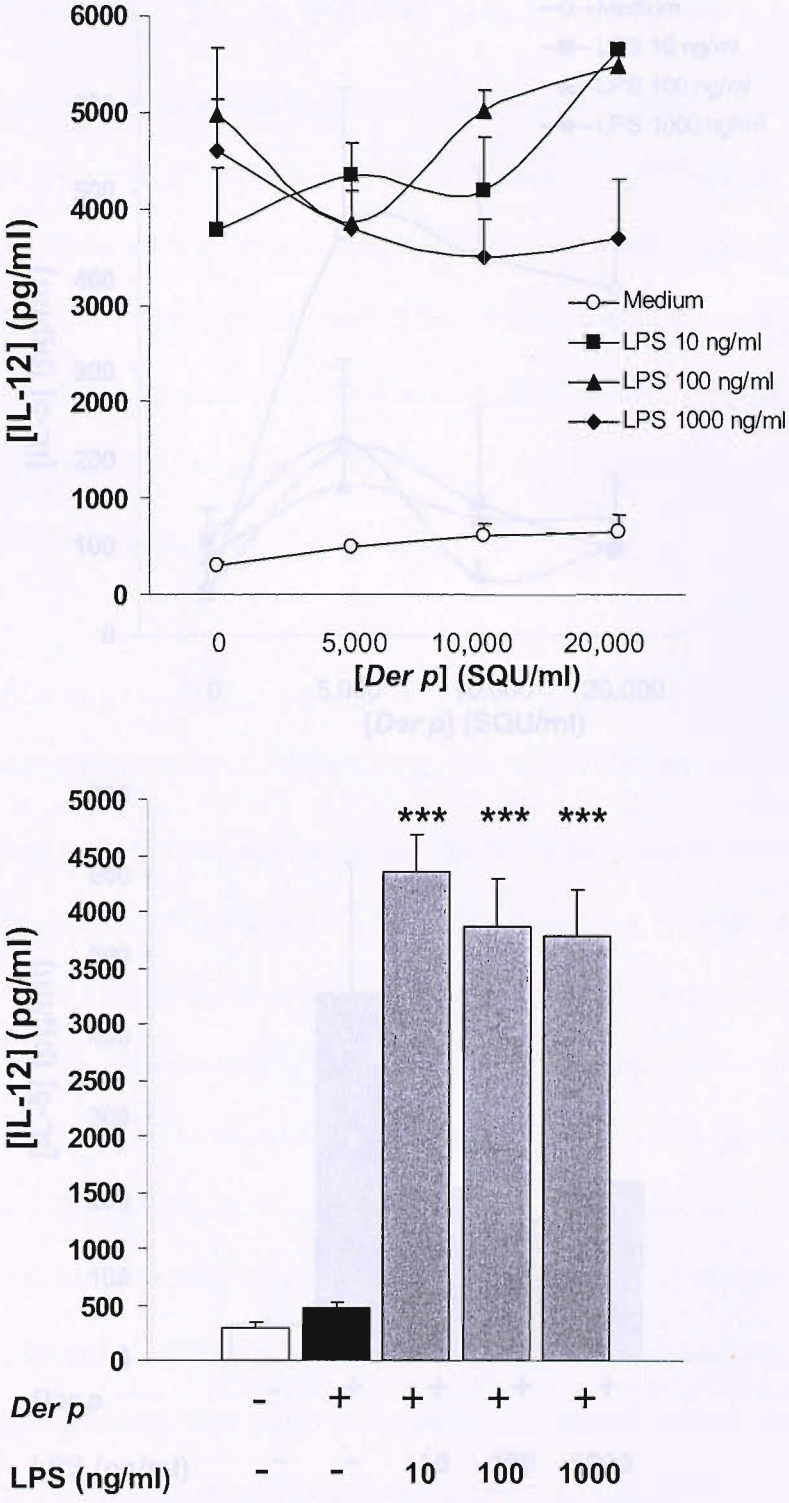


FIGURE 6.3-3: *Effects of LPS and Der p co-exposure on IL-12 synthesis in PBMC from atopic asthmatic donors (n=8). Upper panel shows responses to LPS 10 nm/ml (■), 100 ng/ml (▲), 1000 ng/ml (♦) given togerther with doubling doses of Der p. Lower panel shows the concentration relationship of LPS's effect on allergen-induced IL-12 synthesis (Der p, 5000 SQU/ml) . Bars depict mean ± SEM, (n=8). *** p<0.001*

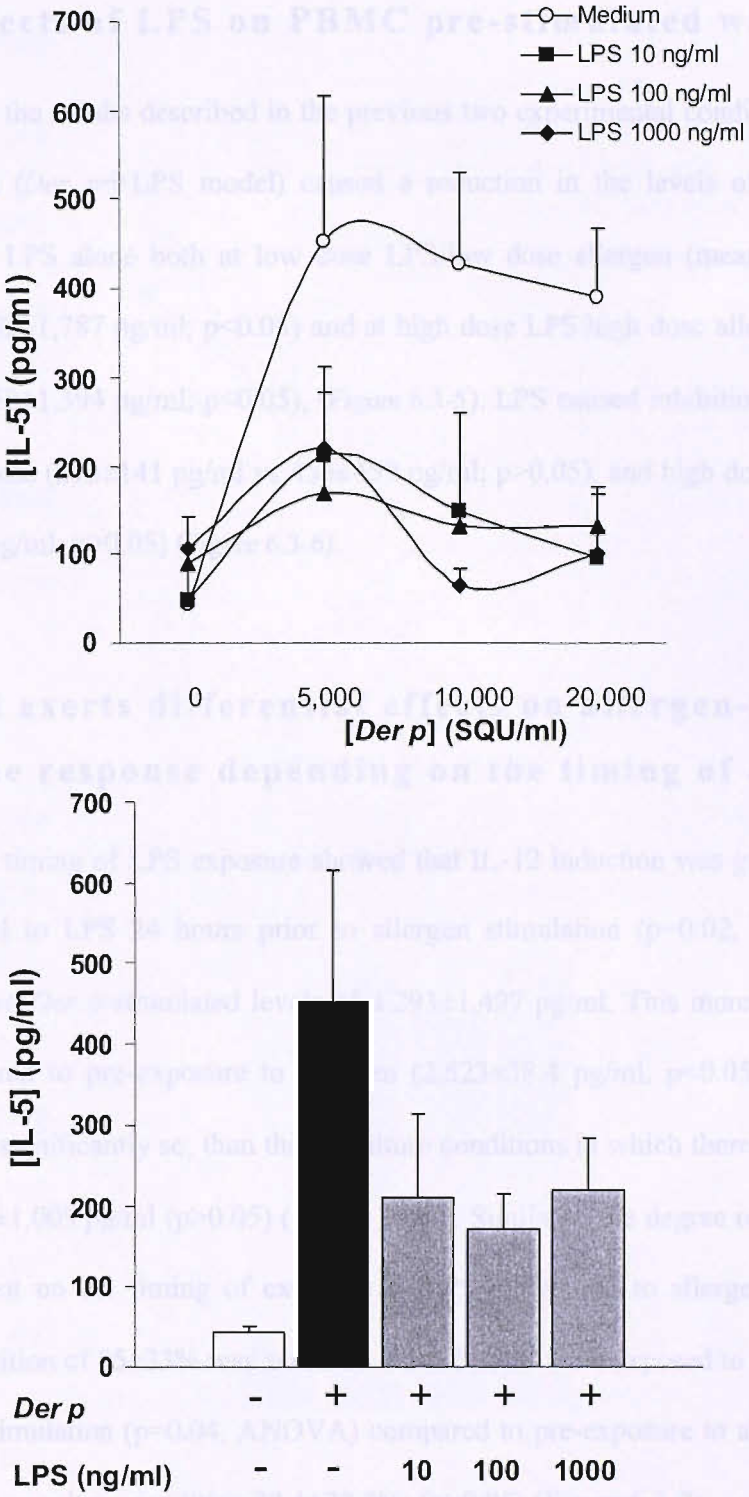


FIGURE 6.3-4: *Effects of LPS and Der p co-exposure on IL-5 synthesis in PBMC from atopic asthmatic donors (n=8). Upper panel shows responses to LPS 10 nm/ml (■), 100 ng/ml (▲), 1000 ng/ml (♦) given togerther with doubling doses of Der p. Lower panel shows to the concentration dependence of LPS's effect on allergen-induced IL-5 synthesis (Der p, 5000 SQU/ml) . Bars depict mean ± SEM, (n=8). p<0.18, repeated measure ANOVA.*

6.3.3 Effects of LPS on PBMC pre-stimulated with allergen

In contrast to the results described in the previous two experimental conditions, pre-exposure with allergen (*Der p*→LPS model) caused a reduction in the levels of IL-12 generation compared to LPS alone both at low dose LPS/low dose allergen (mean±SD 3,006±1,229 pg/ml vs 4,202±1,787 pg/ml; $p<0.05$) and at high dose LPS/high dose allergen (2,750±1,994 pg/ml vs 5,050±1,394 pg/ml; $p<0.05$), (Figure 6.3-5). LPS caused inhibition of IL-5 secretion both at low dose (293±141 pg/ml vs 453±459 pg/ml; $p>0.05$), and high dose (237±143 pg/ml vs 453±459 pg/ml; $p>0.05$) (Figure 6.3-6).

6.3.4LPS exerts differential effects on allergen-induced cytokine response depending on the timing of exposure

The different timing of LPS exposure showed that IL-12 induction was greater when PBMC were exposed to LPS 24 hours prior to allergen stimulation ($p=0.02$, ANOVA) with an increase above *Der p*-stimulated levels of 4,293±1,497 pg/ml. This increase was significant when compared to pre-exposure to allergen (2,523±38.4 pg/ml; $p<0.05$) and was greater, although not significantly so, than the co-culture conditions in which there was an increase in IL-12 (3,874±1,009 pg/ml ($p>0.05$) (Figure 6.3-7). Similarly, the degree of inhibition of IL-5 was dependent on the timing of exposure to LPS in relation to allergen stimulation. The greatest inhibition of 55±33% was achieved when PBMC were exposed to LPS 24-hours prior to allergen stimulation ($p=0.04$, ANOVA) compared to pre-exposure to allergen 5.7±38.4%, ($p<0.05$) and co-culture condition 32.4±37.2%; ($p>0.05$) (Figure 6.3-7).

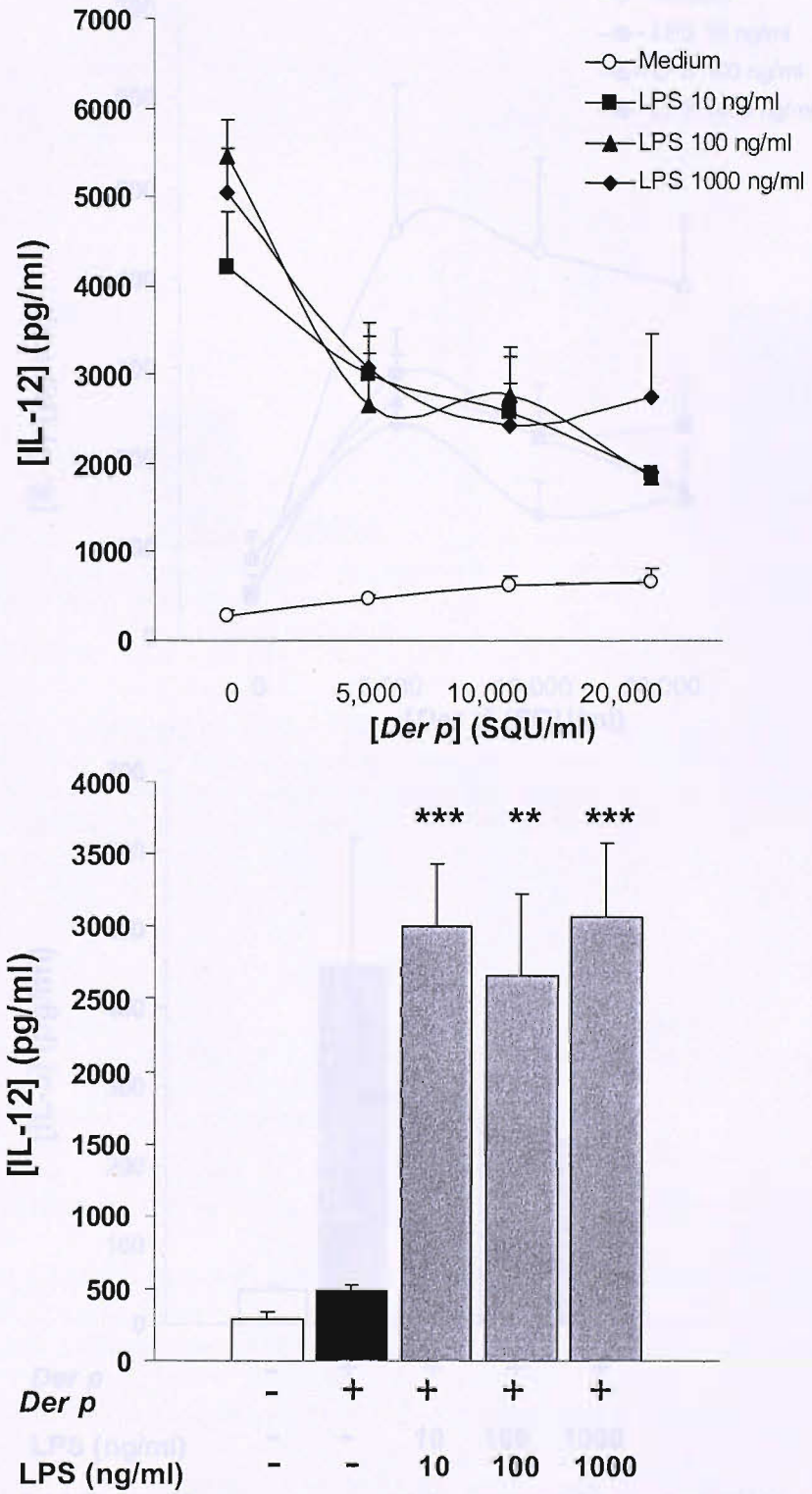


FIGURE 6.3-5: *Effects of Der p pre-exposure on IL-12 synthesis in LPS-stimulated PBMC from atopic asthmatic donors (n=8). Upper panel shows responses to LPS 10 nm/ml (■), 100 ng/ml (▲), 1000 ng/ml (♦) given 24 hours before challenge with doubling doses of Der p. Lower panel shows to the concentration relationship of LPS's effect on subsequent allergen challenge (Der p, 5000 SQU/ml). Bars depict mean ± SEM, (n=8). **, p<0.01; *** p<0.001*

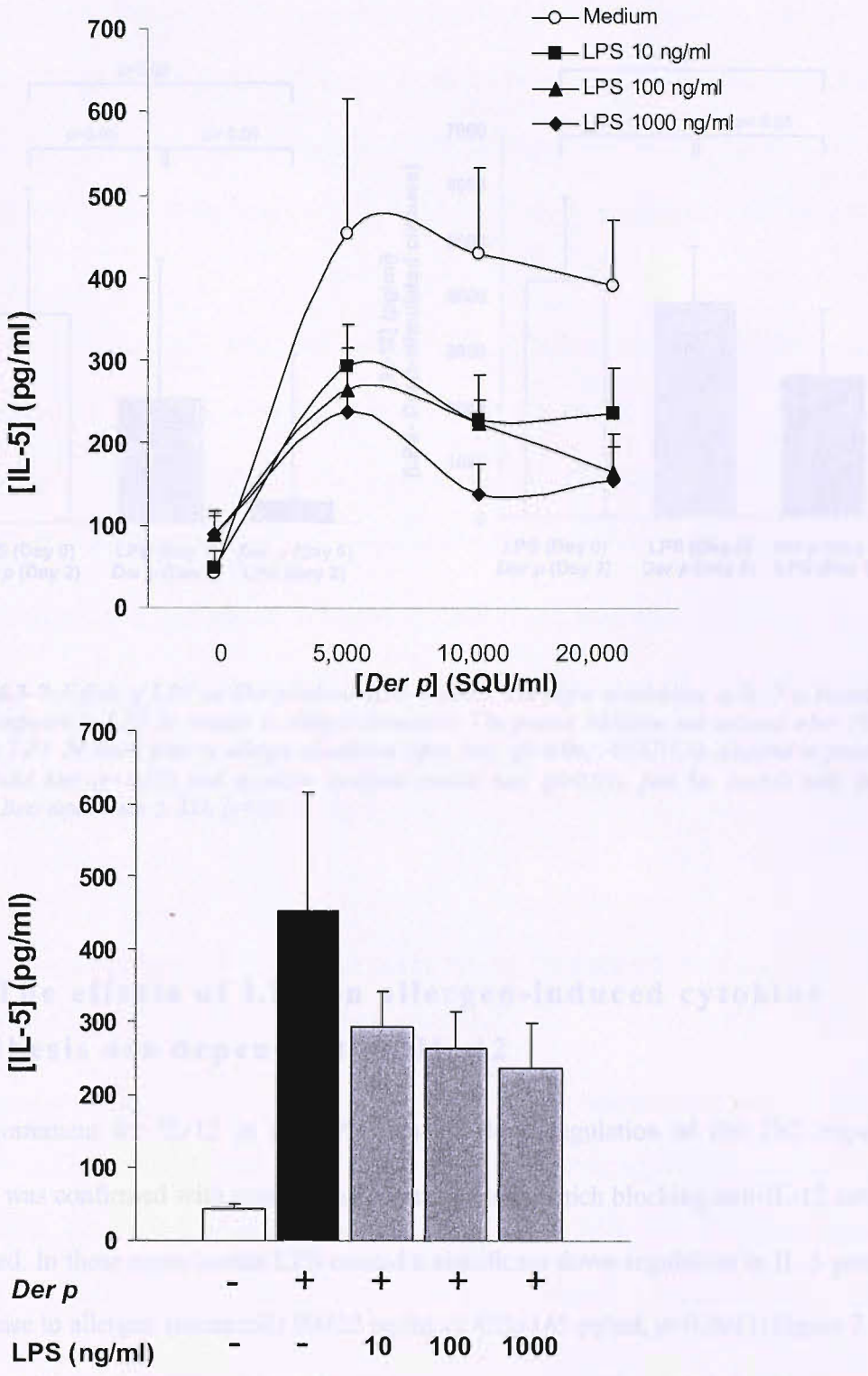


FIGURE 6.3-6: *Effects of Der p pre-exposure on IL-5 synthesis in LPS-stimulated PBMC from atopic asthmatic donors (n=8). Upper panel shows responses to LPS 10 nm/ml (■), 100 ng/ml (▲), 1000 ng/ml (♦) given 24 hours before challenge with doubling doses of Der p. Lower panel shows to the concentration relationship of LPS's effect on subsequent allergen challenge (Der p, 5000 SQU/ml).*

Bars depict mean ± SEM, (n=8). p<0.30, repeated measure ANOVA

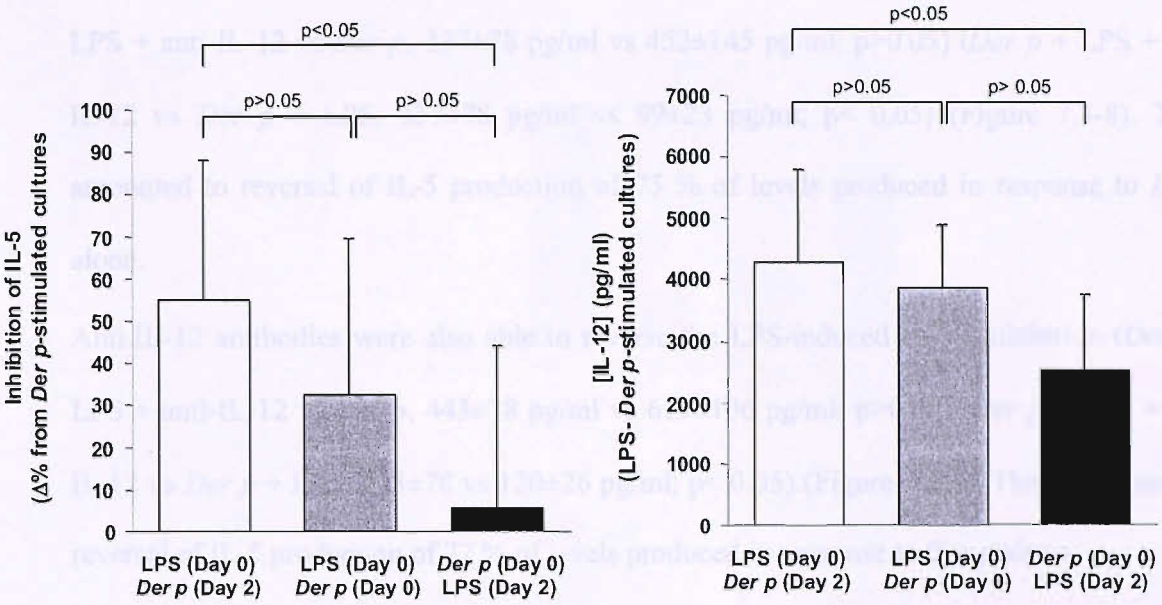


FIGURE 6.3-7: *Effects of LPS on Der p-induced IL-5 synthesis. The degree of inhibition of IL-5 is dependent on the timing of exposure to LPS in relation to allergen stimulation. The greatest inhibition was achieved when PBMC were exposed to LPS 24 hours prior to allergen stimulation (open bar), (p=0.04, ANOVA) compared to pre-exposure to allergen (solid bar) (p<0.05) and co-culture condition (shaded bar) (p>0.05), post hoc analysis with Bonferroni's correction. Bars depict mean ± SD, (n=8).*

6.3.5 The effects of LPS on allergen-induced cytokine synthesis are dependent on IL-12

The requirement for IL-12 in the LPS induced down-regulation of the Th2 response to allergen was confirmed with neutralising experiments in which blocking anti-IL-12 antibodies were used. In these experiments LPS caused a significant down-regulation in IL-5 generation in response to allergen (mean±SD 99±22 pg/ml vs 453±145 pg/ml; p<0.001) (Figure 7.3-8) as well as IL-13 (119±26 pg/ml vs 619±106 pg/ml; p<0.01) (Figure 7.3-8). In contrast, the concentration of IFN-γ increased (472±81 pg/ml vs 303±115 pg/ml; p<0.05) (Figure 7.3-8).

Anti-IL-12 antibodies were able to partially restore IL-5 concentrations in culture (*Der p* + LPS + anti-IL-12 vs *Der p*, 337±78 pg/ml vs 452±145 pg/ml; p>0.05) (*Der p* + LPS + anti-IL-12 vs *Der p* + LPS, 337±78 pg/ml vs 99±23 pg/ml; p< 0.05) (Figure 7.3-8). These amounted to reversal of IL-5 production of 75 % of levels produced in response to *Der p* alone.

Anti-IL-12 antibodies were also able to reverse the LPS-induced IL-13 inhibition (*Der p* + LPS + anti-IL-12 vs *Der p*, 443±78 pg/ml vs 619±106 pg/ml; p>0.05) (*Der p* + LPS + anti-IL-12 vs *Der p* + LPS, 443±78 vs 120±26 pg/ml; p< 0.05) (Figure 7.3-8). These amounted to reversal of IL-5 production of 72 % of levels produced in response to *Der p* alone.

IFN-γ synthesis was completely inhibited by anti-IL-12 antibodies, showing that in this system, IFN-γ production was entirely IL-12 dependent (*Der p* + LPS + anti-IL-12 vs *Der p*, 85±16 pg/ml vs 303±115 pg/ml; p>0.05) (*Der p* + LPS + anti-IL-12 vs *Der p* + LPS, 85±16 pg/ml vs 472±81 pg/ml; p<0.01) (Figure 7.3-8).

6.3.6 The effects of LPS on allergen-induced cytokine synthesis are not dose-dependent

As demonstrated above the effects of LPS in any set of experimental conditions (LPS→*Der p*; LPS+*Der p*; *Der p*→LPS) were not dependent on the concentration used and no differential effects on IL-12 and IL-5 were demonstrated using low vs high dose of LPS, (Figure 6.3-1, Figure 6.3-2, Figure 6.3-3, Figure 6.3-4, Figure 6.3-5, Figure 6.3-6, Figure 6.3-7).

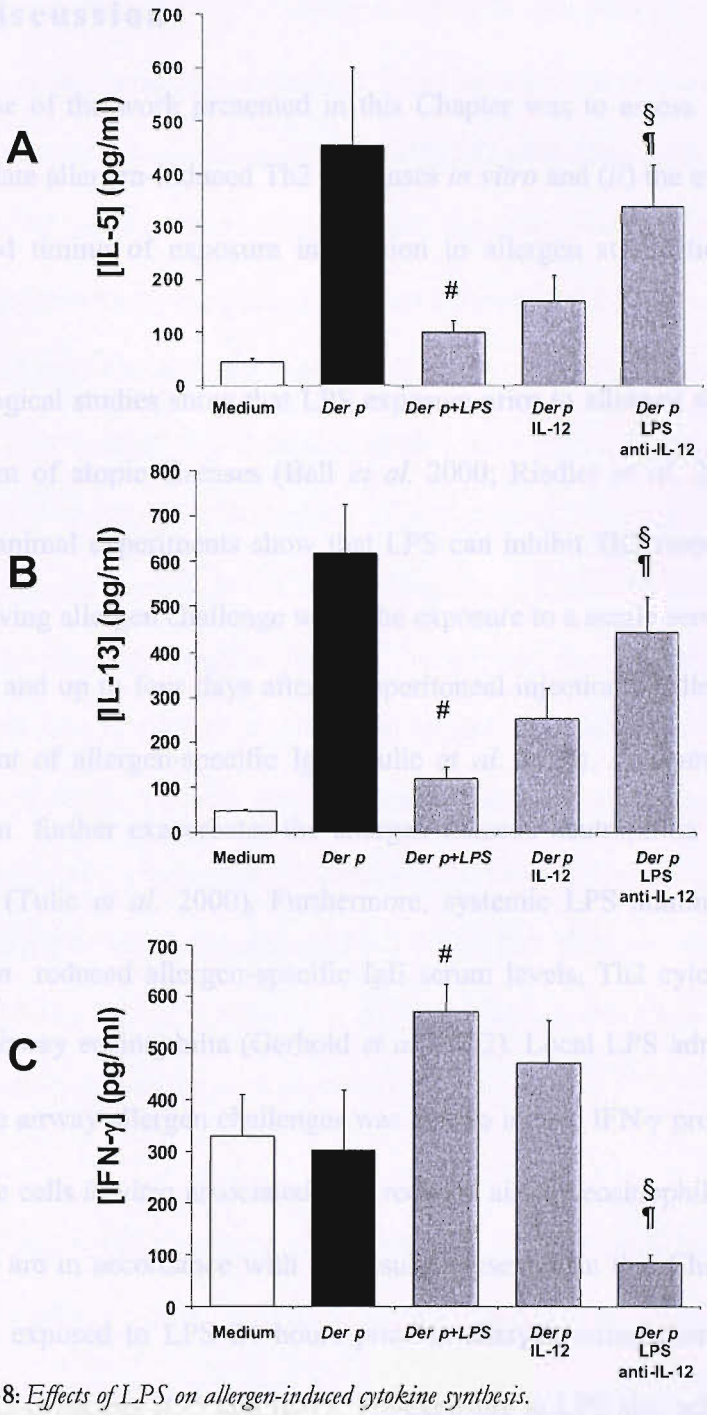


FIGURE 6.3-8: *Effects of LPS on allergen-induced cytokine synthesis.*

(A) *IL-5 synthesis by PBMC from atopic asthmatic donors (n=8) was inhibited by LPS (10 ng/ml). # (LPS+Der p vs Der p), $p<0.01$. Anti-IL-12 antibodies were able to restore IL-5 concentrations in culture. ¶ (Der p + LPS+anti-IL-12 vs Der p) p=ns; § (Der p + LPS+anti-IL-12 vs Der p+LPS), $p<0.05$.*

(B) *IL-13 synthesis by PBMC from atopic asthmatic donors (n=8) was inhibited by LPS (10 ng/ml). # (LPS+Der p vs Der p), $p<0.01$. Anti-IL-12 antibodies were able to restore IL-5 concentrations in culture. ¶ (Der p + LPS+anti-IL-12 vs Der p) p=ns; § (Der p + LPS+anti-IL-12 vs Der p+LPS), $p<0.05$.*

(C) *IFN-γ synthesis by PBMC from atopic asthmatic donors (n=8) was enhanced by LPS (10 ng/ml). # (LPS+Der p vs Der p), $p<0.05$. Anti-IL-12 antibodies completely inhibited IFN-γ synthesis. ¶ (Der p + LPS+anti-IL-12 vs Der p) p=ns; § (Der p + LPS+anti-IL-12 vs Der p+LPS), $p<0.01$. Bars depict mean ± SD, (n=8).*

6.3.7 Discussion

The purpose of the work presented in this Chapter was to assess (*i*) the ability of LPS to down-regulate allergen-induced Th2 responses *in vitro* and (*ii*) the effect of the concentration of LPS and timing of exposure in relation to allergen stimulation on *in vitro* cytokine response.

Epidemiological studies show that LPS exposure prior to allergen sensitisation prevents the development of atopic diseases (Ball *et al.* 2000; Riedler *et al.* 2001; Bolte *et al.* 2002). Similarly, animal experiments show that LPS can inhibit Th2 responses, IgE synthesis and BHR following allergen challenge while the exposure to a single aerosol exposure to LPS one day before and up to four days after intraperitoneal injection of allergen protects against the development of allergen-specific IgE (Tulic *et al.* 2000). In contrast, LPS exposure after sensitisation further exacerbates the allergen-induced neutrophilia with no effect on serum IgE levels (Tulic *et al.* 2000). Furthermore, systemic LPS administration before allergen sensitisation reduced allergen-specific IgE serum levels, Th2 cytokine production and the extent of airway eosinophilia (Gerhold *et al.* 2002). Local LPS administration to sensitized mice before airway allergen challenges was able to induce IFN-γ production by peribronchial lymph node cells *in vitro* associated with reduced airway eosinophilia (Gerhold *et al.* 2002). These data are in accordance with the results presented in this Chapter which show that if PBMC are exposed to LPS 24 hours prior to allergen stimulation, LPS is able to down-regulate Th2 cytokines IL-5 and IL-13. Pre-exposure to LPS also achieved the greatest IL-12 synthesis throughout the concentration-range of LPS.

While the data on LPS pre-exposure seem to accord with a protective effect of LPS on the development of the allergic phenotype *in vivo* or *in vitro*, the data on co-administration LPS and allergen are conflicting. In contrast to animal data showing that the co-administration of

LPS and allergen decreases IgE production and BHR following allergen challenge (Watanabe *et al.* 2003), in this experimental model I found no statistical difference in the IL-5 and IL-13 inhibition between co-colture experiments (PBMC stimulated with allergen and LPS at the same time) and pre-stimulation with allergen. However, other animal studies using different routes of administration and types of LPS have shown that LPS in addition to allergen has no effect on Th2 response (Nakstad *et al.* 1999), can decrease Th1 response but not Th2 cytokine production (Lauw *et al.* 2000) or can amplify IL-5 synthesis (Matsui *et al.* 2001). Other studies have even shown a synergistic effect between LPS and IL-4 in the synthesis and release of the chemotactic factor for eosinophils, eotaxin (Nonaka *et al.* 2004), with the promotion of tissue eosinophilia through the increase of eosinophil chemotaxis via CCR3 receptor activation (Penido *et al.* 2001) and enhanced eosinophil survival (Meerschaert *et al.* 2000). The discrepancy among published studies and between these and the data presented in this thesis may reflect differences in experimental models (human *vs* animal; whole blood vs PBMC), difference in the stimulus used (CD3/CD28 vs allergen) and differences in the strain of LPS used (e.g. *H. influenzae vs E.coli*), as well as the route of administration (intra-venously; intra-peritoneally; aerosol; *ex vivo*, *in vitro*).

The second aim of this Chapter was to test the hypothesis that different doses of LPS exerted a differential effect on allergen-induced T cell response. Epidemiological studies suggest that the level of exposure to LPS could be as important as the timing of exposure in preventing allergic response. Epidemiological data in humans support a differential effect of the level of endotoxin exposure and the incidence of lung disease and severity (Eisenbarth *et al.* 2002; Liu 2002). Eisenbarth *et al*, showed that allergen exposure in the presence of high dose LPS fails to induce Th2 cells, but instead induces IL-12 production and a Th1 response. By contrast,

low dose LPS is not sufficient to induce Th1 cells but is required to induce Th2 inflammation (Eisenbarth *et al.* 2002). The concentration-response data in this Chapter do not support the concept of differential regulatory properties of LPS on T cells. Indeed, there was no difference in the degree of inhibition IL-5 between the lowest and the highest dose of LPS used in culture.

It is possible that the lowest concentration of LPS used in this Chapter was already supra-maximal, with consequent loss of concentration-dependency. It is also difficult to compare the equivalence between LPS concentration (ng/ml) used *in vitro* and LPS activity measured in Endotoxin Units as reported in epidemiological studies. The other possible explanation is that different routes of administration may elicit different patterns of cytokine responses responsible of Th2 down-regulation (e.g. IL-10 or TGF-β production). Furthermore, the dose of LPS in the environment can be related to the presence of different doses of other compounds such as bacterial DNA, which in turn may have a differential effect on Th1/Th2 regulation.

The fact that LPS exposure upregulates the production of IL-12 and Th1 cytokines in human T cells (Kobayashi *et al.* 1989) and dendritic cells (Jotwani *et al.* 2003) is well known. For this reason, LPS has been used as a stimulus to induce Th1 responses *in vitro* (Itazawa *et al.* 2003). Human explant models also confirm that LPS can upregulate the number of $CD3^+$ cells, and stimulates T cell proliferation and the production of IL-12 (Tulic *et al.* 2002). Animal studies show that the effects of LPS on allergen sensitisation and eosinophilic airway inflammation are inhibited by administration of anti-IL-12 antibodies before LPS exposure (Gerhold *et al.* 2002). These animal studies are in accordance with the results of neutralisation experiments presented in this thesis which show that the effects of LPS are, at least in these

experimental conditions, IL-12 dependent as demonstrated by the fact that anti-IL-12 antibodies reverse the inhibitory effects of LPS on IL-5 and IL-13 synthesis.

By contrast, other studies show that the level of IL-12 production by DC did not correlate with Th1 development, and the capacity of LPS to reduce Th2-dependent eosinophilic airway inflammation was IL-12-independent, suggesting that LPS reduces sensitisation to inhaled antigen by reducing the DC-driven Th2 development (Kuipers *et al.* 2003). Intranasal administration of neutralizing anti-IL-12 at the time of high-dose LPS challenge reduced lung IL-12 and IFN-γ but did not affect the levels of Th2-type cytokines or BHR, suggesting that the amelioration of airway hyperresponsiveness observed in LPS-treated, allergen-sensitised mice is coincident with an immune deviation of the lung inflammatory response, independent of IL-12 (Lundy *et al.* 2003).

Another aspect of the effects of endotoxin on allergic phenotype is the fact that the relationship between the two may not necessarily be one of cause and effect. In other words endotoxin may represent a surrogate marker of other molecular bacterial structures (e.g. bacterial DNA, heat shock protein) that can directly activate the innate immune system by signalling through pattern recognition receptors such as toll-like receptors (TLRs) (Song *et al.* 2003). This is supported by Roy *et al.* who report that stimulation with both endotoxin and farm barn DNA rich in bacterial DNA synergistically potentiated IL-10 and IL-12 responses (Roy *et al.* 2003) whereas dust from urban homes (low in bacterial DNA content) did not potentiate IL-10 and IL-12 responses (Roy *et al.* 2003).

Along with immune deviation with activation of Th1 response, immune regulation with activation of Treg cells can take place and has been described in association with LPS

exposure (Caramalho *et al.* 2003). This hypothesis has not been specifically tested in this Chapter.

The importance of studying the interaction between LPS and allergens has wider implications which go beyond understanding the immunological basis of the 'hygiene hypothesis', and are of importance in the treatment of allergic diseases. Indeed, non-toxic LPS analogues (Monophosphoryl lipid A, MPL®, Corixa Corporation, Seattle, WA, USA) have been developed and are being tested in clinical trials as adjuvants in immunotherapy.

Monophosphoryl lipid A (MPL®), is a 3-deacylated MPL, derived from the lipopolysaccharide (LPS) of *Salmonella minnesota* R595. Whilst MPL retains the immunostimulatory properties of the parent molecule it does not have its inherent toxicity. MPL can promote Th1 responses, which are mediated by the induction of IFN-γ in a IL-12-dependent fashion, while down-regulating the Th2-response, as shown by a reduction in IL-5 synthesis. The results presented in this Chapter, show also that the pattern of cytokines elicited by LPS in allergen-stimulated cultures is similar to that of the synthetic lipid A Similarly to my study, the effects of MPL®, in allergen-stimulated cultures, were dependent on IL-12 but not on the synthesis of IL-10.

In conclusion, I have shown that LPS, when administered before allergen stimulation, down-regulates Th2 cytokine response and achieves the greatest increase in IL-12 production and reduction in IL-5. These effects seem IL-12 mediated, although involvement of Treg cells and IL-10 has not been tested and cannot be excluded.

CHAPTER SEVEN

Conclusions and future directions

7.1 CONCLUSIONS AND FUTURE DIRECTIONS

Down-regulation of allergen-induced T cell responses by blocking IL-4

Epidemiological studies have shown an increase in the prevalence of asthma and other related allergic disorders with a striking difference in prevalence between nations and between urban and rural environments. As described in Chapter one, allergic asthma is the result of a type 2 T helper (Th2) cell-mediated immune response against 'innocuous' environmental antigens (allergens). Numerous studies now support the critical role of Th2 cytokines, such as IL-4, IL-5, IL-9 and IL-13, in the initiation, maintenance and amplification of human allergic inflammation (Romagnani 2004).

Th2 cells represent one end of the spectrum of T helper cell polarisation in response to antigens; on the other end of the spectrum are Th1 cells, which synthesise IFN-γ. T helper cells are thought to be able to cross-regulate each other and based on this concept a number of possible treatments for allergic disorders have been tested with the aim either of potentiating Th1 responses (e.g. administration of IL-12 or IFN-γ), which in turn, down-regulates the Th2 response to allergen, or of neutralising Th2 cytokines (anti-IL-5, anti-IL-4) or their receptors.

Based on the 'Th2 hypothesis' of allergy I have used a soluble form of IL-4 receptor (sIL-4R), which, by competing with the membrane-bound counterpart, binds IL-4 in solution and prevents the post-receptor signalling cascade leading to cell activation and gene transcription. With the experiments described in Chapter three, I have shown that sIL-4R was able to down-regulate Th2 cytokine responses to allergen *in vitro,* as shown by an inhibition of IL-5 and IL-13 to a degree comparable to blocking antibodies against IL-4 and IL-4R. Soluble IL-4R, at the concentration used to maximally inhibit IL-5 did not up-regulate IFN-γ production. An

increase in the concentration of IFN-γ, was however demonstrated when higher concentrations of sIL-4 were used.

The down-regulation of Th2 cytokines was not secondary to a reduced survival of PBMC with sIL-4R, as demonstrated by proliferation assays. This could mean that IFN-γ generation may not be the *sine qua non* for Th2 down-regulation. Alternatively, it is possible that the two phenomena can both occur but each at different concentrations of sIL-4R, and different mechanisms may be involved.

Although these experiments clearly show the effectiveness of sIL-4R at down-regulating an allergen-induced Th2 response *in vitro*, further experiments are necessary to clarify the mechanisms responsible for such an inhibition. In particular, understanding whether sIL-4R is able to modulate the expression of transcription factors such as STAT6, GATA-3 and c-maf, which control the generation of the Th2 response, and what are the effects on the Th1 transcription factor t-bet could elucidate which mechanism – up-regulation of Th1 or down-regulation of Th2 – is prevalent in this system and accounts for the inhibition in IL-5 and IL-13. Furthermore, the differential effects exerted by different concentrations of sIL-4R deserve further attention to better understand whether sIL-4R can inhibit Th2 transcription factors at low concentration while higher concentrations are necessary to up-regulate Th1 transcription factors. The latter scheme could have an important clinical repercussion because it would the selection of a dose able to reduce the exaggerated Th2 response without the potentially harmful consequences of inducing a Th1-mediated immune response. Flow cytometry experiments staining cells for intracellular cytokines (IL-4, IL-5, IL-12, IFN-γ) in association with surface markers for T cells (CD3, CD4, CD8) could give us data on the population of cells involved in the observed changes in immune response following treatment with sIL-4R. Intracellular cytokine staining by FACS will also clarify whether the inhibition of IL-4 causes

a reduction in the number of cells secreting IL-4 and IL-5, as well as IFN-γ, or the intensity of cytokine synthesis per cell. Further studies are needed on the effects of sIL-4R on the activation of transcription factors for Th2 cytokines – GATA-3 and STAT6. As an IL-4 antagonist, sIL-4R should be able to block GATA-3 and STAT6 activation and prevent their binding to DNA responsive elements. Furthermore, the ability of sIL-4R to inhibit preferentially the IL-4R cytokine transcription pathway rather than the apoptotic pathway should be further evaluated. Analysis of the effects of IL-4 inbibition on the bcl-2 family of pro- and anti-apoptotic molecules as well as on Caspases during cytokine activation (Caspase-1, -4, -5, and -13), apoptosis initiation (Caspase-2, -8, -9, -and -10), and apoptosis execution (Caspase-3, -6, and -7) will provide us with a better understanding of the molecular actions of sIL-4R.

The ultimate goal should be to understand whether, and to what extent, the effects of sIL-4R, are dependent on allergen specific cells or it has a by-standard effect and therefore can down-regulate Th2 response to multiple allergens in poly-sensitised atopic patients. This should be tested in *in vito* or in *in vivo* animal experiments using a single dose of sIL-4R followed by repeated challenge experiments with different allergens and analysing the T cell phenotype and cytokine response. In the presence of a significant by-stander effect, we would expect a Th2 cytokine production which remains low for different allergens.

Experiments in which animals sensitised and challenged with an allergen are treated with sIL-4R, then receive a T cell transfer (using cells from animals sensitised to a different allergen). On re-challenge with the second allergen we would also expect a low Th2 activation compared to untreated, challenged animals.

Down-regulation of Th2 responses by mechanisms mimicking those believed responsible of the 'Hygiene Hypothesis'

Over the last decade, studies have shown that modifications of the pattern of microbial exposure during childhood, are represent a critical factor underlying the rising prevalence of atopic disorders ('hygiene hypothesis'). Although most of the initial evidence came from the observation on the inverse relationship between allergy and infectious diseases such as hepatitis A, measles and tuberculosis, it appears now that the microbes more likely to contribute to the 'hygiene hypothesis' and prevent allergic diseases are the innocuous environmental commensal bacteria that have evolved with the human race (Rook *et al.* 2003; Rook *et al.* 2004). These 'old friends' are likely to be represented by saprophytic mycobacteria, lactobacilli and helminths (Rook *et al.* 2003; Rook *et al.* 2004).

To test the hypothesis that the non-pathogenic *Mycobacterium vaccae* could reduce airway inflammation, bronchial hyperresponsiveness following allergen challenge and the Th2 cytokine response of *in vitro*-challenged PBMC, I conducted a clinical trial where *M. vaccae* (in the form of SRL172) was administered intradermally. As described in Chapter four *M. vaccae* was able to cause a reduction in the area under the curve (AUC) of FEV_1 plotted against time during the LAR and a decrease in the maximum fall in FEV_1, although this failed to reach conventional statistical significance versus placebo. The *M. vaccae* treated group showed a trend towards reduction in IL-5 synthesis *in vitro* and serum IgE levels three weeks post-treatment.

This study was the first to test *M. vaccae* as a treatment for asthma. Some elements of the design of this trial could be modified to improve future clinical trials with *M. vaccae.* In particular, using the data generated by this study, a more accurate calculation of sample size can be obtained. It is possible that larger clinical studies are necessary to reduce the noise-to-

signal ratio and show a significant difference between *M. vaccae* and placebo. When I started this study, no data were available on the time-course, dose and number of administration (single vs. multiple injections) of *M. vaccae* needed to demonstrate a change in the clinical and immunological outcome parameters. Therefore, a larger trial, possibly with three arms (placebo vs. single dose vs. multiple doses), could clarify the influence of these factors on the observed results.

To understand the mechanisms responsible for the observed changes in the allergen challenge parameters as well as the tendency towards down-regulation in IL-5 and IgE, I studied the *in vitro* effects of *M. vaccae* (SRP299) on cytokine production by PBMC from mild atopic asthmatics. The results showed that SRP299 caused a potent inhibition of allergen-induced IL-5 and IL-13 synthesis. This effect did not involve IL-12 induction but did involve IL-10, providing further evidence of the role of *M. vaccae* in the induction of regulatory T cells. Furthermore, the results in Chapter five showed that the inhibitory effect of *M. vaccae* may involve the generation of prostaglandins.

The immunological mechanisms underlying the effects of *M. vaccae* are still not entirely understood. Similarly, it remains unclear which components of *M. vaccae* are responsible for the immunomodulatory effects of the mycobacterium.

A possible mechanism is a shift of allergen-specific responses from the Th2 to the Th1 phenotype (i.e. immune deviation) caused by the production of Th1-polarising cytokines by cells of the innate immune system (Romagnani 1994; Romagnani 2004). More recently, the importance of activity of T regulatory (Treg) cells (i.e. immune suppression/regulation) has been emphasised (Yazdanbakhsh *et al.* 2002; Walker *et al.* 2003).

The interactions of ligands of *M. vaccae* with several receptors on host cells play a crucial role in the immune response. As discussed in Chapter one, mycobacteria express several

molecules that can cause cellular activation through pattern recognition receptors such as the mannose receptor, Toll-like receptor (TLR) 2 and/or TLR 4 on APC. *M. vaccae* express mycobacterial heat-shock proteins and lipoarabinomannan (LAM). The type of LAM expressed by *M. vaccae* is currently unknown. This is of importance since certain types of LAM activate TLR4 with generation of IL-12, others preferentially activate TLR2, generating both IL-12 and IL-10 synthesis, while the mannosilated LAM (ManLAM) signals through the c-lectin DC-SIGN, stimulating the synthesis of the regulatory cytokine IL-10 and TGF-β. The fact that delipidated fractions of *M. vaccae* are ineffective *in vitro* and *in vivo* in down-regulating Th2 cytokines and improving asthma symptoms (Shirtcliffe *et al.* 2001; Shirtcliffe *et al.* 2003), wheras lypoglicans are able to suppress airway inflammation and down-regulate Th2-mediated inflammation (Sayers *et al.* 2004), indicates that lipids are important components of the *M. vaccae* preparations and significantly contribute to its role as an immunomodulator.

Other important components of *M. vaccae* could be represented by heat shock proteins and bacterial DNA. Furthermore, whether LPS is present in the preparation of *M. vaccae* and its role on *in vitro* regulation of cytokine profile is not known and deserves clarification.

There is evidence that mycobacterial heat-shock proteins (Hsp) can stimulate $CD4^+CD25^+$ regulatory T cells (Tregs) from $CD4^+CD25^-$ T cells. These newly formed $CD4^+CD25^+$ T cells express Treg-cell markers — such as GITR (glucocorticoid-induced TNF-receptor-related protein), CTLA-4 (cytotoxic T-lymphocyte antigen 4) and CD30 — and high levels of the transcription factor FOXP3. Moreover, these $CD4^+CD25^+$ T cells are capable of suppressing *in vitro* immune responses through the generation of IL-10, TGF-β.

HSPs can activate APCs via cell-surface receptors such as CD14, CD40, scavenger receptors CD36, CD91 and LOX1 (lectin-type oxidized LDL receptor 1) and can signal through TLR2

on both APC and T cells and therefore affect Th1/Th2 phenotype (Vabulas *et al.* 2002; van Eden *et al.* 2005).

It is also possible that small quantities of bacterial DNA (CpG oligodeoxynucleotides) can be released, activating TLR9 and contributing to IL-12/IFN-γ production and the inhibition of the allergic Th2 response both *in vivo* and *in vitro*.

The hygiene hypothesis has evolved to propose that harmful infections *per se* may not be as critical as the exposure to microbial burden in the absence of infections.

In the last decade, a series of epidemiological studies have consistently shown a reduced risk of atopic disease and allergic sensitisation in children from farming families (reviewed in (Braun-Fahrlander 2003)). In particular, exposure to livestock and stables and pets was shown to confer protection from allergy. This effect was thought due to higher exposure to bacterial lipopolysaccharide (LPS) endotoxin associated with the presence of animals. LPS is a potent inducer of IL-12 and IFN-γ, which are cytokines that stimulate Th1-mediated immunity and have the potential to down-regulate the production of Th2 cytokines such as IL-4, IL-5 and IL-13.

The above immunological mechanisms elicited by endotoxin give a framework to hypothesise how natural microbial exposures might have an immune modulatory and atopy-protective effect without the harm of infections. However, LPS has been associated with both a protective effect on allergy and a detrimental effect in terms of promoting asthma exacerbations. This apparent paradox could be explained in terms of different timing and dose of LPS in relation to allergen stimulation.

The aim of my study presented in Chapter six was to assess the effect of LPS on allergen-induced cytokine response by PBMC from patients with mild atopic asthma. In particular, three aspects were evaluated: 1) the effect of increasing concentrations of LPS on allergen-

stimulated PBMC, 2) the effect of timing of LPS exposure in relation to allergen challenge *in vitro*, and 3) the importance of the generation of a Th1 response in preventing Th2 cytokine generation in response to allergen. The results of these experiments show that LPS, when administered before allergen stimulation, down-regulates Th2 cytokine response, achieves the greatest increase in IL-12 production and reduction in IL-5. These effects seem IL-12 mediated although involvement of Treg cells and IL-10 has not been tested and cannot be excluded.

In Figure 7.1-1, I have summarised a model describing the effects of the exposure to mycobacteria and LPS on allergen-induced T cell activation and cytokine response. In the model, the consequences of blocking IL-4 using sIL-4R are also illustrated.

Future studies will be necessary to address some of the points raised by the *in vitro* studies carried out with both *M. vaccae* and LPS. In particular, it is important to identify all the components of SRP299 and SRL172 (lipids, glycolipids, proteins, DNA) that account for the immunoregulatory properties exhibited by *M. vaccae* and then test them individually in *in vitro* systems like the one described in this thesis. It is also important to understand the pattern of receptors involved in the immunoresponse to SRP299 ans SRL172. In particular, the relationship between TLR4 and TLR2 needs to be better understood and the relative role of these two receptors should be tested with blocking antibodies against TLR.

Very little is known regarding the role of DC-SIGN, described as receptor for ManLAM of virulent mycobacteria, in the recognition of saprophytic mycobacteria such as *M. vaccae*. Studying the cellular distribution and regulation of expressions of this molecule after stimulation with allergen and *M. vaccae,* in the presence or absence of antibodies against DC-SIGN, would clarify whether DC-SIGN plays any role in IL-10 generation induced by the non-virulent M. *vaccae*.

For both *M. vaccae* and LPS, a detailed description of the cells involved in the inhibition of the Th2 response is necessary, with particular regard to Treg cells, secreting IL-10 and TGF-β. To this purpose, staining for IL-10 and TGF-β together with newly identified markers specifically expressed by Treg cells (Table 7.1-1), will clarify the cellular source of IL-10 and TGF-β . Understanding whether the expression of these molecules is modulated by *M. vaccae* and LPS and their relationship with the Th1/Th2 effector cytokines, may be extremely helpful in understanding the immunoregulatory action of bacteria.

The studies presented in this thesis adds further controversy to the debate regarding which immunological mechanism is responsible for the 'hygiene hypothesis'. There is a dispute between those who sustain 'immune deviation' (i.e. the lack of shift Th2 to Th1) as the prevalent mechanism resulting in the allergy epidemic (Romagnani 2004), while others believe that 'immune regulation' (i.e. the lack of activation of Treg cells) is the prevalent pathogenic mechanism (Rook *et al.* 2003). A better understanding of this question is important not only from a theoretical point of view, but also because of its therapeutic implications.

Surface molecules	Comments
Foxp3	The forkhead transcription factor Foxp3 is specifically expressed in mouse $CD4^+CD25^+$ T cells and is required for their development and function, and appears to be the most specific molecular marker available to date. However, Foxp3 expression in humans may not be specific for $CD4^+CD25^+$ cells
GITR	Nonactivated $CD4^+CD25^+$ T cells constitutively express GITR, whose engagement was presumed to abrogate regulatory T cell-mediated suppression
CTLA-4	CTLA-4 regulates $CD4^+CD25^+$ T cell function by two distinct mechanisms, one during functional development of $CD4^+CD25^+$ T cells and the other during the effector phase, when the CTLA-4 signaling pathway is required for suppression
4-1BB	The 4-1BB costimulator receptor is able to induce the proliferation of the $CD4^+CD25^+$ T cells both *in intro* and *in vivo*. The 4-1BB-expanded $CD4^+CD25^+$ T cells are functional, as they remain suppressive to other T cells in coculture
LAG-3	LAG-3 is selectively expressed on $CD4^+CD25^+$ T cells, which modulates both the *in vitro* and *in vivo* suppressive function of $CD4^+CD25^+$ T cells, and ectopic expression of LAG-3 is sufficient to confer regulatory activity
Neuropilin-1	Neuropilin-1 is constitutively expressed on the surface of $CD4^+CD25^+$ T cells independently of their activation status, and the expression of neuropilin-1 correlates with *in vitro* suppressor function of $CD4^+CD25^+$ T cells

GITR, glucocorticoid-induced tumor necrosis factor receptor; CTLA-4, cytotoxic lymphocyte associated antigen-4.

TABLE 7.1-1: *Surface molecules expressed on Tregs cells (From (Shi et al. 2005))*

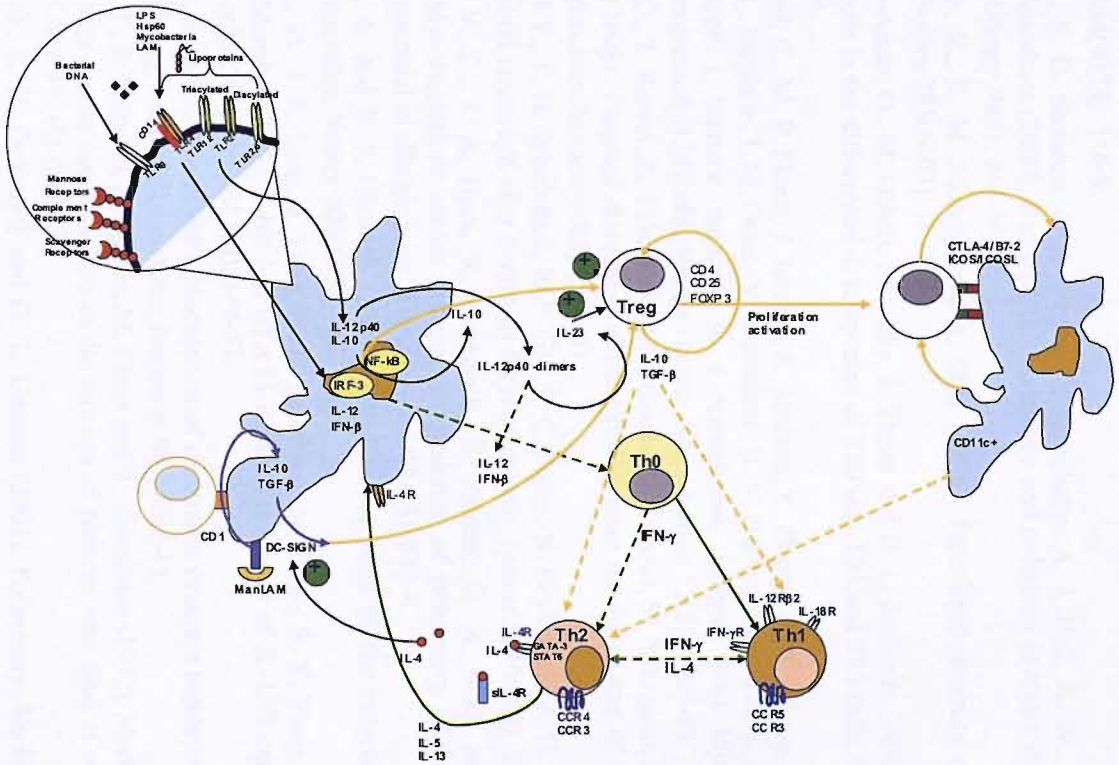


Figure 7.1-1 *Model summarising the cellular and molecular mechanisms involved in the immunomodulatory action of M.vaccae, LPS and sIL4R.*
Mycobacteria can bind to multiple receptors and the immunological consequences of the interactions between APC and mycobacteria depend on the type of receptor involved. Interaction with TLR4 leads to NF-kB and IRF-3 activation leading to IL-12, and IFN-β and g production. IFN-γ stimulates Th0 cells to produce more IFN-γ and differentiate into effector Th1 cells (expressing Th1 markers and receptors for IL-12 and IFN-γ) that, in turn can down-regulate Th2 response.
On the contrary, stimulation of TLR2 leads to formation of IL-12p40 homodimers that antagonise IL-12 and stimulate the synthesis of IL-23. IL-23 induces the upregulation of pro-inflammatory cytokine IL-17 and the up-regulation of IL-10 and possibly proliferation of regulatory T cells (expressing CD4, CD25, FOXP3). Tregs can down-regulate both Th1 and Th2 response to antigens and induce maturation and proliferation of DC (CD11c), through contact with specific receptors (CTLA-4/B7.2; ICOS/ICOSL) which have an inhibitory role on Th2 cytokine generation upon allergen stimulation. In the presence of mannosilated Eparabinimannans (ManLAM) a c-lectine type receptor, DC-SIGN is activated, which stimulates the synthesis of the regulatory cytokines IL-10 and TGF-β. The expression of DC-SIGN seems up-regulated by the Th2 cytokine IL-4. In the presence of a Th2 inflammation, the main cytokine that drives Th2 proliferation and differentiation is IL-4. This model illustrates that in the presence of sIL-4R, IL-4 is bound by sIL-4R, preventing the activation of the membrane-bound IL-4R. This will down-regulate Th2 cytokine generation and remove the cross-regulatory activity of IL-4 on Th1 cells.
Overall, by potentiating Treg and Th1 activity it is theoretically possible to control the exaggerated Th2 activation characteristic of allergic diseases. (YELLOW LINES: effects of regulatory cytokines; GREEN LINES: effects of cross-regulation Th1/Th2; SOLID LINES: stimulation; DASHED LINES: inhibition)

REFERENCES

(1999). Immunotherapy with Mycobacterium vaccae in patients with newly diagnosed pulmonary tuberculosis: a randomised controlled trial. Durban Immunotherapy Trial Group. *Lancet* 354(9173): 116-9.

Aaby, P., S. O. Shaheen, C. B. Heyes, A. Goudiaby, A. J. Hall, A. W. Shiell, H. Jensen and A. Marchant (2000). Early BCG vaccination and reduction in atopy in Guinea-Bissau. *Clin Exp Allergy* 30(5): 644-50.

Abbas, A. K., K. M. Murphy and A. Sher (1996). Functional diversity of helper T lymphocytes. *Nature* 383(6603): 787-93.

Abehsira-Amar, O., M. Gibert, M. Joliy, J. Theze and D. L. Jankovic (1992). IL-4 plays a dominant role in the differential development of Th0 into Th1 and Th2 cells. *J Immunol* 148(12): 3820-9.

Abou-Zeid, C., M. P. Gares, J. Inwald, R. Janssen, Y. Zhang, D. B. Young, C. Hetzel, J. R. Lamb, S. L. Baldwin, I. M. Orme, V. Yeremeev, B. V. Nikonenko and A. S. Apt (1997). Induction of a type 1 immune response to a recombinant antigen from Mycobacterium tuberculosis expressed in Mycobacterium vaccae. *Infect Immun* 65(5): 1856-62.

Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi and S. Akira (1998). Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9(1): 143-50.

Adams, J. F., E. H. Scholvinck, R. P. Gie, P. C. Potter, N. Beyers and A. D. Beyers (1999). Decline in total serum IgE after treatment for tuberculosis. *Lancet* 353(9169): 2030-3.

Adams, V. C., J. R. Hunt, R. Martinelli, R. Palmer, G. A. Rook and L. R. Brunet (2004). Mycobacterium vaccae induces a population of pulmonary CD11c+ cells with regulatory potential in allergic mice. *Eur J Immunol* 34(3): 631-8.

Aderem, A. and R. J. Ulevitch (2000). Toll-like receptors in the induction of the innate immune response. *Nature* 406(6797): 782-7.

Afkarian, M., J. R. Sedy, J. Yang, N. G. Jacobson, N. Cereb, S. Y. Yang, T. L. Murphy and K. M. Murphy (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 3(6): 549-57.

Agarwal, S. and A. Rao (1998). Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* 9(6): 765-75.

Aikawa, T., S. Shimura, H. Sasaki, M. Ebina and T. Takishima (1992). Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 101(4): 916-921.

Akbari, O., R. H. DeKruyff and D. T. Umetsu (2001). Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2(8): 725-31.

Akbari, O., G. J. Freeman, E. H. Meyer, E. A. Greenfield, T. T. Chang, A. H. Sharpe, G. Berry, R. H. DeKruyff and D. T. Umetsu (2002). Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* 8(9): 1024-32.

Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Crameri, S. Thunberg, G. Deniz, R. Valenta, H. Fiebig, C. Kegel, R. Disch, C. B. Schmidt-Weber, K. Blaser and C. A. Akdis (2004). Immune Responses in Healthy and Allergic Individuals Are Characterized by a Fine Balance between Allergen-specific T Regulatory 1 and T Helper 2 Cells. *J Exp Med* 199(11): 1567-75.

Akiba, H., Y. Miyahira, M. Atsuta, K. Takeda, C. Nohara, T. Futagawa, H. Matsuda, T. Aoki, H. Yagita and K. Okumura (2000). Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. *J Exp Med* 191(2): 375-80.

Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski and A. Zychlinsky (1999). Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* 285(5428): 736-9.

Alm, J. S., G. Lilja, G. Pershagen and A. Scheynius (1997). Early BCG vaccination and development of atopy. *Lancet* 350(9075): 400-3.

Alm, J. S., C. B. Sanjeevi, E. N. Miller, P. Dabadghao, G. Lilja, G. Pershagen, J. M. Blackwell and A. Scheynius (2002). Atopy in children in relation to BCG vaccination and genetic polymorphisms at SLC11A1 (formerly NRAMP1) and D2S1471. *Genes Immun* 3(2): 71-7.

Altare, F., A. Durandy, D. Lammas, J. F. Emile, S. Lamhamedi, F. Le Deist, P. Drysdale, E. Jouanguy, R. Doffinger, F. Bernaudin, O. Jeppsson, J. A. Gollob, E. Meinl, A. W. Segal, A. Fischer, D. Kumararatne and J. L. Casanova (1998). Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* 280(5368): 1432-5.

Anderson, G. P. and A. J. Coyle (1994). TH2 and 'TH2-like' cells in allergy and asthma: pharmacological perspectives. *Trends Pharmacol Sci* 15(9): 324-32.

Arkwright, P. D., J. M. Chase, S. Babbage, V. Pravica, T. J. David and I. V. Hutchinson (2001). Atopic dermatitis is associated with a low-producer transforming growth factor beta(1) cytokine genotype. *J Allergy Clin Immunol* 108(2): 281-4.

Arkwright, P. D. and T. J. David (2001). Intradermal administration of a killed Mycobacterium vaccae suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderate-to-severe disease. *J Allergy Clin Immunol* 107(3): 531-4.

Arkwright, P. D. and T. J. David (2003). Effect of Mycobacterium vaccae on atopic dermatitis in children of different ages. *Br J Dermatol* 149(5): 1029-34.

Arkwright, P. D., C. Fujisawa, A. Tanaka and H. Matsuda (2005). Mycobacterium vaccae reduces scratching behavior but not the rash in NC mice with eczema: a randomized, blinded, placebo-controlled trial. *J Invest Dermatol* 124(1): 140-3.

Ashkar, S., G. F. Weber, V. Panoutsakopoulou, M. E. Sanchirico, M. Jansson, S. Zawaideh, S. R. Rittling, D. T. Denhardt, M. J. Glimcher and H. Cantor (2000). Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* 287(5454): 860-4.

Askenase, P. W. (2000). Gee whiz: CpG DNA allergy therapy! *J Allergy Clin Immunol* 106(1 Pt 1): 37-40.

Assersohn, L., B. E. Souberbielle, M. E. O'Brien, C. D. Archer, R. Mendes, R. Bass, K. V. Bromelow, R. D. Palmer, E. Bouilloux, D. A. Kennard and I. E. Smith (2002). A randomized pilot study of SRL172 (Mycobacterium vaccae) in patients with small cell lung cancer (SCLC) treated with chemotherapy. *Clin Oncol (R Coll Radiol)* 14(1): 23-7.

Atamas, S. P., J. Choi, V. V. Yurovsky and B. White (1996). An alternative splice variant of human IL-4, IL-4 delta 2, inhibits IL-4-stimulated T cell proliferation. *J Immunol* 156(2): 435-41.

Aversa, G., J. Punnonen, B. G. Cocks, R. de Waal Malefyt, F. Vega, Jr., S. M. Zurawski, G. Zurawski and J. E. de Vries (1993). An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: support for a common component shared by IL-4 and IL-13 receptors. *J Exp Med* 178(6): 2213-8.

Awomoyi, A. A., A. Marchant, J. M. Howson, K. P. McAdam, J. M. Blackwell and M. J. Newport (2002). Interleukin-10, polymorphism in SLC11A1 (formerly NRAMP1), and susceptibility to tuberculosis. *J Infect Dis* 186(12): 1808-14.

Azzawi, M., B. Bradley, P. K. Jeffery, A. J. Frew, A. J. Wardlaw, G. Knowles, B. Assoufi, J. V. Collins, S. Durham and A. B. Kay (1990). Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am Rev Respir Dis* 142(6 Pt 1): 1407-13.

Bach, E. A., M. Aguet and R. D. Schreiber (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 15: 563-91.

Bach, J. F. (2002). The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 347(12): 911-20.

Bachert, C., P. Prohaska and U. Pipkorn (1990). IgE-positive mast cells on the human nasal mucosal surface in response to allergen exposure. *Rhinology* 28(3): 149-58.

Baldini, M., I. C. Lohman, M. Halonen, R. P. Erickson, P. G. Holt and F. D. Martinez (1999). A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am J Respir Cell Mol Biol* 20(5): 976-83.

Baldrick, P., D. Richardson, G. Elliott and A. W. Wheeler (2002). Safety evaluation of monophosphoryl lipid A (MPL): an immunostimulatory adjuvant. *Regul Toxicol Pharmacol* 35(3): 398-413.

Baldrick, P., D. Richardson and A. W. Wheeler (2001). Safety evaluation of a glutaraldehyde modified tyrosine adsorbed housedust mite extract containing monophosphoryl lipid A (MPL) adjuvant: a new allergy vaccine for dust mite allergy. *Vaccine* 20(5-6): 737-43.

Baldrick, P., D. Richardson, A. W. Wheeler and S. R. Woroniecki (2004). Safety evaluation of a new allergy vaccine containing the adjuvant monophosphoryl lipid A (MPL) for the treatment of grass pollen allergy. *J Appl Toxicol* 24(4): 261-8.

Baldridge, J. R. and R. T. Crane (1999). Monophosphoryl lipid A (MPL) formulations for the next generation of vaccines. *Methods* 19(1): 103-7.

Ball, T. M., J. A. Castro-Rodriguez, K. A. Griffith, C. J. Holberg, F. D. Martinez and A. L. Wright (2000). Siblings, Day-Care Attendance, and the Risk of Asthma and Wheezing during Childhood. *N Engl J Med* 343(8): 538-543.

Barclay, R., V. Furst and I. Smith (1992). A simple and rapid method for the detection and identification of mycobacteria using mycobactin. *J Med Microbiol* 37(4): 286-90.

Barnes, P. J., K. F. Chung and C. P. Page (1998). Inflammatory mediators of asthma: an update. *Pharmacol Rev* 50(4): 515-96.

Beasley, R., W. R. Roche, J. A. Roberts and S. T. Holgate (1989). Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 139(3): 806-17.

Beasley, R., P. Shirtcliffe, J. L. Harper, S. Holt and G. Le Gros (2002). Mycobacterium-based vaccines for the prevention of allergic disease: a progress report. *Clin Exp Allergy* 32(8): 1128-30.

Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach and D. L. Sacks (2002). CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature* 420(6915): 502-7.

Bellinghausen, I., B. Klostermann, J. Knop and J. Saloga (2003). Human CD4+CD25+ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. *J Allergy Clin Immunol* 111(4): 862-8.

Benson, M., I. L. Strannegard, G. Wennergren and O. Strannegard (2000). Increase of the soluble IL-4 receptor (IL-4sR) and positive correlation between IL-4sR and IgE in nasal fluids from school children with allergic rhinitis. *Allergy Asthma Proc* 21(2): 89-95.

Bentley, A. M., Q. Meng, D. S. Robinson, Q. Hamid, A. B. Kay and S. R. Durham (1993). Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am J Respir Cell Mol Biol* 8(1): 35-42.

Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation. *Nature* 321(6067): 209-13.

Bjorksten, B. (1994). Risk factors in early childhood for the development of atopic diseases. *Allergy* 49(6): 400-7.

Blackwell, J. M., G. F. Black, C. Sharples, S. S. Soo, C. S. Peacock and N. Miller (1999). Roles of Nramp1, HLA, and a gene(s) in allelic association with IL-4, in determining T helper subset differentiation. *Microbes Infect* 1(1): 95-102.

Blum, H., M. Wolf, K. Enssle, M. Rollinghoff and A. Gessner (1996). Two distinct stimulus-dependent pathways lead to production of soluble murine interleukin-4 receptor. *J Immunol* 157(5): 1846-53.

Bochner, B. S., B. J. Undem and L. M. Lichtenstein (1994). Immunological Aspects of Allergic Asthma. *Annu. Rev. Immunol.* 12(1): 295-335.

Bochud, P.-Y., T. R. Hawn and A. Aderem (2003). Cutting Edge: A Toll-Like Receptor 2 Polymorphism That Is Associated with Lepromatous Leprosy Is Unable to Mediate Mycobacterial Signaling. *J Immunol* 170(7): 3451-3454.

Boehm, U., T. Klamp, M. Groot and J. C. Howard (1997). Cellular responses to interferon-gamma. *Annu Rev Immunol* 15: 749-95.

Boenickse, R. and E. Juhasz (1964). [Description of the New Species Mycobacterium Vaccae N. Sp.]. *Zentralbl Bakteriol [Orig]* 192: 133-5.

Boguniewicz, M., R. J. Martin, D. Martin, U. Gibson, A. Celniker, M. Williams and D. Y. Leung (1995). The effects of nebulized recombinant interferon-gamma in asthmatic airways. *J Allergy Clin Immunol* 95(1 Pt 1): 133-5.

Boguniewicz, M., L. C. Schneider, H. Milgrom, D. Newell, N. Kelly, P. Tam, A. E. Izu, H. S. Jaffe, L. R. Bucalo and D. Y. Leung (1993). Treatment of steroid-dependent asthma with recombinant interferon-gamma. *Clin Exp Allergy* 23(9): 785-90.

Bolte, G., S. Krauss-Etschmann, N. Konstantopoulos, W. Bischof, B. Fahlbusch, D. J. Schendel, H. E. Wichmann and J. Heinrich (2002). Different effects of endotoxin versus mite and cat allergen exposure on T-cell differentiation in infants. *J Allergy Clin Immunol* 110(4): 634-40.

Bonicke, R. (1962). [Present status of methods for the routine differentiation of various species of Mycobacterium.]. *Ann Soc Belg Med Trop* 42: 403-39.

Borish, L. C., H. S. Nelson, J. Corren, G. Bensch, W. W. Busse, J. B. Whitmore and J. M. Agosti (2001). Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. *J Allergy Clin Immunol* 107(6): 963-70.

Borish, L. C., H. S. Nelson, M. J. Lanz, L. Claussen, J. B. Whitmore, J. M. Agosti and L. Garrison (1999). Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. *Am J Respir Crit Care Med* 160(6): 1816-23.

Bottcher, M. F., B. Bjorksten, S. Gustafson, T. Voor and M. C. Jenmalm (2003). Endotoxin levels in Estonian and Swedish house dust and atopy in infancy. *Clin Exp Allergy* 33(3): 295-300.

Bousquet, J., P. Chanez, J. Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard and et al. (1990). Eosinophilic inflammation in asthma. *N Engl J Med* 323(15): 1033-9.

Bousquet, J., P. K. Jeffery, W. W. BUSSE, M. JOHNSON and A. M. VIGNOLA (2000). Asthma. From Bronchoconstriction to Airways Inflammation and Remodeling. *Am. J. Respir. Crit. Care Med.* 161(5): 1720-1745.

Boussiotis, V. A., E. Y. Tsai, E. J. Yunis, S. Thim, J. C. Delgado, C. C. Dascher, A. Berezovskaya, D. Rousset, J.-M. Reynes and A. E. Goldfeld (2000). IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. *J. Clin. Invest.* 105(9): 1317-1325.

Bradding, P., I. H. Feather, S. Wilson, P. G. Bardin, C. H. Heusser, S. T. Holgate and P. H. Howarth (1993). Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol* 151(7): 3853-65.

Bradley, B. L., M. Azzawi, M. Jacobson, B. Assoufi, J. V. Collins, A. M. Irani, L. B. Schwartz, S. R. Durham, P. K. Jeffery and A. B. Kay (1991). Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 88(4): 661-74.

Braun-Fahrlander, C. (2003). Environmental exposure to endotoxin and other microbial products and the decreased risk of childhood atopy: evaluating developments since April 2002. *Curr Opin Allergy Clin Immunol* 3(5): 325-9.

Braun-Fahrlander, C., M. Gassner, L. Grize, U. Neu, F. H. Sennhauser, H. S. Varonier, J. C. Vuille and B. Wuthrich (1999). Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. *Clin Exp Allergy* 29(1): 28-34.

Braun-Fahrlander, C., J. Riedler, U. Herz, W. Eder, M. Waser, L. Grize, S. Maisch, D. Carr, F. Gerlach, A. Bufe, R. P. Lauener, R. Schierl, H. Renz, D. Nowak and E. von Mutius (2002). Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 347(12): 869-77.

Breivik, T. and G. A. Rook (2000). Prevaccination with SRL172 (heat-killed Mycobacterium vaccae) inhibits experimental periodontal disease in Wistar rats. *Clin Exp Immunol* 120(3): 463-7.

Breivik, T. and G. A. Rook (2003). Oral treatment with SRP299 (killed Mycobacterium vaccae) inhibits experimental periodontal disease in Wistar rats. *J Clin Periodontol* 30(11): 931-6.

Brelvik, T. and G. A. Rook (2002). Treatment with SRL172 (heat-killed Mycobacterium vaccae) inhibits progression of established experimental periodontal disease in Wistar rats. *J Periodontal Res* 37(3): 210-4.

Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski and R. L. Modlin (1999). Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* 285(5428): 732-6.

Briken, V., S. A. Porcelli, G. S. Besra and L. Kremer (2004). Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. *Mol Microbiol* 53(2): 391-403.

Broide, D. and E. Raz (1999). DNA-Based immunization for asthma. *Int Arch Allergy Immunol* 118(2-4): 453-6.

Broide, D., J. Schwarze, H. Tighe, T. Gifford, M. D. Nguyen, S. Malek, J. Van Uden, E. Martin-Orozco, E. W. Gelfand and E. Raz (1998). Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 161(12): 7054-62.

Broide, D. and P. Sriramarao (2001). Eosinophil trafficking to sites of allergic inflammation. *Immunol Rev* 179: 163-72.

Broide, D. H., M. M. Paine and G. S. Firestein (1992). Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J Clin Invest* 90(4): 1414-24.

Brusselle, G., J. Kips, G. Joos, H. Bluethmann and R. Pauwels (1995). Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am. J. Respir. Cell Mol. Biol.* 12(3): 254-259.

Bryan, S. A., B. J. O'Connor, S. Matti, M. J. Leckie, V. Kanabar, J. Khan, S. J. Warrington, L. Renzetti, A. Rames, J. A. Bock, M. J. Boyce, T. T. Hansel, S. T. Holgate and P. J. Barnes (2000). Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356(9248): 2149-53.

Bulut, Y., E. Faure, L. Thomas, O. Equils and M. Arditi (2001). Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *J Immunol* 167(2): 987-94.

Burney, P. G., S. Chinn and R. J. Rona (1990). Has the prevalence of asthma increased in children? Evidence from the national study of health and growth 1973-86. *BMJ* 300(6735): 1306-10.

Busse, W. W. and R. F. Lemanske (2001). Asthma. *N Engl J Med* 344(5): 350-362.

Buttner, C., A. Lun, T. Splettstoesser, G. Kunkel and H. Renz (2003). Monoclonal anti-interleukin-5 treatment suppresses eosinophil but not T-cell functions. *Eur Respir J* 21(5): 799-803.

Calhoun, W. J., N. N. Jarjour, G. J. Gleich, C. A. Stevens and W. W. Busse (1993). Increased airway inflammation with segmental versus aerosol antigen challenge. *Am Rev Respir Dis* 147(6 Pt 1): 1465-71.

Cambi, A. and C. G. Figdor (2005). Levels of complexity in pathogen recognition by C-type lectins. *Curr Opin Immunol* 17(4): 345-51.

Camporota, L., A. Corkhill, H. Long, J. Lordan, L. Stanciu, N. Tuckwell, A. Cross, J. L. Stanford, G. A. Rook, S. T. Holgate and R. Djukanovic (2003). The effects of Mycobacterium vaccae on allergen-induced airway responses in atopic asthma. *Eur Respir J* 21(2): 287-93.

Car, B. D., V. M. Eng, J. M. Lipman and T. D. Anderson (1999). The toxicology of interleukin-12: a review. *Toxicol Pathol* 27(1): 58-63.

Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury and J. Demengeot (2003). Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med* 197(4): 403-11.

Caramori, G., S. Lim, K. Ito, K. Tomita, T. Oates, E. Jazrawi, K. F. Chung, P. J. Barnes and I. M. Adcock (2001). Expression of GATA family of transcription factors in T-cells, monocytes and bronchial biopsies. *Eur Respir J* 18(3): 466-73.

Carballido, J. M., G. Aversa, D. Schols, J. Punnonen and J. E. de Vries (1995). Inhibition of human IgE synthesis in vitro and in SCID-hu mice by an interleukin-4 receptor antagonist. *Int Arch Allergy Immunol* 107(1-3): 304-7.

Carballido, J. M., D. Schols, R. Namikawa, S. Zurawski, G. Zurawski, M. G. Roncarolo and J. E. de Vries (1995). IL-4 induces human B cell maturation and IgE synthesis in SCID-hu mice. Inhibition of ongoing IgE production by in vivo treatment with an IL-4/IL-13 receptor antagonist. *J Immunol* 155(9): 4162-70.

Casale, T. B., D. Wood, H. B. Richerson, S. Trapp, W. J. Metzger, D. Zavala and G. W. Hunninghake (1987). Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are associated with methacholine bronchial hyperresponsiveness. *J Clin Invest* 79(4): 1197-203.

Celedon, J. C., A. A. Litonjua, L. Ryan, T. Platts-Mills, S. T. Weiss and D. R. Gold (2002). Exposure to cat allergen, maternal history of asthma, and wheezing in first 5 years of life. *Lancet* 360(9335): 781-2.

Chai, H., R. S. Farr, L. A. Froehlich, D. A. Mathison, J. A. McLean, R. R. Rosenthal, A. L. Sheffer, S. L. Spector and R. G. Townley (1975). Standardization of bronchial inhalation challenge procedures. *J Allergy Clin Immunol* 56(4): 323-7.

Chamaillard, M., M. Hashimoto, Y. Horie, J. Masumoto, S. Qiu, L. Saab, Y. Ogura, A. Kawasaki, K. Fukase, S. Kusumoto, M. A. Valvano, S. J. Foster, T. W. Mak, G. Nunez and N. Inohara (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 4(7): 702-7.

Chan, S. C., M. A. Brown, T. M. Willcox, S. H. Li, S. R. Stevens, D. Tara and J. M. Hanifin (1996). Abnormal IL-4 gene expression by atopic dermatitis T lymphocytes is reflected in altered nuclear protein interactions with IL-4 transcriptional regulatory element. *J Invest Dermatol* 106(5): 1131-6.

Chapoval, A. I., J. Ni, J. S. Lau, R. A. Wilcox, D. B. Flies, D. Liu, H. Dong, G. L. Sica, G. Zhu, K. Tamada and L. Chen (2001). B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat Immunol* 2(3): 269-74.

Chen, Q., N. Ghilardi, H. Wang, T. Baker, M. H. Xie, A. Gurney, I. S. Grewal and F. J. de Sauvage (2000). Development of Th1-type immune responses requires the type I cytokine receptor TCCR. *Nature* 407(6806): 916-20.

Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady and S. M. Wahl (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198(12): 1875-86.

Chen, Z. W. (2005). Immune regulation of gammadelta T cell responses in mycobacterial infections. *Clin Immunol* 116(3): 202-7.

Chilton, P. M. and R. Fernandez-Botran (1997). Regulation of the expression of the soluble and membrane forms of the murine IL-4 receptor. *Cell Immunol* 180(2): 104-15.

Cho, H. J., M.-D. Nguyen, Y. Chiu and E. Raz (2000). T helper independent activation of antigen specific cytotoxic lymphocytes and exogenous antigen presentation by immunostimulatory sequence DNA-based vaccines (abstract). *J Allergy Clin Immunol* 105: S163.

Christodoulopoulos, P., L. Cameron, Y. Nakamura, C. Lemiere, S. Muro, M. Dugas, L. P. Boulet, M. Laviolette, R. Olivenstein and Q. Hamid (2001). TH2 cytokine-associated transcription factors in atopic and nonatopic asthma: evidence for differential signal transducer and activator of transcription 6 expression. *J Allergy Clin Immunol* 107(4): 586-91.

Chua, A. O., R. Chizzonite, B. B. Desai, T. P. Truitt, P. Nunes, L. J. Minetti, R. R. Warrier, D. H. Presky, J. F. Levine, M. K. Gately and et al. (1994). Expression cloning of a human IL-12 receptor component. A new member of the cytokine receptor superfamily with strong homology to gp130. *J Immunol* 153(1): 128-36.

Chung, K. F. and P. J. Barnes (1999). Cytokines in asthma. *Thorax* 54(9): 825-57.

Clutterbuck, E. J., E. M. Hirst and C. J. Sanderson (1989). Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GMCSF. *Blood* 73(6): 1504-12.

Cockcroft, D. W. and K. Y. Murdock (1987). Comparative effects of inhaled salbutamol, sodium cromoglycate, and beclomethasone dipropionate on allergen-induced early asthmatic responses, late asthmatic responses, and increased bronchial responsiveness to histamine. *J Allergy Clin Immunol* 79(5): 734-40.

Cockcroft, D. W., R. E. Ruffin, J. Dolovich and F. E. Hargreave (1977). Allergen-induced increase in non-allergic bronchial reactivity. *Clin Allergy* 7(6): 503-13.

Cohn, L., C. Herrick, N. Niu, R. Homer and K. Bottomly (2001). IL-4 promotes airway eosinophilia by suppressing IFN-gamma production: defining a novel role for IFN-gamma in the regulation of allergic airway inflammation. *J Immunol* 166(4): 2760-7.

Cohn, L., R. J. Homer, H. MacLeod, M. Mohrs, F. Brombacher and K. Bottomly (1999). Th2-Induced Airway Mucus Production Is Dependent on IL-4R{alpha}, But Not on Eosinophils. *J Immunol* 162(10): 6178-6183.

Cohn, L., R. J. Homer, A. Marinov, J. Rankin and K. Bottomly (1997). Induction of Airway Mucus Production By T Helper 2 (Th2) Cells: A Critical Role For Interleukin 4 In Cell Recruitment But Not Mucus Production. *J. Exp. Med.* 186(10): 1737-1747.

Constant, S. L. and K. Bottomly (1997). Induction of Th1 and Th2 CD4+ T cell responses: The Alternative Approaches. *Annu. Rev. Immunol.* 15(1): 297-322.

Cook, D. N., J. W. Hollingsworth, Jr. and D. A. Schwartz (2003). Toll-like receptors and the genetics of innate immunity. *Curr Opin Allergy Clin Immunol* 3(6): 523-9.

Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell and I. M. Orme (1993). Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med* 178(6): 2243-7.

Cooper, A. M., J. Magram, J. Ferrante and I. M. Orme (1997). Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *J Exp Med* 186(1): 39-45.

Corlan, E., C. Marica, C. Macavei, J. L. Stanford and C. A. Stanford (1997). Immunotherapy with Mycobacterium vaccae in the treatment of tuberculosis in Romania. 1. Newly-diagnosed pulmonary disease. *Respir Med* 91(1): 13-9.

Corlan, E., C. Marica, C. Macavei, J. L. Stanford and C. A. Stanford (1997). Immunotherapy with Mycobacterium vaccae in the treatment of tuberculosis in Romania. 2. Chronic or relapsed disease. *Respir Med* 91(1): 21-9.

Corrigan, C. J. and A. B. Kay (1990). CD4 T-lymphocyte activation in acute severe asthma. Relationship to disease severity and atopic status. *Am Rev Respir Dis* 141(4 Pt 1): 970-7.

Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, J. P. Wiener-Kronish and R. M. Locksley (1996). Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J Exp Med* 183(1): 109-17.

Coyle, A. J., G. Le Gros, C. Bertrand, S. Tsuyuki, C. H. Heusser, M. Kopf and G. P. Anderson (1995). Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol* 13(1): 54-9.

Crispe, I. N. (1994). Fatal interactions: Fas-induced apoptosis of mature T cells. *Immunity* 1(5): 347-9.

Dabbagh, K., K. Takeyama, H. M. Lee, I. F. Ueki, J. A. Lausier and J. A. Nadel (1999). IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *J Immunol* 162(10): 6233-7.

Daher, S., L. M. Santos, D. Sole, M. G. De Lima, C. K. Naspitz and C. C. Musatti (1995). Interleukin-4 and soluble CD23 serum levels in asthmatic atopic children. *J Investig Allergol Clin Immunol* 5(5): 251-4.

Dahl, K. E., H. Shiratsuchi, B. D. Hamilton, J. J. Ellner and Z. Toossi (1996). Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of Mycobacterium tuberculosis. *Infect Immun* 64(2): 399-405.

D'Ambrosio, D., M. Cippitelli, M. G. Cocciolo, D. Mazzeo, P. D. Lucia, R. Lang, F. Sinigaglia and P. Panina-Bordignon (1998). Inhibition of IL-12 Production by 1,25-Dihydroxyvitamin D3 . Involvement of NF-kappa B Downregulation in Transcriptional Repression of the p40 Gene. *J. Clin. Invest.* 101(1): 252-262.

Das, J., C. H. Chen, L. Yang, L. Cohn, P. Ray and A. Ray (2001). A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat Immunol* 2(1): 45-50.

de, B. and P. Garner (2001). Mycobacterium vaccae immunotherapy for treating tuberculosis. *Cochrane Database Syst Rev*(1): CD001166.

de Bruyn, G. and P. Garner (2000). Mycobacterium vaccae immunotherapy for treating tuberculosis. *Cochrane Database Syst Rev*(2): CD001166.

de Bruyn, G. and P. Garner (2003). Mycobacterium vaccae immunotherapy for treating tuberculosis. *Cochrane Database Syst Rev*(1): CD001166.

de Jong, R., F. Altare, I. A. Haagen, D. G. Elferink, T. Boer, P. J. van Breda Vriesman, P. J. Kabel, J. M. Draaisma, J. T. van Dissel, F. P. Kroon, J. L. Casanova and T. H. Ottenhoff (1998). Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science* 280(5368): 1435-8.

De Monchy, J. G., H. F. Kauffman, P. Venge, G. H. Koeter, H. M. Jansen, H. J. Sluiter and K. De Vries (1985). Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am Rev Respir Dis* 131(3): 373-6.

De Sanctis, G. T., A. Itoh, F. H. Green, S. Qin, T. Kimura, J. K. Grobholz, T. R. Martin, T. Maki and J. M. Drazen (1997). T-lymphocytes regulate genetically determined airway hyperresponsiveness in mice. *Nat Med* 3(4): 460-2.

de Vries, J. E. (1998). The role of IL-13 and its receptor in allergy and inflammatory responses. *J Allergy Clin Immunol* 102(2): 165-9.

Del Prete, G. F., M. De Carli, M. M. D'Elios, P. Maestrelli, M. Ricci, L. Fabbri and S. Romagnani (1993). Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol* 23(7): 1445-9.

Dent, A. L., A. L. Shaffer, X. Yu, D. Allman and L. M. Staudt (1997). Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* 276(5312): 589-92.

Dent, G. (1999). Sch-55700. *Curr Opin Cardiovascular, Pulmonary Renal Invest Drugs* 1: 535-540.

Devos, R., Y. Guisez, S. Cornelis, A. Verhee, J. Van der Heyden, M. Manneberg, H. W. Lahm, W. Fiers, J. Tavernier and G. Plaetinck (1993). Recombinant soluble human interleukin-5 (hIL-5) receptor molecules. Cross-linking and stoichiometry of binding to IL-5. *J Biol Chem* 268(9): 6581-7.

Devos, R., Y. Guisez, G. Plaetinck, S. Cornelis, J. Tavernier, J. van der Heyden, L. H. Foley and J. E. Scheffler (1994). Covalent modification of the interleukin-5 receptor by isothiazolones leads to inhibition of the binding of interleukin-5. *Eur J Biochem* 225(2): 635-40.

Di Luzio, N. R. and T. J. Friedmann (1973). Bacterial endotoxins in the environment. *Nature* 244(5410): 49-51.

Djukanovic, R., W. R. Roche, J. W. Wilson, C. R. Beasley, O. P. Twentyman, R. H. Howarth and S. T. Holgate (1990). Mucosal inflammation in asthma. *Am Rev Respir Dis* 142(2): 434-57.

Dlugovitzky, D., O. Bottasso, J. C. Dominino, E. Valentini, R. Hartopp, M. Singh, C. Stanford and J. Stanford (1999). Clinical and serological studies of tuberculosis patients in Argentina receiving immunotherapy with Mycobacterium vaccae (SRL 172). *Respir Med* 93(8): 557-62.

Dobrovolskaia, M. A., A. E. Medvedev, K. E. Thomas, N. Cuesta, V. Toshchakov, T. Ren, M. J. Cody, S. M. Michalek, N. R. Rice and S. N. Vogel (2003). Induction of in vitro reprogramming by Toll-like receptor (TLR)2 and TLR4 agonists in murine macrophages: effects of TLR "homotolerance" versus "heterotolerance" on NF-kappa B signaling pathway components. *J Immunol* 170(1): 508-19.

Donaldson, D. D., M. J. Whitters, L. J. Fitz, T. Y. Neben, H. Finnerty, S. L. Henderson, R. M. O'Hara, Jr., D. R. Beier, K. J. Turner, C. R. Wood and M. Collins (1998). The Murine IL-13 Receptor α2: Molecular Cloning, Characterization, and Comparison with Murine IL-13 Receptor {alpha}1. *J Immunol* 161(5): 2317-2324.

Drachenberg, K. J., M. Heinzkill, E. Urban and S. R. Woroniecki (2003). Efficacy and tolerability of short-term specific immunotherapy with pollen allergoids adjuvanted by monophosphoryl lipid A (MPL) for children and adolescents. *Allergol Immunopathol (Madr)* 31(5): 270-7.

Drennan, M. B., D. Nicolle, V. J. Quesniaux, M. Jacobs, N. Allie, J. Mpagi, C. Fremond, H. Wagner, C. Kirschning and B. Ryffel (2004). Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. *Am J Pathol* 164(1): 49-57.

Dubois, G. R., R. C. Schweizer, C. Versluis, C. A. Bruijnzeel-Koomen and P. L. Bruijnzeel (1998). Human eosinophils constitutively express a functional interleukin-4 receptor: interleukin-4 - induced priming of chemotactic responses and induction of PI-3 kinase activity. *Am J Respir Cell Mol Biol* 19(4): 691-9.

Duez, C., A. Tomkinson, L. D. Shultz, D. L. Bratton and E. W. Gelfand (2001). Fas deficiency delays the resolution of airway hyperresponsiveness after allergen sensitization and challenge. *J Allergy Clin Immunol* 108(4): 547-56.

Dunnill, M. S. (1971). The pathology of asthma. *Ciba Found Study Group* 38: 35-46.

Dunnill, M. S., G. R. Massarella and J. A. Anderson (1969). A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* 24(2): 176-9.

Eaton, J. D., M. J. Perry, S. Nicholson, M. Guckian, N. Russell, M. Whelan and R. S. Kirby (2002). Allogeneic whole-cell vaccine: a phase I/II study in men with hormone-refractory prostate cancer. *BJU Int* 89(1): 19-26.

ECRHS (1996). Variations in the prevalence of respiratory symptoms, self-reported asthma attacks, and use of asthma medication in the European Community Respiratory Health Survey (ECRHS). *Eur Respir J* 9(4): 687-95.

Egan, R. W., D. Athwal, M. W. Bodmer, J. M. Carter, R. W. Chapman, C. C. Chou, M. A. Cox, J. S. Emtage, X. Fernandez, N. Genatt, S. R. Indelicato, C. H. Jenh, W. Kreutner, T. T. Kung, P. J. Mauser, M. Minnicozzi, N. J. Murgolo, S. K. Narula, M. E. Petro, A. Schilling, S. Sehring, D. Stelts, S. Stephens, S. S. Taremi, J. Zurcher and et al. (1999). Effect of Sch 55700, a humanized monoclonal antibody to human interleukin-5, on eosinophilic responses and bronchial hyperreactivity. *Arzneimittelforschung* 49(9): 779-90.

Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick and K. Bottomly (2002). Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 196(12): 1645-51.

Eldridge, M. W. and D. B. Peden (2000). Allergen provocation augments endotoxin-induced nasal inflammation in subjects with atopic asthma. *J Allergy Clin Immunol* 105(3): 475-81.

El-Mezayen, R. E. and T. Matsumoto (2004). In vitro responsiveness to IL-18 in combination with IL-12 or IL-2 by PBMC from patients with bronchial asthma and atopic dermatitis. *Clin Immunol* 111(1): 61-8.

Erb, K. J., J. W. Holloway, A. Sobeck, H. Moll and G. Le Gros (1998). Infection of mice with Mycobacterium bovis-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J Exp Med* 187(4): 561-9.

Ernst, J. D. (1998). Macrophage receptors for Mycobacterium tuberculosis. *Infect Immun* 66(4): 1277-81.

Ernst, P. and Y. Cormier (2000). Relative scarcity of asthma and atopy among rural adolescents raised on a farm. *Am J Respir Crit Care Med* 161(5): 1563-6.

Evans, J. T., C. W. Cluff, D. A. Johnson, M. J. Lacy, D. H. Persing and J. R. Baldridge (2003). Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529. *Expert Rev Vaccines* 2(2): 219-29.

Fahy, J. V., J. Liu, H. Wong and H. A. Boushey (1994). Analysis of cellular and biochemical constituents of induced sputum after allergen challenge: a method for studying allergic airway inflammation. *J Allergy Clin Immunol* 93(6): 1031-9.

Fernandez-Botran, R. (2000). Soluble cytokine receptors: novel immunotherapeutic agents. *Expert Opin Investig Drugs* 9(3): 497-514.

Fernandez-Botran, R., P. M. Chilton, Y. Ma, J. L. Windsor and N. E. Street (1996). Control of the production of soluble interleukin-4 receptors: implications in immunoregulation. *J Leukoc Biol* 59(4): 499-504.

Fernandez-Botran, R., P. M. Chilton, Y. Ma, J. L. Windsor and N. E. Street (1997). The production of soluble interleukin 4 receptors is preferentially regulated by the murine Th2 cell subset. *Cytokine* 9(3): 166-77.

Filipiak, B., J. Heinrich, T. Schafer, J. Ring and H. E. Wichmann (2001). Farming, rural lifestyle and atopy in adults from southern Germany--results from the MONICA/KORA study Augsburg. *Clin Exp Allergy* 31(12): 1829-38.

Finkelman, F., I. Katona, J. Urban, Jr, J. Holmes, J. Ohara, A. Tung, J. Sample and W. Paul (1988). IL-4 is required to generate and sustain in vivo IgE responses. *J Immunol* 141(7): 2335-2341.

Finkelman, F. D., K. B. Madden, S. C. Morris, J. M. Holmes, N. Boiani, I. M. Katona and C. R. Maliszewski (1993). Anti-cytokine antibodies as carrier proteins. Prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. *J Immunol* 151(3): 1235-44.

Finotto, S., G. T. De Sanctis, H. A. Lehr, U. Herz, M. Buerke, M. Schipp, B. Bartsch, R. Atreya, E. Schmitt, P. R. Galle, H. Renz and M. F. Neurath (2001). Treatment of allergic airway inflammation and hyperresponsiveness by antisense-induced local blockade of GATA-3 expression. *J Exp Med* 193(11): 1247-60.

Finotto, S., M. F. Neurath, J. N. Glickman, S. Qin, H. A. Lehr, F. H. Green, K. Ackerman, K. Haley, P. R. Galle, S. J. Szabo, J. M. Drazen, G. T. De Sanctis and L. H. Glimcher (2002).

Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 295(5553): 336-8.

Fitch, P. S., V. Brown, B. C. Schock, M. Ennis and M. D. Shields (2003). Interleukin-4 and interleukin-4 soluble receptor alpha levels in bronchoalveolar lavage from children with asthma. *Ann Allergy Asthma Immunol* 90(4): 429-33.

Fitzgerald, K. A., E. M. Palsson-McDermott, A. G. Bowie, C. A. Jefferies, A. S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M. T. Harte, D. McMurray, D. E. Smith, J. E. Sims, T. A. Bird and L. A. O'Neill (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413(6851): 78-83.

Flo, T. H., O. Halaas, S. Torp, L. Ryan, E. Lien, B. Dybdahl, A. Sundan and T. Espevik (2001). Differential expression of Toll-like receptor 2 in human cells. *J Leukoc Biol* 69(3): 474-81.

Flood-Page, P., A. Menzies-Gow, S. Phipps, S. Ying, A. Wangoo, M. S. Ludwig, N. Barnes, D. Robinson and A. B. Kay (2003). Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 112(7): 1029-36.

Fluckiger, A. C., F. Briere, G. Zurawski, J. M. Bridon and J. Banchereau (1994). IL-13 has only a subset of IL-4-like activities on B chronic lymphocytic leukaemia cells. *Immunology* 83(3): 397-403.

Flynn, J. L. and J. Chan (2001). Immunology of tuberculosis. *Annu Rev Immunol* 19: 93-129.

Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart and B. R. Bloom (1993). An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med* 178(6): 2249-54.

Flynn, J. L., M. M. Goldstein, K. J. Triebold, J. Sypek, S. Wolf and B. R. Bloom (1995). IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection. *J Immunol* 155(5): 2515-24.

Fontenot, J. D., M. A. Gavin and A. Y. Rudensky (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4(4): 330-6.

Foster, P. S., M. Martinez-Moczygemba, D. P. Huston and D. B. Corry (2002). Interleukins-4, -5, and -13: emerging therapeutic targets in allergic disease. *Pharmacol Ther* 94(3): 253-64.

Francis, J. N., S. J. Till and S. R. Durham (2003). Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 111(6): 1255-61.

Fujimoto, K., K. Kubo, Y. Matsuzawa and M. Sekiguchi (1997). Eosinophil cationic protein levels in induced sputum correlate with the severity of bronchial asthma. *Chest* 112(5): 1241-7.

Fujiwara, H., S. H. Hanissian, A. Tsytsykova and R. S. Geha (1997). Homodimerization of the human interleukin 4 receptor alpha chain induces Cvarepsilon germline transcripts in B cells in the absence of the interleukin 2 receptor gamma chain. *PNAS* 94(11): 5866-5871.

Gagliardi, M. C., R. Teloni, F. Giannoni, M. Pardini, V. Sargentini, L. Brunori, L. Fattorini and R. Nisini (2005). Mycobacterium bovis Bacillus Calmette-Guerin infects DC-SIGN- dendritic cell and causes the inhibition of IL-12 and the enhancement of IL-10 production. *J Leukoc Biol* 78(1): 106-13.

Gale, E. A. (2002). A missing link in the hygiene hypothesis? *Diabetologia* 45(4): 588-94.

Gately, M. K., L. M. Renzetti, J. Magram, A. S. Stern, L. Adorini, U. Gubler and D. H. Presky (1998). The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol* 16: 495-521.

Gavett, S. H., D. J. O'Hearn, C. L. Karp, E. A. Patel, B. H. Schofield, F. D. Finkelman and M. Wills-Karp (1997). Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol* 272(2 Pt 1): L253-61.

Gavett, S. H., D. J. O'Hearn, X. Li, S. K. Huang, F. D. Finkelman and M. Wills-Karp (1995). Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 182(5): 1527-36.

Gehring, U., W. Bischof, B. Fahlbusch, H. E. Wichmann and J. Heinrich (2002). House dust endotoxin and allergic sensitization in children. *Am J Respir Crit Care Med* 166(7): 939-44.

Geijtenbeek, T. B., S. J. Van Vliet, E. A. Koppel, M. Sanchez-Hernandez, C. M. Vandenbroucke-Grauls, B. Appelmelk and Y. Van Kooyk (2003). Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* 197(1): 7-17.

Gereda, J. E., M. D. Klinnert, M. R. Price, D. Y. Leung and A. H. Liu (2001). Metropolitan home living conditions associated with indoor endotoxin levels. *J Allergy Clin Immunol* 107(5): 790-6.

Gereda, J. E., D. Y. M. Leung, A. Thatayatikom, J. E. Streib, M. R. Price, M. D. Klinnert and A. H. Liu (2000). Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *The Lancet* 355(9216): 1680-1683.

Gerhold, K., K. Blumchen, A. Bock, C. Seib, P. Stock, T. Kallinich, M. Lohning, U. Wahn and E. Hamelmann (2002). Endotoxins prevent murine IgE production, T(H)2 immune responses, and development of airway eosinophilia but not airway hyperreactivity. *J Allergy Clin Immunol* 110(1): 110-6.

Germann, T., E. Rude, F. Mattner and M. K. Gately (1995). The IL-12 p40 homodimer as a specific antagonist of the IL-12 heterodimer. *Immunol Today* 16(10): 500-1.

Gerosa, F., C. Nisii, S. Righetti, R. Micciolo, M. Marchesini, A. Cazzadori and G. Trinchieri (1999). CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients. *Clin Immunol* 92(3): 224-34.

Gerrard, J. W., C. A. Geddes, P. L. Reggin, C. D. Gerrard and S. Horne (1976). Serum IgE levels in white and metis communities in Saskatchewan. *Ann Allergy* 37(2): 91-100.

Gessner, A., K. Schroppel, A. Will, K. H. Enssle, L. Lauffer and M. Rollinghoff (1994). Recombinant soluble interleukin-4 (IL-4) receptor acts as an antagonist of IL-4 in murine cutaneous Leishmaniasis. *Infect Immun* 62(10): 4112-7.

Gibson, P. G., P. J. Manning, P. M. O'Byrne, A. Girgis-Gabardo, J. Dolovich, J. A. Denburg and F. E. Hargreave (1991). Allergen-induced asthmatic responses. Relationship between increases in airway responsiveness and increases in circulating eosinophils, basophils, and their progenitors. *Am Rev Respir Dis* 143(2): 331-5.

Giembycz, M. A. and M. A. Lindsay (1999). Pharmacology of the eosinophil. *Pharmacol Rev* 51(2): 213-340.

Gong, J. H., M. Zhang, R. L. Modlin, P. S. Linsley, D. Iyer, Y. Lin and P. F. Barnes (1996). Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression. *Infect Immun* 64(3): 913-8.

Gordon, R. E. and M. M. Smith (1953). Rapidly growing, acid fast bacteria. I. Species' descriptions of Mycobacterium phlei Lehmann and Neumann and Mycobacterium smegmatis (Trevisan) Lehmann and Neumann. *J Bacteriol* 66(1): 41-8.

Gould, H. J., B. J. Sutton, A. J. Beavil, R. L. Beavil, N. McCloskey, H. A. Coker, D. Fear and L. Smurthwaite (2003). The biology of IGE and the basis of allergic disease. *Annu Rev Immunol* 21: 579-628. Epub 2001 Dec 19.

Gruber, C., G. Meinlschmidt, R. Bergmann, U. Wahn and K. Stark (2002). Is early BCG vaccination associated with less atopic disease? An epidemiological study in German preschool children with different ethnic backgrounds. *Pediatr Allergy Immunol* 13(3): 177-81.

Grunewald, S. M., S. Kunzmann, B. Schnarr, J. Ezernieks, W. Sebald and A. Duschl (1997). A murine interleukin-4 antagonistic mutant protein completely inhibits interleukin-4-induced cell proliferation, differentiation, and signal transduction. *J Biol Chem* 272(3): 1480-3.

Grunewald, S. M., A. Werthmann, B. Schnarr, C. E. Klein, E. B. Brocker, M. Mohrs, F. Brombacher, W. Sebald and A. Duschl (1998). An antagonistic IL-4 mutant prevents type I allergy in the mouse: inhibition of the IL-4/IL-13 receptor system completely abrogates humoral immune

response to allergen and development of allergic symptoms in vivo. *J Immunol* 160(8): 4004-9.

Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley and D. B. Corry (1998). Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282(5397): 2261-3.

Guerardel, Y., E. Maes, V. Briken, F. Chirat, Y. Leroy, C. Locht, G. Strecker and L. Kremer (2003). Lipomannan and lipoarabinomannan from a clinical isolate of Mycobacterium kansasii: novel structural features and apoptosis-inducing properties. *J Biol Chem* 278(38): 36637-51.

Guerardel, Y., E. Maes, E. Elass, Y. Leroy, P. Timmerman, G. S. Besra, C. Locht, G. Strecker and L. Kremer (2002). Structural study of lipomannan and lipoarabinomannan from Mycobacterium chelonae. Presence of unusual components with alpha 1,3-mannopyranose side chains. *J Biol Chem* 277(34): 30635-48.

Gunshin, H., B. Mackenzie, U. V. Berger, Y. Gunshin, M. F. Romero, W. F. Boron, S. Nussberger, J. L. Gollan and M. A. Hediger (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388(6641): 482-8.

Guo, C., M. Liu, S. Galli, B. Bochner, A. Kagey-Sobotka and L. Lichtenstein (1994). Identification of IgE-bearing cells in the late-phase response to antigen in the lung as basophils. *Am. J. Respir. Cell Mol. Biol.* 10(4): 384-390.

Gupta, R., A. Sheikh, D. P. Strachan and H. R. Anderson (2004). Burden of allergic disease in the UK: secondary analyses of national databases. *Clin Exp Allergy* 34(4): 520-526.

Hadley, E. A., F. I. Smillie, M. A. Turner, A. Custovic, A. Woodcock and P. D. Arkwright (2005). Effect of Mycobacterium vaccae on cytokine responses in children with atopic dermatitis. *Clin Exp Immunol* 140(1): 101-8.

Haglind, P. and R. Rylander (1987). Occupational exposure and lung function measurements among workers in swine confinement buildings. *J Occup Med* 29(11): 904-7.

Hahn, C., M. Teufel, U. Herz, H. Renz, K. J. Erb, G. Wohlleben, E. B. Brocker, A. Duschl, W. Sebald and S. M. Grunewald (2003). Inhibition of the IL-4/IL-13 receptor system prevents allergic sensitization without affecting established allergy in a mouse model for allergic asthma. *J Allergy Clin Immunol* 111(6): 1361-9.

Hannen, M., U. Banning, H. Bonig, Y. Kim, D. Shin, I. Lorenz, K. Seeger and D. Korholz (1999). Cytokine-mediated regulation of granulocyte colony-stimulating factor production. *Scand J Immunol* 50(5): 461-8.

Hansen, G., G. Berry, R. H. DeKruyff and D. T. Umetsu (1999). Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 103(2): 175-83.

Hansen, G., G. Berry, R. H. DeKruyff and D. T. Umetsu (1999). Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced aitway hyperreactivity but causes severe airway inflammation. *J Clin Invest* 103: 175.

Happel, K. I., P. J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L. J. Quinton, A. R. Odden, J. E. Shellito, G. J. Bagby, S. Nelson and J. K. Kolls (2005). Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. *J Exp Med* 202(6): 761-9.

Happel, K. I., E. A. Lockhart, C. M. Mason, E. Porretta, E. Keoshkerian, A. R. Odden, S. Nelson and A. J. Ramsay (2005). Pulmonary interleukin-23 gene delivery increases local T-cell immunity and controls growth of Mycobacterium tuberculosis in the lungs. *Infect Immun* 73(9): 5782-8.

Hasday, J. D., R. Bascom, J. J. Costa, T. Fitzgerald and W. Dubin (1999). Bacterial endotoxin is an active component of cigarette smoke. *Chest* 115(3): 829-35.

Heaney, M. L. and D. W. Golde (1996). Soluble cytokine receptors. *Blood* 87(3): 847-57.

Heaney, M. L. and D. W. Golde (1998). Soluble receptors in human disease. *J Leukoc Biol* 64(2): 135-46.

Heath, V. L., L. Showe, C. Crain, F. J. Barrat, G. Trinchieri and A. O'Garra (2000). Cutting edge: ectopic expression of the IL-12 receptor-beta 2 in developing and committed Th2 cells does not affect the production of IL-4 or induce the production of IFN-gamma. *J Immunol* 164(6): 2861-5.

Heldwein, K. A. and M. J. Fenton (2002). The role of Toll-like receptors in immunity against mycobacterial infection. *Microbes Infect* 4(9): 937-44.

Henderson, R. A., S. C. Watkins and J. L. Flynn (1997). Activation of human dendritic cells following infection with Mycobacterium tuberculosis. *J Immunol* 159(2): 635-43.

Henderson, W. R., Jr., E. Y. Chi and C. R. Maliszewski (2000). Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. *J Immunol* 164(2): 1086-95.

Herz, U., K. Gerhold, C. Gruber, A. Braun, U. Wahn, H. Renz and K. Paul (1998). BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. *J Allergy Clin Immunol* 102(5): 867-74.

Hilkens, C. M., H. Vermeulen, R. J. van Neerven, F. G. Snijdewint, E. A. Wierenga and M. L. Kapsenberg (1995). Differential modulation of T helper type 1 (Th1) and T helper type 2 (Th2) cytokine secretion by prostaglandin E2 critically depends on interleukin-2. *Eur J Immunol* 25(1): 59-63.

Hilton, D. J., J.-G. Zhang, D. Metcalf, W. S. Alexander, N. A. Nicola and T. A. Willson (1996). Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. *PNAS* 93(1): 497-501.

Hirsch, C. S., J. J. Ellner, R. Blinkhorn and Z. Toossi (1997). In vitro restoration of T cell responses in tuberculosis and augmentation of monocyte effector function against Mycobacterium tuberculosis by natural inhibitors of transforming growth factor beta. *Proc Natl Acad Sci U S A* 94(8): 3926-31.

Ho, I. C., M. R. Hodge, J. W. Rooney and L. H. Glimcher (1996). The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell* 85(7): 973-83.

Ho, I.-C., D. Lo and L. H. Glimcher (1998). c-maf Promotes T Helper Cell Type 2 (Th2) and Attenuates Th1 Differentiation by Both Interleukin 4-dependent and -independent Mechanisms. *J. Exp. Med.* 188(10): 1859-1866.

Hobbs, K., J. Negri, M. Klinnert, L. J. Rosenwasser and L. Borish (1998). Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma. *Am J Respir Crit Care Med* 158(6): 1958-62.

Hoeck, J. and M. Woisetschlager (2001). STAT6 mediates eotaxin-1 expression in IL-4 or TNF-alpha-induced fibroblasts. *J Immunol* 166(7): 4507-15.

Hogan, S. P., P. S. Foster, X. Tan and A. J. Ramsay (1998). Mucosal IL-12 gene delivery inhibits allergic airways disease and restores local antiviral immunity. *Eur J Immunol* 28(2): 413-23.

Holgate, S. T. (1993). The 1992 Cournand Lecture. Asthma: past, present and future. *Eur Respir J* 6(10): 1507-20.

Holgate, S. T. (1997). Asthma: a dynamic disease of inflammation and repair. *Ciba Found Symp* 206: 5-28.

Holgate, S. T. (1999). The epidemic of allergy and asthma. *Nature* 402(6760 Suppl): B2-4.

Holt, P. G. (1995). Environmental factors and primary T-cell sensitisation to inhalant allergens in infancy: reappraisal of the role of infections and air pollution. *Pediatr Allergy Immunol* 6(1): 1-10.

Holt, P. G. (1996). Infections and the development of allergy. *Toxicol Lett* 86(2-3): 205-10.

Holt, P. G. and C. A. Jones (2000). The development of the immune system during pregnancy and early life. *Allergy* 55(8): 688-97.

Holt, P. G. and C. Macaubas (1997). Development of long-term tolerance versus sensitisation to environmental allergens during the perinatal period. *Curr Opin Immunol* 9(6): 782-7.

Holt, P. G., C. McMenamin, M. A. Schon-Hegrad, D. Strickland, D. Nelson, L. Wilkes, N. Bilyk, J. Oliver, B. J. Holt and P. G. McMenamin (1991). Immunoregulation of asthma: control of T-lymphocyte activation in the respiratory tract. *Eur Respir J Suppl* 13: 6s-15s.

Holtzman, M. J., D. Sampath, M. Castro, D. C. Look and S. Jayaraman (1996). The one-two of T helper cells: does interferon-gamma knock out the Th2 hypothesis for asthma? *Am J Respir Cell Mol Biol* 14(4): 316-8.

Hopfenspirger, M. T. and D. K. Agrawal (2002). Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-presensitized mice. *J Immunol* 168(5): 2516-22.

Hopfenspirger, M. T., S. K. Parr, R. J. Hopp, R. G. Townley and D. K. Agrawal (2001). Mycobacterial antigens attenuate late phase response, airway hyperresponsiveness, and bronchoalveolar lavage eosinophilia in a mouse model of bronchial asthma. *Int Immunopharmacol* 1(9-10): 1743-51.

Hopkin, J. M. (1997). Mechanisms of enhanced prevalence of asthma and atopy in developed countries. *Curr Opin Immunol* 9(6): 788-92.

Hopkin, J. M., B. Ferry, P. Coull, P. Antrobus, T. Enomoto, T. Yamashita, F. Kurimoto, J. L. Stanford, T. Shirakawa and G. A. W. Rook (1998). Mycobacterial Immunisation in grass pollen asthma and rhinitis. *Thorax* 53(Suppl. 4): 63.

Horak, F., Jr., M. Studnicka, C. Gartner, A. Veiter, E. Tauber, R. Urbanek and T. Frischer (2002). Parental farming protects children against atopy: longitudinal evidence involving skin prick tests. *Clin Exp Allergy* 32(8): 1155-9.

Hori, S., T. Nomura and S. Sakaguchi (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299(5609): 1057-61.

Hori, S. and S. Sakaguchi (2004). Foxp3: a critical regulator of the development and function of regulatory T cells. *Microbes Infect* 6(8): 745-51.

Horner, A., K. Takabayashi, J. Burger, A. Ronaghy, N. Cinman, H. Spielgeberg and E. Raz (2000). Immunostimulatory sequence oligodeoxynucleotide induces an innate type I cytokine response from human PBMCs and inhibits IL-4 dependent CD23 expression and IgE production (abstract). *J Allergy Clin Immunol* 105: S69.

Horner, A. A., G. F. Widhopf, J. A. Burger, K. Takabayashi, N. Cinman, A. Ronaghy, H. L. Spiegelberg and E. Raz (2001). Immunostimulatory DNA inhibits IL-4-dependent IgE synthesis by human B cells. *J Allergy Clin Immunol* 108(3): 417-23.

Horng, T., G. M. Barton, R. A. Flavell and R. Medzhitov (2002). The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* 420(6913): 329-33.

Horng, T., G. M. Barton and R. Medzhitov (2001). TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol* 2(9): 835-41.

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda and S. Akira (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 162(7): 3749-52.

Hrouda, D., B. Baban, W. D. Dunsmuir, R. S. Kirby and A. G. Dalgleish (1998). Immunotherapy of advanced prostate cancer: a phase I/II trial using Mycobacterium vaccae (SRL172). *Br J Urol* 82(4): 568-73.

Hrouda, D., B. E. Souberbielle, J. Kayaga, C. M. Corbishley, R. S. Kirby and A. G. Dalgleish (1998). Mycobacterium vaccae (SRL172): a potential immunological adjuvant evaluated in rat prostate cancer. *Br J Urol* 82(6): 870-6.

Hsieh, C. S., A. B. Heimberger, J. S. Gold, A. O'Garra and K. M. Murphy (1992). Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proc Natl Acad Sci U S A* 89(13): 6065-9.

Huang, F. P., W. Niedbala, X. Q. Wei, D. Xu, G. J. Feng, J. H. Robinson, C. Lam and F. Y. Liew (1998). Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. *Eur J Immunol* 28(12): 4062-70.

Huang, T. J., P. A. MacAry, P. Eynott, A. Moussavi, K. C. Daniel, P. W. Askenase, D. M. Kemeny and K. F. Chung (2001). Allergen-specific Th1 cells counteract efferent Th2 cell-dependent bronchial hyperresponsiveness and eosinophilic inflammation partly via IFN-γ. *J Immunol* 166(1): 207-17.

Hultmark, D. (1994). Macrophage differentiation marker MyD88 is a member of the Toll/IL-1 receptor family. *Biochem Biophys Res Commun* 199(1): 144-6.

Humbert, M., S. R. Durham, S. Ying, P. Kimmitt, J. Barkans, B. Assoufi, R. Pfister, G. Menz, D. S. Robinson, A. B. Kay and C. J. Corrigan (1996). IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity. *Am J Respir Crit Care Med* 154(5): 1497-504.

Hunt, J. R., R. Martinelli, V. C. Adams, G. A. Rook and L. R. Brunet (2005). Intragastric administration of Mycobacterium vaccae inhibits severe pulmonary allergic inflammation in a mouse model. *Clin Exp Allergy* 35(5): 685-90.

Huygen, K., D. Abramowicz, P. Vandenbussche, F. Jacobs, J. De Bruyn, A. Kentos, A. Drowart, J. P. Van Vooren and M. Goldman (1992). Spleen cell cytokine secretion in Mycobacterium bovis BCG-infected mice. *Infect Immun* 60(7): 2880-6.

Ihle, J. N. (1995). The Janus protein tyrosine kinase family and its role in cytokine signaling. *Adv Immunol* 60: 1-35.

Inman, M. D., R. Watson, D. W. Cockcroft, B. J. Wong, F. E. Hargreave and P. M. O'Byrne (1995). Reproducibility of allergen-induced early and late asthmatic responses. *J Allergy Clin Immunol* 95(6): 1191-5.

Inohara, N. and G. Nunez (2003). NODs: intracellular proteins involved in inflammation and apoptosis. *Nat Rev Immunol* 3(5): 371-82.

ISAAC (1998). Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. *Lancet* 351(9111): 1225-32.

Itazawa, T., Y. Adachi, Y. Okabe, M. Hamamichi, Y. S. Adachi, M. Toyoda, M. Morohashi and T. Miyawaki (2003). Developmental changes in interleukin-12-producing ability by monocytes and their relevance to allergic diseases. *Clin Exp Allergy* 33(4): 525-30.

Jacobs, C. A., M. P. Beckmann, K. Mohler, C. R. Maliszewski, W. C. Fanslow and D. H. Lynch (1993). Pharmacokinetic parameters and biodistribution of soluble cytokine receptors. *Int Rev Exp Pathol* 34(Pt B): 123-35.

Jaffar, Z., K. S. Wan and K. Roberts (2002). A key role for prostaglandin I2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. *J Immunol* 169(10): 5997-6004.

Jagielo, P., P. Thorne, J. Watt, K. Frees, T. Quinn and D. Schwartz (1996). Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. *Chest* 110(1): 263-270.

Janefjiord, C. and M. Jenmalm (2001). PHA-induced IL-12Rβ2 mRNA expression in atopic and non-atopic children. *Clin Exp Allergy*.

Janeway, C. A., Jr. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* 13(1): 11-6.

Jang, A. S. and M. H. Son (2002). The association of airway hyperresponsiveness and tuberculin responses. *Allergy* 57(4): 341-5.

Jang, S., S. Uematsu, S. Akira and P. Salgame (2004). IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on TLR2-mediated recognition. *J Immunol* 173(5): 3392-7.

Janssen, R., A. Kruisselbrink, L. Hoogteijling, J. R. Lamb, D. B. Young and J. E. Thole (2001). Analysis of recombinant mycobacteria as T helper type 1 vaccines in an allergy challenge model. *Immunology* 102(4): 441-9.

Jarjour, N., W. Calhoun, E. Kelly, G. Gleich, L. Schwartz and W. Busse (1997). The immediate and late allergic response to segmental bronchopulmonary provocation in asthma. *Am. J. Respir. Crit. Care Med.* 155(5): 1515-1521.

Jenkins, M. K., A. Khoruts, E. Ingulli, D. L. Mueller, S. J. McSorley, R. L. Reinhardt, A. Itano and K. A. Pape (2001). In vivo activation of antigen-specific CD4 T cells. *Annu Rev Immunol* 19: 23-45.

Jiang, H., M. B. Harris and P. Rothman (2000). IL-4/IL-13 signaling beyond JAK/STAT. *J Allergy Clin Immunol* 105(6 Pt 1): 1063-70.

Johnson, J. L., A. J. Nunn, P. B. Fourie, L. P. Ormerod, R. D. Mugerwa, A. Mwinga, C. Chintu, B. Ngwira, P. Onyebujoh and A. Zumla (2004). Effect of Mycobacterium vaccae (SRL172) immunotherapy on radiographic healing in tuberculosis. *Int J Tuberc Lung Dis* 8(11): 1348-54.

Jones, C. A., J. A. Holloway, E. J. Popplewell, N. D. Diaper, J. W. Holloway, G. H. Vance, J. A. Warner and J. O. Warner (2002). Reduced soluble CD14 levels in amniotic fluid and breast milk are associated with the subsequent development of atopy, eczema, or both. *J Allergy Clin Immunol* 109(5): 858-66.

Jonuleit, H., E. Schmitt, G. Schuler, J. Knop and A. H. Enk (2000). Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 192(9): 1213-22.

Jotwani, R., B. Pulendran, S. Agrawal and C. W. Cutler (2003). Human dendritic cells respond to Porphyromonas gingivalis LPS by promoting a Th2 effector response in vitro. *Eur J Immunol* 33(11): 2980-6.

Jouanguy, E., R. Doffinger, S. Dupuis, A. Pallier, F. Altare and J. L. Casanova (1999). IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. *Curr Opin Immunol* 11(3): 346-51.

Jung, T., N. Schrader, M. Hellwig, K. H. Enssle and C. Neumann (1999). Soluble human interleukin-4 receptor is produced by activated T cells under the control of metalloproteinases. *Int Arch Allergy Immunol* 119(1): 23-30.

Jung, T., K. Wagner, C. Neumann and C. H. Heusser (1999). Enhancement of human IL-4 activity by soluble IL-4 receptors *in vitro*. *Eur J Immunol* 29(3): 864-71.

Jutel, M., M. Akdis, F. Budak, C. Aebischer-Casaulta, M. Wrzyszcz, K. Blaser and C. A. Akdis (2003). IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 33(5): 1205-14.

Kalliomaki, M. and E. Isolauri (2003). Role of intestinal flora in the development of allergy. *Curr Opin Allergy Clin Immunol* 3(1): 15-20.

Kaplan, M. H. and M. J. Grusby (1998). Regulation of T helper cell differentiation by STAT molecules. *J Leukoc Biol* 64(1): 2-5.

Kaplan, M. H., U. Schindler, S. T. Smiley and M. J. Grusby (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4(3): 313-9.

Kaplan, M. H., Y. L. Sun, T. Hoey and M. J. Grusby (1996). Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382(6587): 174-7.

Kaplan, M. H., A. L. Wurster and M. J. Grusby (1998). A signal transducer and activator of transcription (Stat)4-independent pathway for the development of T helper type 1 cells. *J Exp Med* 188(6): 1191-6.

Karlsson, M. R., J. Rugtveit and P. Brandtzaeg (2004). Allergen-responsive CD4+CD25+ Regulatory T Cells in Children who Have Outgrown Cow's Milk Allergy. *J Exp Med* 199(12): 1679-88.

Karras, J. G., K. McGraw, R. A. McKay, S. R. Cooper, D. Lerner, T. Lu, C. Walker, N. M. Dean and B. P. Monia (2000). Inhibition of antigen-induced eosinophilia and late phase airway hyperresponsiveness by an IL-5 antisense oligonucleotide in mouse models of asthma. *J Immunol* 164(10): 5409-15.

Kauffmann, F., M. P. Oryszczyn and J. Maccario (2002). The protective role of country living on skin prick tests, immunoglobulin E and asthma in adults from the Epidemiological study on the Genetics and Environment of Asthma, bronchial hyper-responsiveness and atopy. *Clin Exp Allergy* 32(3): 379-86.

Kaufmann, S. H. E. (2003). Immunity to Intracellular Bacteria. Fundamental Immunology. W. E. Paul. Philadelphia, Lippincott Williams & Wilkins: 1229-1261.

Kawai, T., O. Adachi, T. Ogawa, K. Takeda and S. Akira (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11(1): 115-22.

Kelly, E. A. B., R. R. Rodriguez, W. W. BUSSE and N. N. JARJOUR (1997). The Effect of Segmental Bronchoprovocation with Allergen on Airway Lymphocyte Function. *Am. J. Respir. Crit. Care Med.* 156(5): 1421-1428.

Khoo, K. H., J. B. Tang and D. Chatterjee (2001). Variation in mannose-capped terminal arabinan motifs of lipoarabinomannans from clinical isolates of Mycobacterium tuberculosis and Mycobacterium avium complex. *J Biol Chem* 276(6): 3863-71.

Kilchherr, E., V. Mandak, K. Wagner and C. H. Heusser (1993). Regulation of human IgE response in hu-PBL-SCID mice. *Cell Immunol* 151(2): 241-56.

Kilpelainen, M., E. O. Terho, H. Helenius and M. Koskenvuo (2000). Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* 30(2): 201-8.

Kim, J. I., I. C. Ho, M. J. Grusby and L. H. Glimcher (1999). The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity* 10(6): 745-751.

Kinjyo, I., T. Hanada, K. Inagaki-Ohara, H. Mori, D. Aki, M. Ohishi, H. Yoshida, M. Kubo and A. Yoshimura (2002). SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* 17(5): 583-91.

Kips, J. C., G. J. Brusselle, G. F. Joos, R. A. Peleman, J. H. Tavernier, R. R. Devos and R. A. Pauwels (1996). Interleukin-12 inhibits antigen-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* 153(2): 535-9.

Kips, J. C., B. J. O'Connor, S. J. Langley, A. Woodcock, H. A. Kerstjens, D. S. Postma, M. Danzig, F. Cuss and R. A. Pauwels (2003). Effect of SCH55700, a humanized anti-human interleukin-5 antibody, in severe persistent asthma: a pilot study. *Am J Respir Crit Care Med* 167(12): 1655-9.

Kline, J. N., J. D. Cowden, G. W. Hunninghake, B. C. Schutte, J. L. Watt, C. L. Wohlford-Lenane, L. S. Powers, M. P. Jones and D. A. Schwartz (1999). Variable airway responsiveness to inhaled lipopolysaccharide. *Am J Respir Crit Care Med* 160(1): 297-303.

Kline, J. N., T. J. Waldschmidt, T. R. Businga, J. E. Lemish, J. V. Weinstock, P. S. Thorne and A. M. Krieg (1998). Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 160(6): 2555-9.

Klintberg, B., N. Berglund, G. Lilja, M. Wickman and M. van Hage-Hamsten (2001). Fewer allergic respiratory disorders among farmers' children in a closed birth cohort from Sweden. *Eur Respir J* 17(6): 1151-7.

Kobayashi, K., L. D. Hernandez, J. E. Galan, C. A. Janeway, Jr., R. Medzhitov and R. A. Flavell (2002). IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 110(2): 191-202.

Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia and G. Trinchieri (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med* 170(3): 827-45.

Koh, Y. Y., R. Dupuis, M. Pollice, K. H. Albertine, J. E. Fish and S. P. Peters (1993). Neutrophils recruited to the lungs of humans by segmental antigen challenge display a reduced chemotactic response to leukotriene B4. *Am J Respir Cell Mol Biol* 8(5): 493-9.

Kohashi, O., J. Kuwata, K. Umehara, F. Uemura, T. Takahashi and A. Ozawa (1979). Susceptibility to adjuvant-induced arthritis among germfree, specific-pathogen-free, and conventional rats. *Infect Immun* 26(3): 791-4.

Kojima, Y., Y. Kinouchi, S. Takahashi, K. Negoro, N. Hiwatashi and T. Shimosegawa (2001). Inflammatory bowel disease is associated with a novel promoter polymorphism of natural resistance-associated macrophage protein 1 (NRAMP1) gene. *Tissue Antigens* 58(6): 379-84.

Konig, B., A. Fischer and W. Konig (1995). Modulation of cell-bound and soluble CD23, spontaneous and ongoing IgE synthesis of human peripheral blood mononuclear cells by soluble IL-4 receptors and the partial antagonistic IL-4 mutant protein IL-4 (Y124D). *Immunology* 85(4): 604-10.

Kopf, M., G. Le Gros, M. Bachmann, M. C. Lamers, H. Bluethmann and G. Kohler (1993). Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 362(6417): 245-8.

Kotze, M. J., J. N. de Villiers, R. N. Rooney, J. J. Grobbelaar, E. P. Mansvelt, C. S. Bouwens, J. Carr, I. Stander and L. du Plessis (2001). Analysis of the NRAMP1 gene implicated in iron transport: association with multiple sclerosis and age effects. *Blood Cells Mol Dis* 27(1): 44-53.

Koul, A., T. Herget, B. Klebl and A. Ullrich (2004). Interplay between mycobacteria and host signalling pathways. *Nat Rev Microbiol* 2(3): 189-202.

Kramer, U., J. Heinrich, M. Wjst and H. E. Wichmann (1999). Age of entry to day nursery and allergy in later childhood. *Lancet* 353(9151): 450-4.

Kramnik, I., D. Radzioch and E. Skamene (1994). T-helper 1-like subset selection in Mycobacterium bovis bacillus Calmette-Guerin-infected resistant and susceptible mice. *Immunology* 81(4): 618-25.

Krause, T. G., A. Hviid, A. Koch, J. Friborg, T. Hjuler, J. Wohlfahrt, O. R. Olsen, B. Kristensen and M. Melbye (2003). BCG vaccination and risk of atopy. *Jama* 289(8): 1012-5.

Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky and D. M. Klinman (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374(6522): 546-9.

Kroger, L., M. Korppi, J. Pelkonen, M. Pietikainen and M. L. Katila (2000). Development of tuberculin reactivity and sensitization to M. scrofulaceum and M. fortuitum in children BCG-vaccinated at birth. *Eur Respir J* 15(2): 382-7.

Kruse, N., B. J. Shen, S. Arnold, H. P. Tony, T. Muller and W. Sebald (1993). Two distinct functional sites of human interleukin 4 are identified by variants impaired in either receptor binding or receptor activation. *Embo J* 12(13): 5121-9.

Kruse, N., H. P. Tony and W. Sebald (1992). Conversion of human interleukin-4 into a high affinity antagonist by a single amino acid replacement. *Embo J* 11(9): 3237-44.

Kruse, S., J. Forster, J. Kuehr and K. A. Deichmann (1999). Characterization of the membrane-bound and a soluble form of human IL-4 receptor alpha produced by alternative splicing. *Int Immunol* 11(12): 1965-70.

Krutzik, S. R. and R. L. Modlin (2004). The role of Toll-like receptors in combating mycobacteria. *Semin Immunol* 16(1): 35-41.

Kuipers, H., D. Hijdra, V. C. De Vries, H. Hammad, J. B. Prins, A. J. Coyle, H. C. Hoogsteden and B. N. Lambrecht (2003). Lipopolysaccharide-induced suppression of airway Th2 responses does not require IL-12 production by dendritic cells. *J Immunol* 171(7): 3645-54.

Kurup, V. P., P. S. Murali, J. Guo, H. Choi, B. Banerjee, J. N. Fink and R. L. Coffman (1997). Anti-interleukin (IL)-4 and -IL-5 antibodies downregulate IgE and eosinophilia in mice exposed to Aspergillus antigens. *Allergy* 52(12): 1215-21.

Lack, G., K. L. Bradley, E. Hamelmann, H. Renz, J. Loader, D. Y. Leung, G. Larsen and E. W. Gelfand (1996). Nebulized IFN-gamma inhibits the development of secondary allergic responses in mice. *J Immunol* 157(4): 1432-9.

Lack, G., H. Renz, J. Saloga, K. L. Bradley, J. Loader, D. Y. Leung, G. Larsen and E. W. Gelfand (1994). Nebulized but not parenteral IFN-gamma decreases IgE production and normalizes airways function in a murine model of allergen sensitization. *J Immunol* 152(5): 2546-54.

Lai, S., J. Molden, K. Liu, J. Puck, M. White and M. Goldsmith (1996). Interleukin-4-specific signal transduction events are driven by homotypic interactions of the interleukin-4 receptor alpha subunit. *EMBO J.* 15(17): 4506-4514.

Lammas, D. A., J. L. Casanova and D. S. Kumararatne (2000). Clinical consequences of defects in the IL-12-dependent interferon-gamma (IFN-gamma) pathway. *Clin Exp Immunol* 121(3): 417-25.

Lane, P. (2000). Role of OX40 signals in coordinating CD4 T cell selection, migration, and cytokine differentiation in T helper (Th)1 and Th2 cells. *J Exp Med* 191(2): 201-6.

Lang, T., E. Prina, D. Sibthorpe and J. M. Blackwell (1997). Nramp1 transfection transfers Ity/Lsh/Bcg-related pleiotropic effects on macrophage activation: influence on antigen processing and presentation. *Infect Immun* 65(2): 380-6.

Lauener, R. P., T. Birchler, J. Adamski, C. Braun-Fahrlander, A. Bufe, U. Herz, E. von Mutius, D. Nowak, J. Riedler, M. Waser and F. H. Sennhauser (2002). Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. *Lancet* 360(9331): 465-6.

Lauw, F. N., T. ten Hove, P. E. Dekkers, E. de Jonge, S. J. van Deventer and T. van Der Poll (2000). Reduced Th1, but not Th2, cytokine production by lymphocytes after in vivo exposure of healthy subjects to endotoxin. *Infect Immun* 68(3): 1014-8.

Lazarevic, V., A. J. Myers, C. A. Scanga and J. L. Flynn (2003). CD40, but not CD40L, is required for the optimal priming of T cells and control of aerosol M. tuberculosis infection. *Immunity* 19(6): 823-35.

Leckie, M. J., T. A. Brinke, J. Khan, Z. Diamant, B. J. O'Connor, C. M. Walls, A. K. Mathur, H. Cowley, F. K. Chung, R. Djukanovic, T. T. Hansel, S. T. Holgate, P. J. Sterk and P. J. Barnes (2000). Effects of an Interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356: 2144-2184.

Leckie, M. J., A. ten Brinke, J. Khan, Z. Diamant, B. J. O'Connor, C. M. Walls, A. K. Mathur, H. C. Cowley, K. F. Chung, R. Djukanovic, T. T. Hansel, S. T. Holgate, P. J. Sterk and P. J. Barnes (2000). Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 356(9248): 2144-8.

Lee, H. J., N. Takemoto, H. Kurata, Y. Kamogawa, S. Miyatake, A. O'Garra and N. Arai (2000). GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. *J Exp Med* 192(1): 105-15.

Lee, S. Y., Y. H. Lee, C. Shin, J. J. Shim, K. H. Kang, S. H. Yoo and K. H. In (2002). Association of Asthma Severity and Bronchial Hyperresponsiveness With a Polymorphism in the Cytotoxic T-Lymphocyte Antigen-4 Gene*. *Chest* 122(1): 171-176.

Leemans, J. C., S. Florquin, M. Heikens, S. T. Pals, R. van der Neut and T. Van Der Poll (2003). CD44 is a macrophage binding site for Mycobacterium tuberculosis that mediates macrophage recruitment and protective immunity against tuberculosis. *J Clin Invest* 111(5): 681-9.

Lehmann, D. and A. Ben-Nun (1992). Bacterial agents protect against autoimmune disease. I. Mice pre-exposed to Bordetella pertussis or Mycobacterium tuberculosis are highly refractory to induction of experimental autoimmune encephalomyelitis. *J Autoimmun* 5(6): 675-90.

Leonard, C., V. Tormey, C. Burke and L. W. Poulter (1997). Allergen-induced cytokine production in atopic disease and its relationship to disease severity. *Am J Respir Cell Mol Biol* 17(3): 368-75.

Leonard, J. P., M. L. Sherman, G. L. Fisher, L. J. Buchanan, G. Larsen, M. B. Atkins, J. A. Sosman, J. P. Dutcher, N. J. Vogelzang and J. L. Ryan (1997). Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 90(7): 2541-8.

Leung, D. Y., R. J. Martin, S. J. Szefler, E. R. Sher, S. Ying, A. B. Kay and Q. Hamid (1995). Dysregulation of interleukin 4, interleukin 5, and interferon gamma gene expression in steroid-resistant asthma. *J Exp Med* 181(1): 33-40.

Lewinsohn, D. M., T. T. Bement, J. Xu, D. H. Lynch, K. H. Grabstein, S. G. Reed and M. R. Alderson (1998). Human purified protein derivative-specific CD4+ T cells use both CD95-dependent and CD95-independent cytolytic mechanisms. *J Immunol* 160(5): 2374-9.

Leynaert, B., C. Neukirch, D. Jarvis, S. Chinn, P. Burney and F. Neukirch (2001). Does living on a farm during childhood protect against asthma, allergic rhinitis, and atopy in adulthood? *Am J Respir Crit Care Med* 164(10 Pt 1): 1829-34.

Li, B., H. Bassiri, M. D. Rossman, P. Kramer, A. F. Eyuboglu, M. Torres, E. Sada, T. Imir and S. R. Carding (1998). Involvement of the Fas/Fas ligand pathway in activation-induced cell death of mycobacteria-reactive human gamma delta T cells: a mechanism for the loss of gamma delta T cells in patients with pulmonary tuberculosis. *J Immunol* 161(3): 1558-67.

Lighvani, A. A., D. M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B. D. Hissong, B. V. Nguyen, M. Gadina, A. Sher, W. E. Paul and J. J. O'Shea (2001). T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A* 98(26): 15137-42.

Lim, S., E. Crawley, P. Woo and P. J. Barnes (1998). Haplotype associated with low interleukin-10 production in patients with severe asthma. *Lancet* 352(9122): 113.

Lin, J. X., T. S. Migone, M. Tsang, M. Friedmann, J. A. Weatherbee, L. Zhou, A. Yamauchi, E. T. Bloom, J. Mietz, S. John and et al. (1995). The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* 2(4): 331-9.

Ling, E. M., T. Smith, X. D. Nguyen, C. Pridgeon, M. Dallman, J. Arbery, V. A. Carr and D. S. Robinson (2004). Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 363(9409): 608-15.

Lipford, G. B., M. Bauer, C. Blank, R. Reiter, H. Wagner and K. Heeg (1997). CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur J Immunol* 27(9): 2340-4.

Liu, A. H. (2002). Endotoxin exposure in allergy and asthma: reconciling a paradox. *J Allergy Clin Immunol* 109(3): 379-92.

Lowenthal, J., B. Castle, J. Christiansen, J. Schreurs, D. Rennick, N. Arai, P. Hoy, Y. Takebe and M. Howard (1988). Expression of high affinity receptors for murine interleukin 4 (BSF-1) on hemopoietic and nonhemopoietic cells. *J Immunol* 140(2): 456-464.

Luft, C., M. Hausding and S. Finotto (2004). Regulation of T cells in asthma: implications for genetic manipulation. *Curr Opin Allergy Clin Immunol* 4(1): 69-74.

Lukacs, N., R. Strieter, S. Chensue and S. Kunkel (1994). Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* 10(5): 526-532.

Lundy, S. K., A. A. Berlin and N. W. Lukacs (2003). Interleukin-12-independent down-modulation of cockroach antigen-induced asthma in mice by intranasal exposure to bacterial lipopolysaccharide. *Am J Pathol* 163(5): 1961-8.

MacAry, P. A., B. J. Holmes and D. M. Kemeny (1997). Generation of rat MHC class-I-restricted ovalbumin-specific IgE inhibitory CD8+ T cell clones. *Int Arch Allergy Immunol* 113(1-3): 279-80.

Macaubas, C. and P. G. Holt (2001). Regulation of cytokine production in T-cell responses to inhalant allergen:GATA-3 expression distinguishes between Th1- and Th2-polarized immunity. *Int Arch Allergy Immunol* 124(1-3): 176-9.

Madden, K., J. Urban, Jr, H. Ziltener, J. Schrader, F. Finkelman and I. Katona (1991). Antibodies to IL-3 and IL-4 suppress helminth-induced intestinal mastocytosis. *J Immunol* 147(4): 1387-1391.

Maeda, N., J. Nigou, J. L. Herrmann, M. Jackson, A. Amara, P. H. Lagrange, G. Puzo, B. Gicquel and O. Neyrolles (2003). The cell surface receptor DC-SIGN discriminates between Mycobacterium species through selective recognition of the mannose caps on lipoarabinomannan. *J Biol Chem* 278(8): 5513-6.

Magram, J., S. E. Connaughton, R. R. Warrier, D. M. Carvajal, C. Y. Wu, J. Ferrante, C. Stewart, U. Sarmiento, D. A. Faherty and M. K. Gately (1996). IL-12-deficient mice are defective in IFN-γ production and type 1 cytokine responses. *Immunity* 4(5): 471-81.

Major, T., G. Wohlleben, B. Reibetanz and K. J. Erb (2002). Application of heat killed Mycobacterium bovis-BCG into the lung inhibits the development of allergen-induced Th2 responses. *Vaccine* 20(11-12): 1532-40.

Maliszewski, C. R., P. J. Morrissey, W. C. Fanslow, T. A. Sato, C. Willis and B. Davison (1992). Delayed allograft rejection in mice transgenic for a soluble form of the IL-4 receptor. *Cell Immunol* 143(2): 434-48.

Maliszewski, C. R., T. A. Sato, B. Davison, C. A. Jacobs, F. D. Finkelman and W. C. Fanslow (1994). In vivo biological effects of recombinant soluble interleukin-4 receptor. *Proc Soc Exp Biol Med* 206(3): 233-7.

Malmberg, P. and K. Larsson (1993). Acute exposure to swine dust causes bronchial hyperresponsiveness in healthy subjects. *Eur Respir J* 6(3): 400-4.

Manfredi, A. A., S. Heltai, P. Rovere, C. Sciorati, C. Paolucci, G. Galati, C. Rugarli, R. Vaiani, E. Clementi and M. Ferrarini (1998). Mycobacterium tuberculosis exploits the CD95/CD95 ligand system of gammadelta T cells to cause apoptosis. *Eur J Immunol* 28(6): 1798-806.

Maraveyas, A., B. Baban, D. Kennard, G. A. Rook, M. Westby, J. M. Grange, P. Lydyard, J. L. Stanford, M. Jones, P. Selby and A. G. Dalgleish (1999). Possible improved survival of patients with stage IV AJCC melanoma receiving SRL 172 immunotherapy: correlation with induction of increased levels of intracellular interleukin-2 in peripheral blood lymphocytes. *Ann Oncol* 10(7): 817-24.

Marchant, A., T. Goetghebuer, M. O. Ota, I. Wolfe, S. J. Ceesay, D. De Groote, T. Corrah, S. Bennett, J. Wheeler, K. Huygen, P. Aaby, K. P. McAdam and M. J. Newport (1999). Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination. *J Immunol* 163(4): 2249-55.

Marshall, J. D., J. J. Eiden, A. Kagey-Sabotka, P. S. Creticos, L. M. Lichtenstein and G. van Nest (2000). ISS conjugated to Amb a 1 allergen promotes a Th2 to Th1 switch in PBMC from ragweed allergic humans (abstract). *J Allergy Clin Immunol* 105: S76.

Martin, R. J., M. Boguniewicz, J. E. Henson, A. C. Celniker, M. Williams, R. C. Giorno and D. Y. Leung (1993). The effects of inhaled interferon gamma in normal human airways. *Am Rev Respir Dis* 148(6 Pt 1): 1677-82.

Martinez, F. D. (1994). Role of viral infections in the inception of asthma and allergies during childhood: could they be protective? *Thorax* 49(12): 1189-91.

Martinez, F. D. and P. G. Holt (1999). Role of microbial burden in aetiology of allergy and asthma. *Lancet* 354 Suppl 2: SII12-5.

Martinez, F. D., D. A. Stern, A. L. Wright, C. J. Holberg, L. M. Taussig and M. Halonen (1995). Association of interleukin-2 and interferon-gamma production by blood mononuclear cells in infancy with parental allergy skin tests and with subsequent development of atopy. *J Allergy Clin Immunol* 96(5 Pt 1): 652-60.

Martins, T. C. and A. P. Aguas (1999). Mechanisms of Mycobacterium avium-induced resistance against insulin-dependent diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice: role of Fas and Th1 cells. *Clin Exp Immunol* 115(2): 248-54.

Mason, D. and F. Powrie (1998). Control of immune pathology by regulatory T cells. *Curr Opin Immunol* 10(6): 649-55.

Matricardi, P. M. (2001). Prevalence of atopy and asthma in eastern versus western Europe: why the difference? *Ann Allergy Asthma Immunol* 87(6 Suppl 3): 24-7.

Matricardi, P. M., B. Bjorksten, S. Bonini, J. Bousquet, R. Djukanovic, S. Dreborg, J. Gereda, H. J. Malling, T. Popov, E. Raz, H. Renz and A. Wold (2003). Microbial products in allergy prevention and therapy. *Allergy* 58(6): 461-71.

Matricardi, P. M., F. Rosmini, L. Ferrigno, R. Nisini, M. Rapicetta, P. Chionne, T. Stroffolini, P. Pasquini and R. D'Amelio (1997). Cross sectional retrospective study of prevalence of atopy among Italian military students with antibodies against hepatitis A virus [see comments]. *BMJ* 314(7086): 999-1003.

Matricardi, P. M., F. Rosmini, S. Riondino, M. Fortini, L. Ferrigno, M. Rapicetta and S. Bonini (2000). Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. *BMJ* 320(7232): 412-7.

Matricardi, P. M. and M. Yazdanbakhsh (2003). Mycobacteria and atopy, 6 years later: a fascinating, still unfinished, business. *Clin Exp Allergy* 33(6): 717-20.

Matsui, E., H. Kaneko, T. Fukao, T. Teramoto, R. Inoue, M. Watanabe, K. Kasahara and N. Kondo (1999). Mutations of the IL-12 receptor beta2 chain gene in atopic subjects. *Biochem Biophys Res Commun* 266(2): 551-5.

Matsui, E., H. Kaneko, T. Teramoto, T. Fukao, R. Inoue, K. Kasahara, M. Takemura, M. Seishima and N. Kondo (2000). Reduced IFNgamma production in response to IL-12 stimulation and/or reduced IL-12 production in atopic patients. *Clin Exp Allergy* 30(9): 1250-6.

Matsui, K., N. Tanaka and A. Nishikawa (2001). Lipopolysaccharide of Haemophilus influenzae induces interleukin-5 mRNA expression in human peripheral blood mononuclear cells. *J Interferon Cytokine Res* 21(6): 439-43.

McIntire, J. J., D. T. Umetsu and R. H. DeKruyff (2004). TIM-1, a novel allergy and asthma susceptibility gene. *Springer Semin Immunopathol* 25(3-4): 335-48.

McIntire, J. J., S. E. Umetsu, O. Akbari, M. Potter, V. K. Kuchroo, G. S. Barsh, G. J. Freeman, D. T. Umetsu and R. H. DeKruyff (2001). Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. *Nat Immunol* 2(12): 1109-16.

McKenzie, G. J., C. L. Emson, S. E. Bell, S. Anderson, P. Fallon, G. Zurawski, R. Murray, R. Grencis and A. N. McKenzie (1998). Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* 9(3): 423-32.

Means, T. K., S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock and M. J. Fenton (1999). Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J Immunol* 163(7): 3920-7.

Medzhitov, R. and C. Janeway, Jr. (2000). Innate immunity. *N Engl J Med* 343(5): 338-44.

Medzhitov, R., P. Preston-Hurlburt and C. A. Janeway, Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388(6640): 394-7.

Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh and C. A. Janeway, Jr. (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* 2(2): 253-8.

Meerschaert, J., W. W. Busse, P. J. Bertics and D. F. Mosher (2000). CD14(+) cells are necessary for increased survival of eosinophils in response to lipopolysaccharide. *Am J Respir Cell Mol Biol* 23(6): 780-7.

Mendes, R., M. E. O'Brien, A. Mitra, A. Norton, R. K. Gregory, A. R. Padhani, K. V. Bromelow, A. R. Winkley, S. Ashley, I. E. Smith and B. E. Souberbielle (2002). Clinical and immunological

assessment of Mycobacterium vaccae (SRL172) with chemotherapy in patients with malignant mesothelioma. *Br J Cancer* 86(3): 336-41.

Menzies-Gow, A. and D. S. Robinson (2002). Eosinophils, eosinophilic cytokines (interleukin-5), and antieosinophilic therapy in asthma. *Curr Opin Pulm Med* 8(1): 33-8.

Metzger, W. J., H. B. Richerson, K. Worden, M. Monick and G. W. Hunninghake (1986). Bronchoalveolar lavage of allergic asthmatic patients following allergen bronchoprovocation. *Chest* 89(4): 477-83.

Michel, O., R. Ginanni, J. Duchateau, F. Vertongen, B. Le Bon and R. Sergysels (1991). Domestic endotoxin exposure and clinical severity of asthma. *Clin Exp Allergy* 21(4): 441-8.

Michel, O., R. Ginanni, B. Le Bon, J. Content, J. Duchateau and R. Sergysels (1992). Inflammatory response to acute inhalation of endotoxin in asthmatic patients. *Am Rev Respir Dis* 146(2): 352-7.

Michel, O., J. Kips, J. Duchateau, F. Vertongen, L. Robert, H. Collet, R. Pauwels and R. Sergysels (1996). Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 154(6 Pt 1): 1641-6.

Mitsuyasu, H., Y. Yanagihara, X. Q. Mao, P. S. Gao, Y. Arinobu, K. Ihara, A. Takabayashi, T. Hara, T. Enomoto, S. Sasaki, M. Kawai, N. Hamasaki, T. Shirakawa, J. M. Hopkin and K. Izuhara (1999). Cutting edge: dominant effect of Ile50Val variant of the human IL-4 receptor alpha-chain in IgE synthesis. *J Immunol* 162(3): 1227-31.

Miyazaki, T., A. Kawahara, H. Fujii, Y. Nakagawa, Y. Minami, Z. J. Liu, I. Oishi, O. Silvennoinen, B. A. Witthuhn, J. N. Ihle and et al. (1994). Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* 266(5187): 1045-7.

Mochizuki, M., J. Bartels, A. I. Mallet, E. Christophers and J.-M. Schroder (1998). IL-4 Induces Eotaxin: A Possible Mechanism of Selective Eosinophil Recruitment in Helminth Infection and Atopy. *J Immunol* 160(1): 60-68.

Monahan, J., N. Siegel, R. Keith, M. Caparon, L. Christine, R. Compton, S. Cusik, J. Hirsch, M. Huynh, C. Devine, J. Polazzi, S. Rangwala, B. Tsai and J. Portanova (1997). Attenuation of IL-5-mediated signal transduction, eosinophil survival, and inflammatory mediator release by a soluble human IL-5 receptor. *J Immunol* 159(8): 4024-34.

Moore, P. E., T. L. Church, D. D. Chism, R. A. Panettieri, Jr. and S. A. Shore (2002). IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am J Physiol Lung Cell Mol Physiol* 282(4): L847-53.

Moser, M. and K. M. Murphy (2000). Dendritic cell regulation of TH1-TH2 development. *Nat Immunol* 1(3): 199-205.

Moser, R., J. Fehr and P. L. Bruijnzeel (1992). IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. *J Immunol* 149(4): 1432-8.

Mosmann, T. R. and R. L. Coffman (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7: 145-73.

Mosmann, T. R. and S. Sad (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 17(3): 138-46.

Mueller, T. D., J. L. Zhang, W. Sebald and A. Duschl (2002). Structure, binding, and antagonists in the IL-4/IL-13 receptor system. *Biochim Biophys Acta* 1592(3): 237-50.

Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang and S. L. Reiner (2001). Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292(5523): 1907-10.

Munk, M. E. and M. Emoto (1995). Functions of T-cell subsets and cytokines in mycobacterial infections. *Eur Respir J Suppl* 20: 668s-675s.

Munk, M. E., P. Mayer, P. Anding, K. Feldmann and S. H. Kaufmann (1996). Increased numbers of interleukin-12-producing cells in human tuberculosis. *Infect Immun* 64(3): 1078-80.

Murata, T., J. Taguchi and R. K. Puri (1998). Interleukin-13 Receptor alpha ' But Not alpha Chain: A Functional Component of Interleukin-4 Receptors. *Blood* 91(10): 3884-3891.

Mwinga, A., A. Nunn, B. Ngwira, C. Chintu, D. Warndorff, P. Fine, J. Darbyshire and A. Zumla (2002). Mycobacterium vaccae (SRL172) immunotherapy as an adjunct to standard antituberculosis treatment in HIV-infected adults with pulmonary tuberculosis: a randomised placebo-controlled trial. *Lancet* 360(9339): 1050-5.

Nakagawa, R., T. Naka, H. Tsutsui, M. Fujimoto, A. Kimura, T. Abe, E. Seki, S. Sato, O. Takeuchi, K. Takeda, S. Akira, K. Yamanishi, I. Kawase, K. Nakanishi and T. Kishimoto (2002). SOCS-1 participates in negative regulation of LPS responses. *Immunity* 17(5): 677-87.

Nakajima, H., H. Sano, T. Nishimura, S. Yoshida and I. Iwamoto (1994). Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 interactions in antigen-induced eosinophil and T cell recruitment into the tissue. *J. Exp. Med.* 179(4): 1145-1154.

Nakamura, Y., O. Ghaffar, R. Olivenstein, R. A. Taha, A. Soussi-Gounni, D. H. Zhang, A. Ray and Q. Hamid (1999). Gene expression of the GATA-3 transcription factor is increased in atopic asthma. *The Journal of Allergy and Clinical Immunology* 103(1): 215-222.

Nakstad, B., H. Kahler and T. Lyberg (1999). Allergen-stimulated expression of CD154 (CD40 ligand) on CD3+ lymphocytes in atopic, but not in nonatopic individuals. Modulation by bacterial lipopolysaccharide. *Allergy* 54(7): 722-9.

Naseer, T., E. M. Minshall, D. Y. Leung, S. Laberge, P. Ernst, R. J. Martin and Q. Hamid (1997). Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy. *Am J Respir Crit Care Med* 155(3): 845-51.

Nelms, K., A. D. Keegan, J. Zamorano, J. J. Ryan and W. E. Paul (1999). The IL-4 receptor: Signaling Mechanisms and Biologic Functions. *Annu. Rev. Immunol.* 17(1): 701-738.

Neurath, M. F., S. Finotto and L. H. Glimcher (2002). The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* 8(6): 567-73.

Nicolai, T., B. Bellach, E. V. Mutius, W. Thefeld and H. Hoffmeister (1997). Increased prevalence of sensitization against aeroallergens in adults in West compared with East Germany. *Clin Exp Allergy* 27(8): 886-92.

Nigou, J., M. Gilleron, B. Cahuzac, J. D. Bounery, M. Herold, M. Thurnher and G. Puzo (1997). The phosphatidyl-myo-inositol anchor of the lipoarabinomannans from Mycobacterium bovis bacillus Calmette Guerin. Heterogeneity, structure, and role in the regulation of cytokine secretion. *J Biol Chem* 272(37): 23094-103.

Nigou, J., C. Zelle-Rieser, M. Gilleron, M. Thurnher and G. Puzo (2001). Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J Immunol* 166(12): 7477-85.

Niimi, A., R. Amitani, K. Suzuki, E. Tanaka, T. Murayama and F. Kuze (1998). Serum eosinophil cationic protein as a marker of eosinophilic inflammation in asthma. *Clin Exp Allergy* 28(2): 233-40.

Nishikomori, R., R. O. Ehrhardt and W. Strober (2000). T helper type 2 cell differentiation occurs in the presence of interleukin 12 receptor beta2 chain expression and signaling. *J Exp Med* 191(5): 847-58.

Noguchi, M., H. Yi, H. M. Rosenblatt, A. H. Filipovich, S. Adelstein, W. S. Modi, O. W. McBride and W. J. Leonard (1993). Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 73(1): 147-57.

Nonaka, M., R. Pawankar, A. Fukumoto, N. Ogihara, A. Sakanushi and T. Yagi (2004). Induction of eotaxin production by interleukin-4, interleukin-13 and lipopolysaccharide by nasal fibroblasts. *Clin Exp Allergy* 34(5): 804-11.

Nouri-Aria, K. T., P. A. Wachholz, J. N. Francis, M. R. Jacobson, S. M. Walker, L. K. Wilcock, S. Q. Staple, R. C. Aalberse, S. J. Till and S. R. Durham (2004). Grass pollen immunotherapy

induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 172(5): 3252-9.

O'Brien, M. E., H. Anderson, E. Kaukel, K. O'Byrne, M. Pawlicki, J. Von Pawel and M. Reck (2004). SRL172 (killed Mycobacterium vaccae) in addition to standard chemotherapy improves quality of life without affecting survival, in patients with advanced non-small-cell lung cancer: phase III results. *Ann Oncol* 15(6): 906-14.

O'Brien, M. E., A. Saini, I. E. Smith, A. Webb, K. Gregory, R. Mendes, C. Ryan, K. Priest, K. V. Bromelow, R. D. Palmer, N. Tuckwell, D. A. Kennard and B. E. Souberbielle (2000). A randomized phase II study of SRL172 (Mycobacterium vaccae) combined with chemotherapy in patients with advanced inoperable non-small-cell lung cancer and mesothelioma. *Br J Cancer* 83(7): 853-7.

Oddo, M., T. Renno, A. Attinger, T. Bakker, H. R. MacDonald and P. R. Meylan (1998). Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. *J Immunol* 160(11): 5448-54.

O'Grady, N. P., H. L. Preas, II, J. Pugin, C. Fiuza, M. Tropea, D. REDA, S. M. BANKS and A. F. SUFFREDINI (2001). Local Inflammatory Responses following Bronchial Endotoxin Instillation in Humans. *Am. J. Respir. Crit. Care Med.* 163(7): 1591-1598.

Ohara, J. and W. E. Paul (1987). Receptors for B-cell stimulatory factor-1 expressed on cells of haematopoietic lineage. *Nature* 325(6104): 537-40.

Ohnishi, T., S. Sur, D. S. Collins, J. E. Fish, G. J. Gleich and S. P. Peters (1993). Eosinophil survival activity identified as interleukin-5 is associated with eosinophil recruitment and degranulation and lung injury twenty-four hours after segmental antigen lung challenge. *J Allergy Clin Immunol* 92(4): 607-15.

Olivenstein, R., R. Taha, E. M. Minshall and Q. A. Hamid (1999). IL-4 and IL-5 mRNA expression in induced sputum of asthmatic subjects: comparison with bronchial wash. *J Allergy Clin Immunol* 103(2 Pt 1): 238-45.

Omenaas, E., H. F. Jentoft, W. M. Vollmer, A. S. Buist and A. Gulsvik (2000). Absence of relationship between tuberculin reactivity and atopy in BCG vaccinated young adults. *Thorax* 55(6): 454-8.

Omenaas, E., H. F. Jentoft, W. M. Vollmer, A. S. Buist and A. Gulsvik (2000). Absence of relationship between tuberculin reactivity and atopy in BCG vaccinated young adults [see comments]. *Thorax* 55(6): 454-8.

Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J. S. Abrams, K. W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J. F. Bazan and R. A. Kastelein (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13(5): 715-25.

O'Shea, J. J. and W. E. Paul (2002). Regulation of T(H)1 differentiation--controlling the controllers. *Nat Immunol* 3(6): 506-8.

Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa and T. Seya (2003). TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 4(2): 161-7.

Oskeritzian, C. A. and L. B. Schwartz (2000). Human mast cells and basophils:heterogeneity and mediators. Asthma & Rhinitis. W. W. Busse and S. T. Holgate. Oxford, Blackwell. **1:** 275-295.

Ota, M. O., M. A. van der Sande, G. E. Walraven, D. Jeffries, O. A. Nyan, A. Marchant and K. P. McAdam (2003). Absence of association between delayed type hypersensitivity to tuberculin and atopy in children in The Gambia. *Clin Exp Allergy* 33(6): 731-6.

Ottenhoff, T. H., T. de Boer, C. E. Verhagen, F. A. Verreck and J. T. van Dissel (2000). Human deficiencies in type 1 cytokine receptors reveal the essential role of type 1 cytokines in immunity to intracellular bacteria. *Microbes Infect* 2(13): 1559-66.

Ouyang, W., S. H. Ranganath, K. Weindel, D. Bhattacharya, T. L. Murphy, W. C. Sha and K. M. Murphy (1998). Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* 9(5): 745-755.

Ownby, D. R., C. C. Johnson and E. L. Peterson (2002). Exposure to dogs and cats in the first year of life and risk of allergic sensitization at 6 to 7 years of age. *Jama* 288(8): 963-72.

Ozdemir, C., T. Akkoc, N. N. Bahceciler, D. Kucukercan, I. B. Barlan and M. M. Basaran (2003). Impact of Mycobacterium vaccae immunization on lung histopathology in a murine model of chronic asthma. *Clin Exp Allergy* 33(2): 266-70.

Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder and A. Aderem (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *PNAS* 97(25): 13766-13771.

Ozmen, S., N. Tomac, A. Uysal, Z. Arslan, N. Kuyucu and A. Yoney (2002). Tuberculin responses in children with allergic diseases. *Allergy* 57(11): 1059-62.

Pahari, A., S. Welch and S. Lingam (2002). BCG, tuberculin skin-test results and asthma prevalence in school children in North London. *Indian Pediatr* 39(3): 254-8.

Panina-Bordignon, P., D. Mazzeo, P. D. Lucia, D. D'Ambrosio, R. Lang, L. Fabbri, C. Self and F. Sinigaglia (1997). Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12. *J Clin Invest* 100(6): 1513-9.

Panina-Bordignon, P., A. Papi, M. Mariani, P. Di Lucia, G. Casoni, C. Bellettato, C. Buonsanti, D. Miotto, C. Mapp, A. Villa, G. Arrigoni, L. M. Fabbri and F. Sinigaglia (2001). The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* 107(11): 1357-64.

Pauwels, R. (1989). The relationship between airway inflammation and bronchial hyperresponsiveness. *Clin Exp Allergy* 19(4): 395-8.

Peat, J. K., W. J. Britton, C. M. Salome and A. J. Woolcock (1987). Bronchial hyperresponsiveness in two populations of Australian schoolchildren. III. Effect of exposure to environmental allergens. *Clin Allergy* 17(4): 291-300.

Peat, J. K., C. M. Salome and A. J. Woolcock (1992). Factors associated with bronchial hyperresponsiveness in Australian adults and children. *Eur Respir J* 5(8): 921-9.

Penido, C., H. C. Castro-Faria-Neto, A. Vieira-de-Abreu, R. T. Figueiredo, A. Pelled, M. A. Martins, P. J. Jose, T. J. Williams and P. T. Bozza (2001). LPS induces eosinophil migration via CCR3 signaling through a mechanism independent of RANTES and Eotaxin. *Am J Respir Cell Mol Biol* 25(6): 707-16.

Phipatanakul, W., J. C. Celedon, B. A. Raby, A. A. Litonjua, D. K. Milton, D. Sredl, S. T. Weiss and D. R. Gold (2004). Endotoxin exposure and eczema in the first year of life. *Pediatrics* 114(1): 13-8.

Piccinni, M. P., L. Beloni, L. Giannarini, C. Livi, G. Scarselli, S. Romagnani and E. Maggi (1996). Abnormal production of T helper 2 cytokines interleukin-4 and interleukin-5 by T cells from newborns with atopic parents. *Eur J Immunol* 26(10): 2293-8.

Platts-Mills, T. A. and M. C. Carter (1997). Asthma and indoor exposure to allergens. *N Engl J Med* 336(19): 1382-4.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton and B. Beutler (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282(5396): 2085-8.

Porcelli, S. A. and R. L. Modlin (1999). The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu Rev Immunol* 17: 297-329.

Prescott, S. L., C. Macaubas, T. Smallacombe, B. J. Holt, P. D. Sly and P. G. Holt (1999). Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 353(9148): 196-200.

Presky, D. H., H. Yang, L. J. Minetti, A. O. Chua, N. Nabavi, C. Y. Wu, M. K. Gately and U. Gubler (1996). A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc Natl Acad Sci U S A* 93(24): 14002-7.

Prigozy, T. I., P. A. Sieling, D. Clemens, P. L. Stewart, S. M. Behar, S. A. Porcelli, M. B. Brenner, R. L. Modlin and M. Kronenberg (1997). The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. *Immunity* 6(2): 187-97.

Prior, J. G., A. A. Khan, K. A. Cartwright, P. A. Jenkins and J. L. Stanford (1995). Immunotherapy with Mycobacterium vaccae combined with second line chemotherapy in drug-resistant abdominal tuberculosis. *J Infect* 31(1): 59-61.

Puccetti, P., A. Mencacci, E. Cenci, R. Spaccapelo, P. Mosci, K. H. Enssle, L. Romani and F. Bistoni (1994). Cure of murine candidiasis by recombinant soluble interleukin-4 receptor. *J Infect Dis* 169(6): 1325-31.

Puggioni, F., S. R. Durham and J. N. Francis (2005). Monophosphoryl lipid A (MPL) promotes allergen-induced immune deviation in favour of Th1 responses. *Allergy* 60(5): 678-84.

Punnonen, J., G. Aversa, B. Cocks, A. McKenzie, S. Menon, G. Zurawski, R. Malefyt and J. de Vries (1993). Interleukin 13 Induces Interleukin 4-Independent IgG4 and IgE Synthesis and CD23 Expression by Human B Cells. *PNAS* 90(8): 3730-3734.

Qin, H. Y., M. W. Sadelain, C. Hitchon, J. Lauzon and B. Singh (1993). Complete Freund's adjuvant-induced T cells prevent the development and adoptive transfer of diabetes in nonobese diabetic mice. *J Immunol* 150(5): 2072-80.

Quesniaux, V., C. Fremond, M. Jacobs, S. Parida, D. Nicolle, V. Yeremeev, F. Bihl, F. Erard, T. Botha, M. Drennan, M. N. Soler, M. Le Bert, B. Schnyder and B. Ryffel (2004). Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes Infect* 6(10): 946-59.

Raetz, C. R. and C. Whitfield (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem* 71: 635-700.

Rao, A. and O. Avni (2000). Molecular aspects of T-cell differentiation. *British Medical Bulletin* 56(4): 969-984.

Ray, A. and L. Cohn (1999). Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. *The Journal of Clinical Investigation* 104(8): 985-993.

Re, F. and J. L. Strominger (2001). Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J Biol Chem* 276(40): 37692-9.

Reichel, M., B. Nelson, P. Greenberg and P. Rothman (1997). The IL-4 receptor alpha-chain cytoplasmic domain is sufficient for activation of JAK-1 and STAT6 and the induction of IL-4-specific gene expression. *J Immunol* 158(12): 5860-5867.

Renz, H., K. Bradley, K. Enssle, J. E. Loader, G. L. Larsen and E. W. Gelfand (1996). Prevention of the development of immediate hypersensitivity and airway hyperresponsiveness following in vivo treatment with soluble IL-4 receptor. *Int Arch Allergy Immunol* 109(2): 167-76.

Renz, H., K. Enssle, L. Lauffer, R. Kurrle and E. W. Gelfand (1995). Inhibition of allergen-induced IgE and IgG1 production by soluble IL-4 receptor. *Int Arch Allergy Immunol* 106(1): 46-54.

Resnick, M. B. and P. F. Weller (1993). Mechanisms of eosinophil recruitment. *Am J Respir Cell Mol Biol* 8(4): 349-55.

Riedler, J., C. Braun-Fahrlander, W. Eder, M. Schreuer, M. Waser, S. Maisch, D. Carr, R. Schierl, D. Nowak and E. von Mutius (2001). Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 358(9288): 1129-33.

Riffo-Vasquez, Y. and D. Spina (2002). Role of cytokines and chemokines in bronchial hyperresponsiveness and airway inflammation. *Pharmacol Ther* 94(3): 185-211.

Rincon, M., J. Anguita, T. Nakamura, E. Fikrig and R. A. Flavell (1997). Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp Med* 185(3): 461-9.

Rissoan, M. C., V. Soumelis, N. Kadowaki, G. Grouard, F. Briere, R. de Waal Malefyt and Y. J. Liu (1999). Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283(5405): 1183-6.

Robertson, D. G., A. T. Kerigan, F. E. Hargreave, R. Chalmers and J. Dolovich (1974). Late asthmatic responses induced by ragweed pollen allergen. *J Allergy Clin Immunol* 54(4): 244-54.

Robinson, D., Q. Hamid, A. Bentley, S. Ying, A. B. Kay and S. R. Durham (1993). Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol* 92(2): 313-24.

Robinson, D., Q. Hamid, S. Ying, A. Bentley, B. Assoufi, S. Durham and A. B. Kay (1993). Prednisolone treatment in asthma is associated with modulation of bronchoalveolar lavage cell interleukin-4, interleukin-5, and interferon-gamma cytokine gene expression. *Am Rev Respir Dis* 148(2): 401-6.

Robinson, D., K. Shibuya, A. Mui, F. Zonin, E. Murphy, T. Sana, S. B. Hartley, S. Menon, R. Kastelein, F. Bazan and A. O'Garra (1997). IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity* 7(4): 571-81.

Robinson, D. S. (2000). Th-2 cytokines in allergic diseases. *British Medical Bulletin* 56(4): 956-968.

Robinson, D. S., B. Assoufi, S. R. Durham and A. B. Kay (1995). Eosinophil cationic protein (ECP) and eosinophil protein X (EPX) concentrations in serum and bronchial lavage fluid in asthma. Effect of prednisolone treatment. *Clin Exp Allergy* 25(11): 1118-27.

Robinson, D. S., A. M. Bentley, A. Hartnell, A. B. Kay and S. R. Durham (1993). Activated memory T helper cells in bronchoalveolar lavage fluid from patients with atopic asthma: relation to asthma symptoms, lung function, and bronchial responsiveness. *Thorax* 48(1): 26-32.

Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham and A. B. Kay (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 326(5): 298-304.

Robinson, D. S., S. Ying, A. M. Bentley, Q. Meng, J. North, S. R. Durham, A. B. Kay and Q. Hamid (1993). Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *The Journal of Allergy and Clinical Immunology* 92(3): 397-403.

Rodriguez, M. R., M. F. Gonzalez-Escribano, F. Aguilar, A. Valenzuela, A. Garcia and A. Nunez-Roldan (2002). Association of NRAMP1 promoter gene polymorphism with the susceptibility and radiological severity of rheumatoid arthritis. *Tissue Antigens* 59(4): 311-5.

Rogge, L., L. Barberis-Maino, M. Biffi, N. Passini, D. H. Presky, U. Gubler and F. Sinigaglia (1997). Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J Exp Med* 185(5): 825-31.

Rogge, L., D. D'Ambrosio, M. Biffi, G. Penna, L. J. Minetti, D. H. Presky, L. Adorini and F. Sinigaglia (1998). The Role of Stat4 in Species-Specific Regulation of Th Cell Development by Type I IFNs. *J Immunol* 161(12): 6567-6574.

Rojas, M., M. Olivier, P. Gros, L. F. Barrera and L. F. Garcia (1999). TNF-alpha and IL-10 modulate the induction of apoptosis by virulent Mycobacterium tuberculosis in murine macrophages. *J Immunol* 162(10): 6122-31.

Rojas, R. E., K. N. Balaji, A. Subramanian and W. H. Boom (1999). Regulation of human CD4(+) alphabeta T-cell-receptor-positive (TCR(+)) and gammadelta TCR(+) T-cell responses to Mycobacterium tuberculosis by interleukin-10 and transforming growth factor beta. *Infect Immun* 67(12): 6461-72.

Romagnani, S. (1994). Regulation of the development of type 2 T-helper cells in allergy. *Curr Opin Immunol* 6(6): 838-46.

Romagnani, S. (1995). Biology of human TH1 and TH2 cells. *J Clin Immunol* 15(3): 121-9.

Romagnani, S. (1997). The Th1/Th2 paradigm. *Immunol Today* 18(6): 263-6.

Romagnani, S. (2004). The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? *Immunology* 112(3): 352-63.

Roman, M., E. Martin-Orozco, J. S. Goodman, M. D. Nguyen, Y. Sato, A. Ronaghy, R. S. Kornbluth, D. D. Richman, D. A. Carson and E. Raz (1997). Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med* 3(8): 849-54.

Rook, G. A., V. Adams, J. Hunt, R. Palmer, R. Martinelli and L. R. Brunet (2004). Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. *Springer Semin Immunopathol* 25(3-4): 237-55.

Rook, G. A., R. Martinelli and L. R. Brunet (2003). Innate immune responses to mycobacteria and the downregulation of atopic responses. *Curr Opin Allergy Clin Immunol* 3(5): 337-42.

Rook, G. A. and J. L. Stanford (1998). Give us this day our daily germs. *Immunol Today* 19(3): 113-6.

Rook, G. A. and J. L. Stanford (1999). Skin-test responses to mycobacteria in atopy and asthma. *Allergy* 54(3): 285-6.

Roth, I., D. B. Corry, R. M. Locksley, J. S. Abrams, M. J. Litton and S. J. Fisher (1996). Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. *J Exp Med* 184(2): 539-48.

Rothenberg, M. E. (1998). Eosinophilia. *N Engl J Med* 338(22): 1592-1600.

Roy, S. R., A. M. Schiltz, A. Marotta, Y. Shen and A. H. Liu (2003). Bacterial DNA in house and farm barn dust. *J Allergy Clin Immunol* 112(3): 571-8.

Russell, S. M., A. D. Keegan, N. Harada, Y. Nakamura, M. Noguchi, P. Leland, M. C. Friedmann, A. Miyajima, R. K. Puri, W. E. Paul and et al. (1993). Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. *Science* 262(5141): 1880-3.

Sabroe, I., R. C. Read, M. K. Whyte, D. H. Dockrell, S. N. Vogel and S. K. Dower (2003). Toll-like receptors in health and disease: complex questions remain. *J Immunol* 171(4): 1630-5.

Saetta, M., A. Di Stefano, C. Rosina, G. Thiene and L. M. Fabbri (1991). Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. *Am Rev Respir Dis* 143(1): 138-43.

Sakaguchi, S. (2003). The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. *J Clin Invest* 112(9): 1310-2.

Sareneva, T., I. Julkunen and S. Matikainen (2000). IFN-alpha and IL-12 induce IL-18 receptor gene expression in human NK and T cells. *J Immunol* 165(4): 1933-8.

Sato, T. A., M. B. Widmer, F. D. Finkelman, H. Madani, C. A. Jacobs, K. H. Grabstein and C. R. Maliszewski (1993). Recombinant soluble murine IL-4 receptor can inhibit or enhance IgE responses in vivo. *J Immunol* 150(7): 2717-23.

Sayers, I., W. Severn, C. B. Scanga, J. Hudson, G. Le Gros and J. L. Harper (2004). Suppression of allergic airway disease using mycobacterial lipoglycans. *J Allergy Clin Immunol* 114(2): 302-9.

Schaible, U. E. and S. H. Kaufmann (2000). CD1 and CD1-restricted T cells in infections with intracellular bacteria. *Trends Microbiol* 8(9): 419-25.

Schleimer, R., S. Sterbinsky, J. Kaiser, C. Bickel, D. Klunk, K. Tomioka, W. Newman, F. Luscinskas, M. Gimbrone, Jr and B. McIntyre (1992). IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. *J Immunol* 148(4): 1086-1092.

Schmekel, B., J. Ahlner, M. Malmstrom and P. Venge (2001). Eosinophil cationic protein (ECP) in saliva: a new marker of disease activity in bronchial asthma. *Respir Med* 95(8): 670-5.

Schnare, M., G. M. Barton, A. C. Holt, K. Takeda, S. Akira and R. Medzhitov (2001). Toll-like receptors control activation of adaptive immune responses. *Nat Immunol* 2(10): 947-50.

Schneerson, R., A. Fattom, S. C. Szu, D. Bryla, J. T. Ulrich, J. A. Rudbach, G. Schiffman and J. B. Robbins (1991). Evaluation of monophosphoryl lipid A (MPL) as an adjuvant. Enhancement of the serum antibody response in mice to polysaccharide-protein conjugates by concurrent injection with MPL. *J Immunol* 147(7): 2136-40.

Schulz, O., H. F. Sewell and F. Shakib (1998). Proteolytic Cleavage of CD25, the alpha Subunit of the Human T Cell Interleukin 2 Receptor, by Der p 1, a Major Mite Allergen with Cysteine Protease Activity. *J. Exp. Med.* 187(2): 271-275.

Schwartz, D. A. (1996). Grain dust, endotoxin, and airflow obstruction. *Chest* 109(3 Suppl): 57S-63S.

Seder, R. A., R. Gazzinelli, A. Sher and W. E. Paul (1993). Interleukin 12 acts directly on $CD4^+$ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci USA* 90(21): 10188-92.

Seder, R. A., W. E. Paul, M. M. Davis and B. Fazekas de St Groth (1992). The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. *J Exp Med* 176(4): 1091-8.

Seder, R. A., W. E. Paul, M. M. Davis and B. Fazekas de St. Groth (1992). The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of $CD4^+$ T cells from T cell receptor transgenic mice. *J Exp Med* 176(4): 1091-8.

Sehmi, R., K. Howie, D. Sutherland, W. Schragge, P. O'Byrne and J. Denburg (1996). Increased levels of CD34+ hemopoietic progenitor cells in atopic subjects. *Am. J. Respir. Cell Mol. Biol.* 15(5): 645-655.

Sengler, C., A. Haider, C. Sommerfeld, S. Lau, M. Baldini, F. Martinez, U. Wahn and R. Nickel (2003). Evaluation of the CD14 C-159 T polymorphism in the German Multicenter Allergy Study cohort. *Clin Exp Allergy* 33(2): 166-9.

Shaheen, S. O., P. Aaby, A. J. Hall, D. J. Barker, C. B. Heyes, A. W. Shiell and A. Goudiaby (1996). Measles and atopy in Guinea-Bissau. *Lancet* 347(9018): 1792-6.

Shakib, F., O. Schulz and H. Sewell (1998). A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol Today* 19(7): 313-6.

Shanafelt, A. B., C. P. Forte, J. J. Kasper, L. Sanchez-Pescador, M. Wetzel, R. Gundel and J. M. Greve (1998). An immune cell-selective interleukin 4 agonist. *Proc Natl Acad Sci U S A* 95(16): 9454-8.

Shaw, T. C., L. H. Thomas and J. S. Friedland (2000). Regulation of IL-10 secretion after phagocytosis of Mycobacterium tuberculosis by human monocytic cells. *Cytokine* 12(5): 483-6.

Sheikh, A., L. Smeeth and R. Hubbard (2003). There is no evidence of an inverse relationship between TH2-mediated atopy and TH1-mediated autoimmune disorders: Lack of support for the hygiene hypothesis. *J Allergy Clin Immunol* 111(1): 131-5.

Shi, H.-Z., J.-M. Deng, H. XU, Z.-X. NONG, C.-Q. XIAO, Z.-M. LIU, S.-M. QIN, H.-X. JIANG, G.-N. LIU and Y.-Q. CHEN (1998). Effect of Inhaled Interleukin-4 on Airway Hyperreactivity in Asthmatics. *Am. J. Respir. Crit. Care Med.* 157(6): 1818-1821.

Shi, H. Z. and X. J. Qin (2005). CD4CD25 regulatory T lymphocytes in allergy and asthma. *Allergy* 60(8): 986-95.

Shirakawa, T., T. Enomoto, S. Shimazu and J. M. Hopkin (1997). The inverse association between tuberculin responses and atopic disorder. *Science* 275(5296): 77-9.

Shirtcliffe, P. M., S. E. Easthope, S. Cheng, M. Weatherall, P. L. Tan, G. Le Gros and R. Beasley (2001). The effect of delipidated deglycolipidated (DDMV) and heat-killed Mycobacterium vaccae in asthma. *Am J Respir Crit Care Med* 163(6): 1410-4.

Shirtcliffe, P. M., S. E. Easthope, M. Weatherall and R. Beasley (2004). Effect of repeated intradermal injections of heat-inactivated Mycobacterium bovis bacillus Calmette-Guerin in adult asthma. *Clin Exp Allergy* 34(2): 207-12.

Shirtcliffe, P. M., A. Goldkorn, M. Weatherall, P. L. Tan and R. Beasley (2003). Pilot study of the safety and effect of intranasal delipidated acid-treated Mycobacterium vaccae in adult asthma. *Respirology* 8(4): 497-503.

Skinner, M. A., S. Yuan, R. Prestidge, D. Chuk, J. D. Watson and P. L. Tan (1997). Immunization with heat-killed Mycobacterium vaccae stimulates CD8+ cytotoxic T cells specific for macrophages infected with Mycobacterium tuberculosis. *Infect Immun* 65(11): 4525-30.

Smit, J., H. Van Loveren, M. Hoekstra, M. Schijf, G. Folkerts and F. Nijkamp (2003). Mycobacterium vaccae administration during allergen sensitization or challenge suppresses asthmatic features. *Clin Exp Allergy* 33(8): 1083-1089.

Smit, J. J., G. Folkerts and F. P. Nijkamp (2004). Mycobacteria, genes and the 'hygiene hypothesis'. *Curr Opin Allergy Clin Immunol* 4(1): 57-62.

Smit, J. J., H. Van Loveren, M. O. Hoekstra, K. Karimi, G. Folkerts and F. P. Nijkamp (2003). The Slc11a1 (Nramp1) gene controls efficacy of mycobacterial treatment of allergic asthma. *J Immunol* 171(2): 754-60.

Smit, J. J., H. van Loveren, M. O. Hoekstra, F. P. Nijkamp and N. Bloksma (2003). Influence of the macrophage bacterial resistance gene, Nramp1 (Slc11a1), on the induction of allergic asthma in the mouse. *Faseb J* 17(8): 958-60.

Smit, J. J., H. Van Loveren, M. O. Hoekstra, M. A. Schijf, G. Folkerts and F. P. Nijkamp (2003). Mycobacterium vaccae administration during allergen sensitization or challenge suppresses asthmatic features. *Clin Exp Allergy* 33(8): 1083-9.

Smit, J. J., H. Van Loveren, M. O. Hoekstra, P. A. Van der Kant, G. Folkerts and F. P. Nijkamp (2003). Therapeutic treatment with heat-killed Mycobacterium vaccae (SRL172) in a mild and severe mouse model for allergic asthma. *Eur J Pharmacol* 470(3): 193-9.

Song, B. J. and A. H. Liu (2003). Metropolitan endotoxin exposure, allergy and asthma. *Curr Opin Allergy Clin Immunol* 3(5): 331-5.

Soo, S. S., B. Villarreal-Ramos, C. M. Anjam Khan, C. E. Hormaeche and J. M. Blackwell (1998). Genetic control of immune response to recombinant antigens carried by an attenuated Salmonella typhimurium vaccine strain: Nramp1 influences T-helper subset responses and protection against leishmanial challenge. *Infect Immun* 66(5): 1910-7.

Spiegelberg, H. L., L. Beck, H. P. Kocher, W. C. Fanslow and A. H. Lucas (1994). Role of interleukin-4 in human immunoglobulin E formation in hu-PBL-SCID mice. *J Clin Invest* 93(2): 711-7.

Spinozzi, F., E. Agea, M. Fizzotti, G. Bassotti, A. Russano, S. Droetto, O. Bistoni, F. Grignani and A. Bertotto (1998). Role of T-helper type 2 cytokines in down-modulation of fas mRNA and receptor on the surface of activated CD4(+) T cells: molecular basis for the persistence of the allergic immune response. *Faseb J* 12(15): 1747-53.

Sprecher, C. A., F. J. Grant, J. W. Baumgartner, S. R. Presnell, S. K. Schrader, T. Yamagiwa, T. E. Whitmore, P. J. O'Hara and D. F. Foster (1998). Cloning and characterization of a novel class I cytokine receptor. *Biochem Biophys Res Commun* 246(1): 82-90.

Srivannaboon, K., A. B. Shanafelt, E. Todisco, C. P. Forte, F. G. Behm, S. C. Raimondi, C. H. Pui and D. Campana (2001). Interleukin-4 variant (BAY 36-1677) selectively induces apoptosis in acute lymphoblastic leukemia cells. *Blood* 97(3): 752-8.

SRPharma Characterisation of Mycobacterium vaccae.

SRPharma www.srpharma.com.

Stanford, J. L. and R. C. Paul (1973). A preliminary report on some studies of environmental mycobacteria. *Ann Soc Belg Med Trop* 53(4): 389-93.

Stanford, J. L., C. A. Stanford, J. M. Grange, N. N. Lan and A. Etemadi (2001). Does immunotherapy with heat-killed Mycobacterium vaccae offer hope for the treatment of multi-drug-resistant pulmonary tuberculosis? *Respir Med* 95(6): 444-7.

Stempel, D. A., W. A. Clyde, Jr., F. W. Henderson and A. M. Collier (1980). Serum IgE levels and the clinical expression of respiratory illnesses. *J Pediatr* 97(2): 185-90.

Stene, L. C. and P. Nafstad (2001). Relation between occurrence of type 1 diabetes and asthma. *Lancet* 357(9256): 607-8.

Stenger, S. and R. L. Modlin (1999). T cell mediated immunity to Mycobacterium tuberculosis. *Curr Opin Microbiol* 2(1): 89-93.

Stenger, S. and R. L. Modlin (2002). Control of Mycobacterium tuberculosis through mammalian Toll-like receptors. *Curr Opin Immunol* 14(4): 452-7.

Stirling, R. G. and K. F. Chung (2000). Future treatments of allergic diseases and asthma. *British Medical Bulletin* 56(4): 1037-1053.

Strachan, D. P. (1989). Hay fever, hygiene, and household size. *Bmj* 299(6710): 1259-60.

Strachan, D. P. (1997). Allergy and family size: a riddle worth solving. *Clin Exp Allergy* 27(3): 235-6.

Strachan, D. P. (2000). Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax* 104: 554-558.

Strachan, D. P., E. M. Taylor and R. G. Carpenter (1996). Family structure, neonatal infection, and hay fever in adolescence. *Arch Dis Child* 74(5): 422-6.

Strannegard, I. L., L. O. Larsson, G. Wennergren and O. Strannegard (1998). Prevalence of allergy in children in relation to prior BCG vaccination and infection with atypical mycobacteria. *Allergy* 53(3): 249-54.

Stumbles, P. A., J. A. Thomas, C. L. Pimm, P. T. Lee, T. J. Venaille, S. Proksch and P. G. Holt (1998). Resting Respiratory Tract Dendritic Cells Preferentially Stimulate T Helper Cell Type 2 (Th2) Responses and Require Obligatory Cytokine Signals for Induction of Th1 Immunity. *J. Exp. Med.* 188(11): 2019-2031.

Sudo, N., S. Sawamura, K. Tanaka, Y. Aiba, C. Kubo and Y. Koga (1997). The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 159(4): 1739-45.

Sugamura, K., H. Asao, M. Kondo, N. Tanaka, N. Ishii, K. Ohbo, M. Nakamura and T. Takeshita (1996). The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu Rev Immunol* 14: 179-205.

Svanes, C., D. Jarvis, S. Chinn and P. Burney (1999). Childhood environment and adult atopy: results from the European Community Respiratory Health Survey. *J Allergy Clin Immunol* 103(3 Pt 1): 415-20.

Szabo, S. J., A. S. Dighe, U. Gubler and K. M. Murphy (1997). Regulation of the interleukin (IL)-12R β 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 185(5): 817-24.

Szabo, S. J., N. G. Jacobson, A. S. Dighe, U. Gubler and K. M. Murphy (1995). Developmental commitment to the Th2 lineage by extinction of IL-12 signaling. *Immunity* 2(6): 665-75.

Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman and L. H. Glimcher (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100(6): 655-69.

Szabo, S. J., B. M. Sullivan, S. L. Peng and L. H. Glimcher (2003). Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol* 21: 713-58.

Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman and L. H. Glimcher (2002). Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 295(5553): 338-42.

Szekeres-Bartho, J., Z. Faust, P. Varga, L. Szereday and K. Kelemen (1996). The immunological pregnancy protective effect of progesterone is manifested via controlling cytokine production. *Am J Reprod Immunol* 35(4): 348-51.

Taams, L. S., M. Vukmanovic-Stejic, J. Smith, P. J. Dunne, J. M. Fletcher, F. J. Plunkett, S. B. Ebeling, G. Lombardi, M. H. Rustin, J. W. Bijlsma, F. P. Lafeber, M. Salmon and A. N. Akbar (2002). Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells. *Eur J Immunol* 32(6): 1621-30.

Tailleux, L., O. Schwartz, J. L. Herrmann, E. Pivert, M. Jackson, A. Amara, L. Legres, D. Dreher, L. P. Nicod, J. C. Gluckman, P. H. Lagrange, B. Gicquel and O. Neyrolles (2003). DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. *J Exp Med* 197(1): 121-7.

Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto and S. Akira (1996). Essential role of Stat6 in IL-4 signalling. *Nature* 380(6575): 627-30.

Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin and S. Akira (2002). Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* 169(1): 10-4.

Tamaoki, J., M. Kondo, N. Sakai, K. Aoshiba, E. Tagaya, J. Nakata, K. Isono and A. Nagai (2000). Effect of suplatast tosilate, a Th2 cytokine inhibitor, on steroid-dependent asthma: a double-blind randomised study. Tokyo Joshi-Idai Asthma Research Group. *Lancet* 356(9226): 273-8.

Tanaka, H., H. Nagai and Y. Maeda (1998). Effect of anti-IL-4 and anti-IL-5 antibodies on allergic airway hyperresponsiveness in mice. *Life Sci* 62(13): PL169-74.

Temann, U., B. Prasad, M. Gallup, C. Basbaum, S. Ho, R. Flavell and J. Rankin (1997). A novel role for murine IL-4 in vivo: induction of MUC5AC gene expression and mucin hypersecretion. *Am. J. Respir. Cell Mol. Biol.* 16(4): 471-478.

Terada, N., A. Konno, H. Tada, K. Shirotori, K. Ishikawa and K. Togawa (1992). The effect of recombinant human interleukin-5 on eosinophil accumulation and degranulation in human nasal mucosa. *J Allergy Clin Immunol* 90(2): 160-8.

Thierfelder, W. E., J. M. van Deursen, K. Yamamoto, R. A. Tripp, S. R. Sarawar, R. T. Carson, M. Y. Sangster, D. A. Vignali, P. C. Doherty, G. C. Grosveld and J. N. Ihle (1996). Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382(6587): 171-4.

Thomas, W. R. (1993). Mite allergens groups I-VII. A catalogue of enzymes. *Clin Exp Allergy* 23(5): 350-3.

Thompson, B. S., P. M. Chilton, J. R. Ward, J. T. Evans and T. C. Mitchell (2005). The low-toxicity versions of LPS, MPL(R) adjuvant and RC529, are efficient adjuvants for CD4+ T cells. *J Leukoc Biol.*

Tighe, H., K. Takabayashi, D. Schwartz, G. Van Nest, S. Tuck, J. J. Eiden, A. Kagey-Sobotka, P. S. Creticos, L. M. Lichtenstein, H. L. Spiegelberg and E. Raz (2000). Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 106(1 Pt 1): 124-34.

Till, S., B. Li, S. Durham, M. Humbert, B. Assoufi, D. Huston, R. Dickason, P. Jeannin, A. B. Kay and C. Corrigan (1995). Secretion of the eosinophil-active cytokines interleukin-5, granulocyte/macrophage colony-stimulating factor and interleukin-3 by bronchoalveolar lavage CD4+ and CD8+ T cell lines in atopic asthmatics, and atopic and non-atopic controls. *Eur J Immunol* 25(10): 2727-31.

Ting, C. N., M. C. Olson, K. P. Barton and J. M. Leiden (1996). Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* 384(6608): 474-478.

Tomita, K., S. Lim, T. Hanazawa, O. Usmani, R. Stirling, K. F. Chung, P. J. Barnes and I. M. Adcock (2002). Attenuated production of intracellular IL-10 and IL-12 in monocytes from patients with severe asthma. *Clin Immunol* 102(3): 258-66.

Tomkinson, A., C. Duez, G. Cieslewicz, J. C. Pratt, A. Joetham, M. C. Shanafelt, R. Gundel and E. W. Gelfand (2001). A murine IL-4 receptor antagonist that inhibits IL-4- and IL-13-induced

responses prevents antigen-induced airway eosinophilia and airway hyperresponsiveness. *J Immunol* 166(9): 5792-800.

Tony, H. P., B. J. Shen, P. Reusch and W. Sebald (1994). Design of human interleukin-4 antagonists inhibiting interleukin-4-dependent and interleukin-13-dependent responses in T-cells and B-cells with high efficiency. *Eur J Biochem* 225(2): 659-65.

Toossi, Z., P. Gogate, H. Shiratsuchi, T. Young and J. J. Ellner (1995). Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. *J Immunol* 154(1): 465-73.

Tournoy, K. G., J. C. Kips and R. A. Pauwels (2002). Is Th1 the solution for Th2 in asthma? *Clin Exp Allergy* 32(1): 17-29.

Trinchieri, G. (1998). Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol* 70: 83-243.

Tsan, M. F. and B. Gao (2004). Endogenous ligands of Toll-like receptors. *J Leukoc Biol.*

Tsuji, S., M. Matsumoto, O. Takeuchi, S. Akira, I. Azuma, A. Hayashi, K. Toyoshima and T. Seya (2000). Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guerin: involvement of toll-like receptors. *Infect Immun* 68(12): 6883-90.

Tukenmez, F., N. N. Bahceciler, I. B. Barlan and M. M. Basaran (1999). Effect of pre-immunization by killed Mycobacterium bovis and vaccae on immunoglobulin E response in ovalbumin-sensitized newborn mice. *Pediatr Allergy Immunol* 10(2): 107-11.

Tulic, M. K., J. J. Manoukian, D. H. Eidelman and Q. Hamid (2002). T-cell proliferation induced by local application of LPS in the nasal mucosa of nonatopic children. *J Allergy Clin Immunol* 110(5): 771-6.

Tulic, M. K., J. L. Wale, P. G. Holt and P. D. Sly (2000). Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. *Am J Respir Cell Mol Biol* 22(5): 604-12.

Tunon-De-Lara, J. M., A. E. Redington, P. Bradding, M. K. Church, J. A. Hartley, A. E. Semper and S. T. Holgate (1996). Dendritic cells in normal and asthmatic airways: expression of the alpha subunit of the high affinity immunoglobulin E receptor (Fc epsilon RI -alpha). *Clin Exp Allergy* 26(6): 648-55.

Tuyt, L. M., W. H. Dokter, M. T. Esselink and E. Vellenga (1995). Divergent effects of IL-10 and IL-4 on the proliferation and growth factor secretion by acute myeloblastic leukemic cells. *Eur Cytokine Netw* 6(4): 231-5.

Twentyman, O. P., J. P. Finnerty, A. Harris, J. Palmer and S. T. Holgate (1990). Protection against allergen-induced asthma by salmeterol. *Lancet* 336(8727): 1338-42.

Umetsu, D. T., J. J. McIntire, O. Akbari, C. Macaubas and R. H. DeKruyff (2002). Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 3(8): 715-20.

Vabulas, R. M., H. Wagner and H. Schild (2002). Heat shock proteins as ligands of toll-like receptors. *Curr Top Microbiol Immunol* 270: 169-84.

van Crevel, R., T. H. Ottenhoff and J. W. van der Meer (2002). Innate immunity to Mycobacterium tuberculosis. *Clin Microbiol Rev* 15(2): 294-309.

van den Biggelaar, A. H., L. C. Rodrigues, R. van Ree, J. S. van der Zee, Y. C. Hoeksma-Kruize, J. H. Souverijn, M. A. Missinou, S. Borrmann, P. G. Kremsner and M. Yazdanbakhsh (2004). Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren. *J Infect Dis* 189(5): 892-900.

van der Pouw Kraan, T. C., L. C. Boeije, E. R. de Groot, S. O. Stapel, A. Snijders, M. L. Kapsenberg, J. S. van der Zee and L. A. Aarden (1997). Reduced production of IL-12 and IL-12-dependent IFN-γ release in patients with allergic asthma. *J Immunol* 158(11): 5560-5.

van der Pouw Kraan, T. C., L. C. Boeije, R. J. Smeenk, J. Wijdenes and L. A. Aarden (1995). Prostaglandin-E_2 is a potent inhibitor of human interleukin 12 production. *J Exp Med* 181(2): 775-9.

van der Pouw Kraan, T. C., A. Snijders, L. C. Boeije, E. R. de Groot, A. E. Alewijnse, R. Leurs and L. A. Aarden (1998). Histamine inhibits the production of interleukin-12 through interaction with H_2 receptors. *J Clin Invest* 102(10): 1866-73.

van Eden, W., R. van der Zee and B. Prakken (2005). Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol* 5(4): 318-30.

van Kooyk, Y. and T. B. Geijtenbeek (2003). DC-SIGN: escape mechanism for pathogens. *Nat Rev Immunol* 3(9): 697-709.

Van Lookeren, C., K. Knol and K. De Vries (1969). House dust provocation in children. *Scand J Respir Dis* 50(1): 76-85.

Van Niekerk, C. H., E. G. Weinberg, S. C. Shore, H. V. Heese and J. Van Schalkwyk (1979). Prevalence of asthma: a comparative study of urban and rural Xhosa children. *Clin Allergy* 9(4): 319-4.

van Strien, R. T., R. Engel, O. Holst, A. Bufe, W. Eder, M. Waser, C. Braun-Fahrlander, J. Riedler, D. Nowak and E. von Mutius (2004). Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health. *J Allergy Clin Immunol* 113(5): 860-7.

Vella, A., T. K. Teague, J. Ihle, J. Kappler and P. Marrack (1997). Interleukin 4 (IL-4) or IL-7 prevents the death of resting T cells: stat6 is probably not required for the effect of IL-4. *J Exp Med* 186(2): 325-30.

Vincent, M. S., D. S. Leslie, J. E. Gumperz, X. Xiong, E. P. Grant and M. B. Brenner (2002). CD1-dependent dendritic cell instruction. *Nat Immunol* 3(12): 1163-8.

Vogelzang, P. F., J. W. van der Gulden, H. Folgering, J. J. Kolk, D. Heederik, L. Preller, M. J. Tielen and C. P. van Schayck (1998). Endotoxin exposure as a major determinant of lung function decline in pig farmers. *Am J Respir Crit Care Med* 157(1): 15-8.

von Mutius, E., C. Braun-Fahrlander, R. Schierl, J. Riedler, S. Ehlermann, S. Maisch, M. Waser and D. Nowak (2000). Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy* 30(9): 1230-4.

von Mutius, E., F. D. Martinez, C. Fritzsch, T. Nicolai, P. Reitmeir and H. H. Thiemann (1994). Skin test reactivity and number of siblings. *Bmj* 308(6930): 692-5.

von Mutius, E., F. D. Martinez, C. Fritzsch, T. Nicolai, G. Roell and H. H. Thiemann (1994). Prevalence of asthma and atopy in two areas of West and East Germany. *Am J Respir Crit Care Med* 149(2 Pt 1): 358-64.

von Mutius, E., N. Pearce, R. Beasley, S. Cheng, O. von Ehrenstein, B. Bjorksten and S. Weiland (2000). International patterns of tuberculosis and the prevalence of symptoms of asthma, rhinitis, and eczema [see comments]. *Thorax* 55(6): 449-53.

von Mutius, E., S. K. Weiland, C. Fritzsch, H. Duhme and U. Keil (1998). Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. *Lancet* 351(9106): 862-6.

Walker, C., W. Bauer, R. K. Braun, G. Menz, P. Braun, F. Schwarz, T. T. Hansel and B. Villiger (1994). Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. *Am J Respir Crit Care Med* 150(4): 1038-48.

Walker, C., E. Bode, L. Boer, T. T. Hansel, K. Blaser and J. C. Virchow, Jr. (1992). Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 146(1): 109-15.

Walker, C., M. K. Kaegi, P. Braun and K. Blaser (1991). Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin Immunol* 88(6): 935-42.

Walker, C., E. Sawicka and G. A. Rook (2003). Immunotherapy with mycobacteria. *Curr Opin Allergy Clin Immunol* 3(6): 481-6.

Walker, C., J. C. J. Virchow, P. L. Bruijnzeel and K. Blaser (1991). T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. *Journal of Immunology (Baltimore, Md.: 1950)* 146(6): 1829-1835.

Wang, C. C. and G. A. Rook (1998). Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed Mycobacterium vaccae. *Immunology* 93(3): 307-13.

Wardlaw, A. J., S. Dunnette, G. J. Gleich, J. V. Collins and A. B. Kay (1988). Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 137(1): 62-9.

Warringa, R. A., R. C. Schweizer, T. Maikoe, P. H. Kuijper, P. L. Bruijnzeel and L. Koendermann (1992). Modulation of eosinophil chemotaxis by interleukin-5. *Am J Respir Cell Mol Biol* 7(6): 631-6.

Watanabe, J., Y. Miyazaki, G. A. Zimmerman, K. H. Albertine and T. M. McIntyre (2003). Endotoxin contamination of ovalbumin suppresses murine immunologic responses and development of airway hyper-reactivity. *J Biol Chem* 278(43): 42361-8.

Watson, J. D. (1998). Genesis Research and Development Corporation. When injected i.p. mixed with ovalbumin, *M. vaccae* drives formation of CD8+ cytotoxic T cells, that will lyse recombinant EL-4 thymoma cells expressing ovalbumin. *Patent application; International publication Number WO 98/08542.*

Webb, A., M. Al-Moundhri, B. E. Souberbielle, A. Norton, K. Priest, C. Ryan, R. Mendes, A. S., I. E. Smith and M. E. R. O'Brien (1998). A randomised study of SRL172 (Mycobacterium vaccae) in patients with advanced lung cancer treated with chemotherapy. *British Jurnal of Cancer.*

Wheeler, A. W., J. S. Marshall and J. T. Ulrich (2001). A Th1-inducing adjuvant, MPL, enhances antibody profiles in experimental animals suggesting it has the potential to improve the efficacy of allergy vaccines. *Int Arch Allergy Immunol* 126(2): 135-9.

Wickens, K., J. M. Lane, P. Fitzharris, R. Siebers, G. Riley, J. Douwes, T. Smith and J. Crane (2002). Farm residence and exposures and the risk of allergic diseases in New Zealand children. *Allergy* 57(12): 1171-9.

Wickens, K. L., J. Crane, T. J. Kemp, S. J. Lewis, W. J. D'Souza, G. M. Sawyer, M. L. Stone, S. J. Tohill, J. C. Kennedy, T. M. Slater and N. E. Pearce (1999). Family size, infections, and asthma prevalence in New Zealand children. *Epidemiology* 10(6): 699-705.

Wills-Karp, M. (1999). Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17(1): 255-281.

Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp and D. D. Donaldson (1998). Interleukin-13: central mediator of allergic asthma. *Science* 282(5397): 2258-61.

Wong, G. W., D. S. Hui, C. M. Tam, H. H. Chan, T. F. Fok, M. Chan-Yeung and C. K. Lai (2001). Asthma, atopy and tuberculin responses in Chinese schoolchildren in Hong Kong. *Thorax* 56(10): 770-3.

Wong, W. S. and D. S. Koh (2000). Advances in immunopharmacology of asthma. *Biochem Pharmacol* 59(11): 1323-35.

Woolcock, A. J. and J. K. Peat (1997). Evidence for the increase in asthma worldwide. *Ciba Found Symp* 206: 122-34; discussion 134-9, 157-9.

Xie, Q., R. Bian, K. Wu and X. Zheng (2002). [Effects of vaccae on airway inflammation and Th1/Th2 cytokines in sensitized mice]. *Zhonghua Jie He He Hu Xi Za Zhi* 25(8): 488-92.

Yamaguchi, Y., T. Suda, J. Suda, M. Eguchi, Y. Miura, N. Harada, A. Tominaga and K. Takatsu (1988). Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J Exp Med* 167(1): 43-56.

Yamamoto, M., S. Sato, H. Hemmi, H. Sanjo, S. Uematsu, T. Kaisho, K. Hoshino, O. Takeuchi, M. Kobayashi, T. Fujita, K. Takeda and S. Akira (2002). Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 420(6913): 324-9.

Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda and S. Akira (2002). Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* 169(12): 6668-72.

Yamamoto, S., T. Yamamoto, S. Shimada, E. Kuramoto, O. Yano, T. Kataoka and T. Tokunaga (1992). DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol Immunol* 36(9): 983-97.

Yang, M., S. P. Hogan, P. J. Henry, K. I. Matthaei, A. N. McKenzie, I. G. Young, M. E. Rothenberg and P. S. Foster (2001). Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin. *Am J Respir Cell Mol Biol* 25(4): 522-30.

Yazdanbakhsh, M., P. G. Kremsner and R. van Ree (2002). Allergy, parasites, and the hygiene hypothesis. *Science* 296(5567): 490-4.

Yemaneberhan, H., Z. Bekele, A. Venn, S. Lewis, E. Parry and J. Britton (1997). Prevalence of wheeze and asthma and relation to atopy in urban and rural Ethiopia. *Lancet* 350(9071): 85-90.

Yilmaz, M., G. Bingol, D. Altintas and S. G. Kendirli (2000). Correlation between atopic diseases and tuberculin responses. *Allergy* 55(7): 664-7.

Yilmaz, M., G. Bingol, D. Altintas and S. G. Kendirli (2000). Correlation between atopic diseases and tuberculin responses [In Process Citation]. *Allergy* 55(7): 664-7.

Ying, S., M. Humbert, J. Barkans, C. J. Corrigan, R. Pfister, G. Menz, M. Larche, D. S. Robinson, S. R. Durham and A. B. Kay (1997). Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* 158(7): 3539-44.

Yoneda, T. and J. J. Ellner (1998). CD4(+) T cell and natural killer cell-dependent killing of Mycobacterium tuberculosis by human monocytes. *Am J Respir Crit Care Med* 158(2): 395-403.

Yoshimoto, T., K. Takeda, T. Tanaka, K. Ohkusu, S. Kashiwamura, H. Okamura, S. Akira and K. Nakanishi (1998). IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J Immunol* 161(7): 3400-7.

Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak and et al. (1999). T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402(6763): 827-32.

Zangrilli, J., J. Shaver, R. Cirelli, S. Cho, C. Garlisi, A. Falcone, F. Cuss, J. Fish and S. Peters (1995). sVCAM-1 levels after segmental antigen challenge correlate with eosinophil influx, IL-4 and IL-5 production, and the late phase response. *Am. J. Respir. Crit. Care Med.* 151(5): 1346-1353.

Zangrilli, J. G., J. R. Shaver, R. A. Cirelli, S. K. Cho, C. G. Garlisi, A. Falcone, F. M. Cuss, J. E. Fish and S. P. Peters (1995). sVCAM-1 levels after segmental antigen challenge correlate with eosinophil influx, IL-4 and IL-5 production, and the late phase response. *Am J Respir Crit Care Med* 151(5): 1346-53.

Zarember, K. A. and P. J. Godowski (2002). Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* 168(2): 554-61.

Zhang, D. H., L. Cohn, P. Ray, K. Bottomly and A. Ray (1997). Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *The Journal of Biological Chemistry* 272(34): 21597-21603.

Zhang, D. H., L. Yang, L. Cohn, L. Parkyn, R. Homer, P. Ray and A. Ray (1999). Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. *Immunity* 11(4): 473-82.

Zheng, W. and R. A. Flavell (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89(4): 587-96.

Zimmerman, B., S. Feanny, J. Reisman, H. Hak, N. Rashed, F. J. McLaughlin and H. Levison (1988). Allergy in asthma. I. The dose relationship of allergy to severity of childhood asthma. *J Allergy Clin Immunol* 81(1): 63-70.

Zuany-Amorim, C., C. Manlius, A. Trifilieff, L. R. Brunet, G. Rook, G. Bowen, G. Pay and C. Walker (2002). Long-term protective and antigen-specific effect of heat-killed Mycobacterium vaccae in a murine model of allergic pulmonary inflammation. *J Immunol* 169(3): 1492-9.

Zuany-Amorim, C., E. Sawicka, C. Manlius, A. Le Moine, L. R. Brunet, D. M. Kemeny, G. Bowen, G. Rook and C. Walker (2002). Suppression of airway eosinophilia by killed Mycobacterium vaccae- induced allergen-specific regulatory T-cells. *Nat Med* 8(6): 625-9.

Zurawski, S. M., P. Chomarat, O. Djossou, C. Bidaud, A. N. McKenzie, P. Miossec, J. Banchereau and G. Zurawski (1995). The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin-13 receptor. *J Biol Chem* 270(23): 13869-78.

APPENDIX 1

Skin prick tests and Histamine challenge

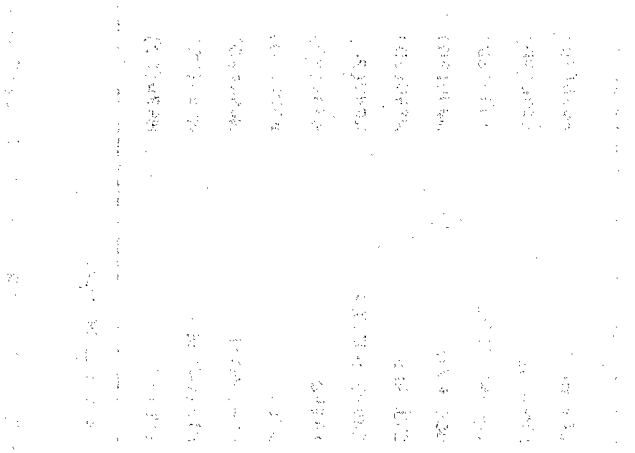


Table 1: Summary of Wheal Size (mm x mm) for each Skin Prick Test

Skin Prick Test		Active	Placebo
SALINE	Mean(SD)	0.5(1.7)	0.3(0.8)
HISTAMINE	Mean(SD)	24.0(14.0)	24.3(9.2)
DUST MITE	Mean(SD)	82.8(70.1)	69.8(66.1)
DUST	Mean(SD)	17.6(18.7)	12.4(10.3)
TREES	Mean(SD)	11.3(19.9)	8.8(14.9)
GRASS POLLEN	Mean(SD)	51.0(70.3)	62.6(48.6)
CAT FUR	Mean(SD)	17.4(20.9)	13.9(16.2)
DOG HAIR	Mean(SD)	3.8(3.8)	3.5(4.9)
ASPERGILLUS	Mean(SD)	1.6 (2.4)	1.1(2.0)
CANDIDA	Mean(SD)	0.8(1.8)	6.5(14.6)
FEATHERS	Mean(SD)	1.0(1.6)	1.3(2.0)

Table 2: Summary of Histamine Challenge Data

Treatment Group	Visit		Histamine Concentration (mg/ml)									
			Baseline	0.03	0.06	0.12	0.25	0.50	1.00	2.00	4.00	8.00
Active Group	VISIT 1	N	12	12	12	12	11	11	7	2	0	0
		Mean	3.93	3.83	3.75	3.58	3.50	3.31	3.25	3.09		
		Median	4.00	3.87	3.74	3.65	3.42	3.28	3.17	3.09		
		Minimum	3.20	3.07	3.20	2.62	2.91	2.67	2.29	2.92		
		Maximum	4.51	4.60	4.56	4.44	4.35	4.35	3.97	3.25		
		Std.Dev	0.421	0.471	0.416	0.493	0.452	0.534	0.572	0.233		
	VISIT 3	N	10	10	10	10	9	7	3	0	0	0
		Mean	3.51	3.43	3.35	3.23	3.08	2.74	2.78			
		Median	3.50	3.43	3.31	3.16	3.06	2.72	2.67			
		Minimum	2.85	2.79	2.47	2.32	2.11	2.07	2.32			
		Maximum	4.29	4.35	4.33	4.39	4.04	3.73	3.35			
		Std.Dev	0.486	0.553	0.604	0.647	0.560	0.537	0.524			
	VISIT 8	N	12	12	12	12	11	6	5	1	1	0
		Mean	3.28	3.27	3.21	3.12	2.84	3.07	2.59	3.39	2.97	
		Median	3.22	3.13	3.05	2.87	2.62	3.11	2.20	3.39	2.97	
		Minimum	2.71	2.60	2.50	2.50	2.10	2.45	2.10	3.39	2.97	
		Maximum	4.20	4.21	4.24	4.23	4.09	3.92	3.67	3.39	2.97	
		Std.Dev	0.500	0.529	0.516	0.551	0.681	0.571	0.680			
Placebo Group	VISIT 1	N	12	12	12	12	12	11	9	5	3	2
		Mean	3.72	3.65	3.63	3.43	3.35	3.15	3.05	2.81	3.03	3.14
		Median	3.57	3.58	3.57	3.44	3.20	2.87	2.85	2.54	3.05	3.14
		Minimum	2.59	2.66	2.51	2.14	2.39	2.21	1.99	1.85	2.15	2.83
		Maximum	5.21	5.00	4.90	4.60	4.65	4.62	4.48	4.23	3.89	3.45
		Std.Dev	0.833	0.753	0.811	0.785	0.765	0.871	0.862	0.936	0.870	0.438
	VISIT 3	N	12	12	12	12	11	7	3	3	2	1
		Mean	3.17	3.10	3.05	2.93	2.73	2.81	2.99	2.64	1.97	1.52
		Median	2.95	2.82	2.77	2.63	2.57	2.60	2.55	2.41	1.97	1.52
		Minimum	1.94	1.95	1.97	1.68	1.53	2.04	2.52	2.36	1.76	1.52
		Maximum	4.45	4.36	4.46	4.36	4.45	4.12	3.91	3.15	2.18	1.52
		Std.Dev	0.818	0.839	0.849	0.854	0.899	0.722	0.794	0.442	0.297	
	VISIT 8	N	11	11	11	11	11	10	6	4	3	1
		Mean	3.31	3.34	3.30	3.21	2.99	2.85	3.26	3.04	3.02	2.37
		Median	2.99	3.06	2.95	2.60	2.48	2.58	3.28	3.28	3.38	2.37
		Minimum	2.40	2.42	2.36	2.02	1.60	1.56	2.10	1.74	2.25	2.37
		Maximum	4.71	4.70	4.68	4.56	4.43	4.18	4.21	3.87	3.43	2.37
		Std.Dev	0.890	0.900	0.918	0.955	0.986	1.001	0.772	1.009	0.667	

Table 3 Table 26: Summary of Changes from Baseline in Histamine Challenge Data

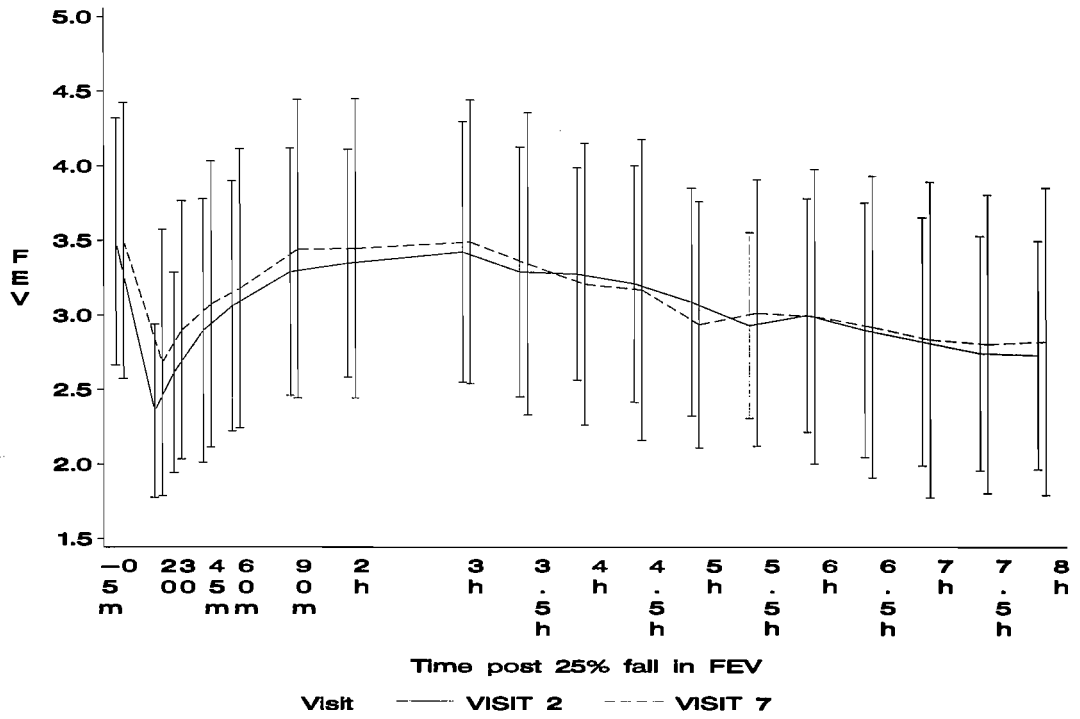
Treatment Group	Visit		Baseline	Histamine Concentration (mg/ml) 0.03	0.06	0.12	0.25	0.50	1.00	2.00	4.00	8.00
Active Group	VISIT 1	N	12	12	12	12	11	11	7	2	0	0
		Mean	3.93	-2.58	-4.52	-8.83	-11.8	-16.8	-20.7	-30.9		
		Median	4.00	-2.14	-5.16	-7.81	-12.8	-18.1	-22.7	-30.9		
		Minimum	3.20	-10.3	-8.29	-25.1	-16.6	-24.9	-28.4	-33.9		
		Maximum	4.51	2.00	1.11	-1.55	-3.55	-3.55	-10.2	-27.9		
		Std.Dev	0.421	2.975	2.753	6.212	3.625	5.983	6.535	4.243		
	VISIT 3	N	10	10	10	10	9	7	3	0	0	0
		Mean	3.51	-2.37	-4.89	-8.47	-13.5	-23.7	-26.5			
		Median	3.50	-1.37	-4.75	-6.25	-12.0	-23.0	-28.2			
		Minimum	2.85	-8.50	-19.3	-24.2	-26.0	-36.6	-29.5			
		Maximum	4.29	1.40	3.23	2.33	0.00	-13.1	-21.9			
		Std.Dev	0.486	3.092	6.155	8.725	8.648	8.115	4.058			
	VISIT 8	N	12	12	12	12	11	6	5	1	1	0
		Mean	3.28	-0.37	-2.35	-5.04	-13.4	-12.1	-25.2	-19.3	-29.3	
		Median	3.22	-1.10	-1.37	-2.80	-9.97	-11.7	-20.8	-19.3	-29.3	
		Minimum	2.71	-4.46	-11.7	-20.3	-27.1	-21.4	-45.8	-19.3	-29.3	
		Maximum	4.20	7.25	2.90	3.99	0.00	-6.67	-12.6	-19.3	-29.3	
		Std.Dev	0.500	3.858	4.438	7.358	10.196	5.640	12.512			
Placebo Group	VISIT 1	N	12	12	12	12	12	11	9	5	3	2
		Mean	3.72	-1.32	-2.40	-7.75	-9.69	-14.7	-17.3	-20.4	-20.1	-23.9
		Median	3.57	-1.03	-1.83	-7.31	-6.70	-14.7	-19.3	-15.1	-17.1	-23.9
		Minimum	2.59	-8.02	-8.92	-17.4	-25.2	-33.0	-23.2	-37.1	-28.1	-26.4
		Maximum	5.21	4.63	2.35	0.00	-0.85	-1.49	-4.48	-9.81	-15.3	-21.4
		Std.Dev	0.833	3.583	3.482	5.457	7.388	9.131	7.342	11.916	6.939	3.571
	VISIT 3	N	12	12	12	12	11	7	3	3	2	1
		Mean	3.17	-2.41	-3.99	-8.28	-15.9	-15.5	-6.57	-16.0	-25.1	-41.5
		Median	2.95	-1.16	-4.27	-7.89	-17.1	-20.5	-4.49	-11.6	-25.1	-41.5
		Minimum	1.94	-17.5	-15.0	-22.4	-46.2	-33.3	-12.1	-29.2	-34.1	-41.5
		Maximum	4.45	6.92	5.00	1.92	4.62	4.62	-3.08	-7.31	-16.2	-41.5
		Std.Dev	0.818	6.512	6.178	7.389	14.045	13.893	4.869	11.604	12.678	
	VISIT 8	N	11	11	11	11	11	10	6	4	3	1
		Mean	3.31	0.96	-0.34	-3.70	-10.7	-17.7	-15.1	-15.7	-23.8	-43.8
		Median	2.99	0.95	1.25	-1.15	-8.02	-15.7	-13.0	-13.6	-21.8	-43.8
		Minimum	2.40	-4.21	-13.7	-16.9	-34.2	-35.0	-29.4	-27.5	-30.8	-43.8
		Maximum	4.71	4.58	6.25	4.17	0.83	-3.24	-2.55	-8.29	-18.7	-43.8
		Std.Dev	0.890	2.616	5.048	6.894	10.119	11.447	10.047	8.354	6.267	

APPENDIX 2

Allergen Challenge Curves

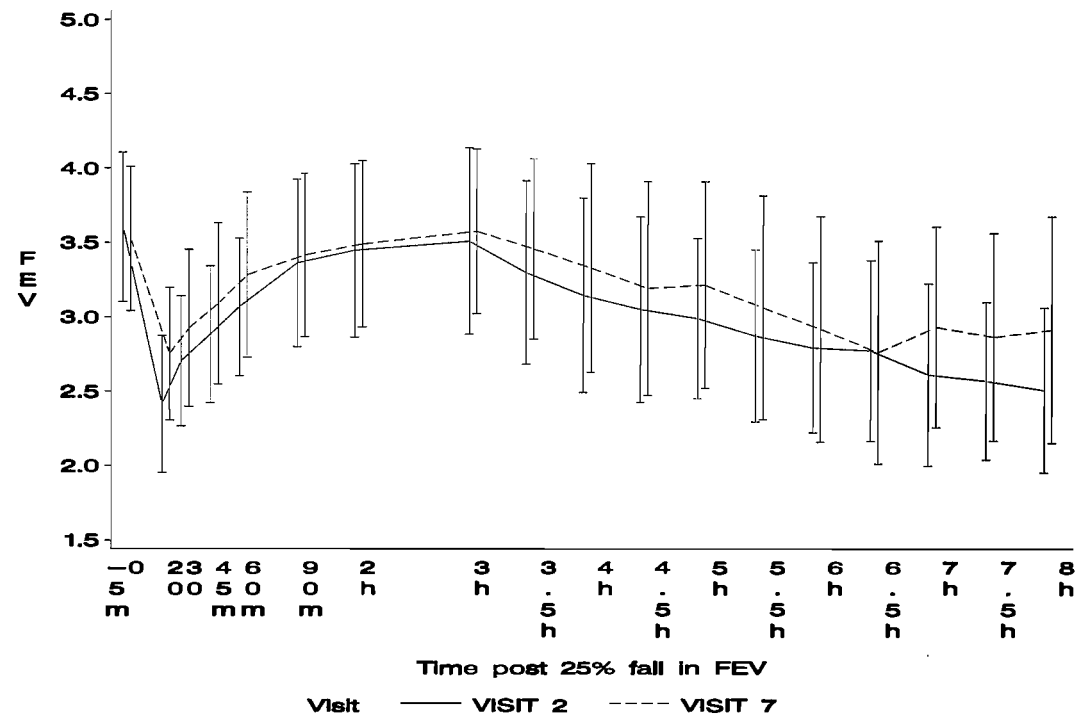
Study SR – AL – 02

Figure 1: Plot of FEV values during Allergen Challenge
Placebo Group



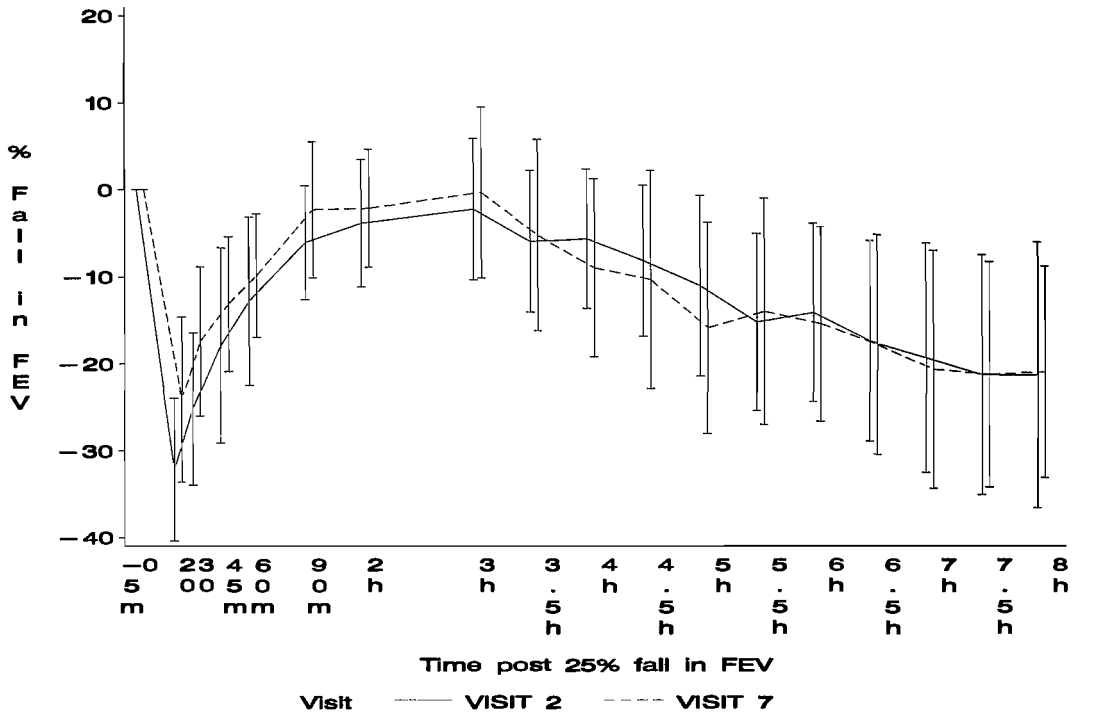
Study SR – AL – 02

Figure 2: Plot of FEV values during Allergen Challenge
Active Group



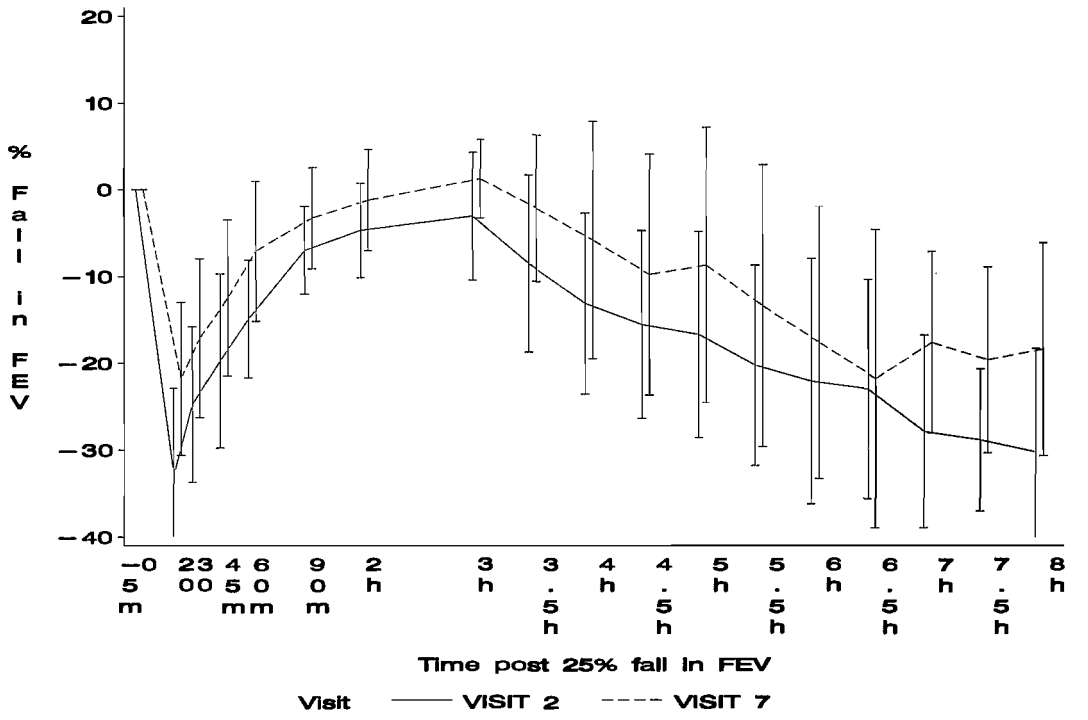
Study SR – AL – 02

Figure 3: Plot of % Fall in FEV values during Allergen Challenge
Placebo Group



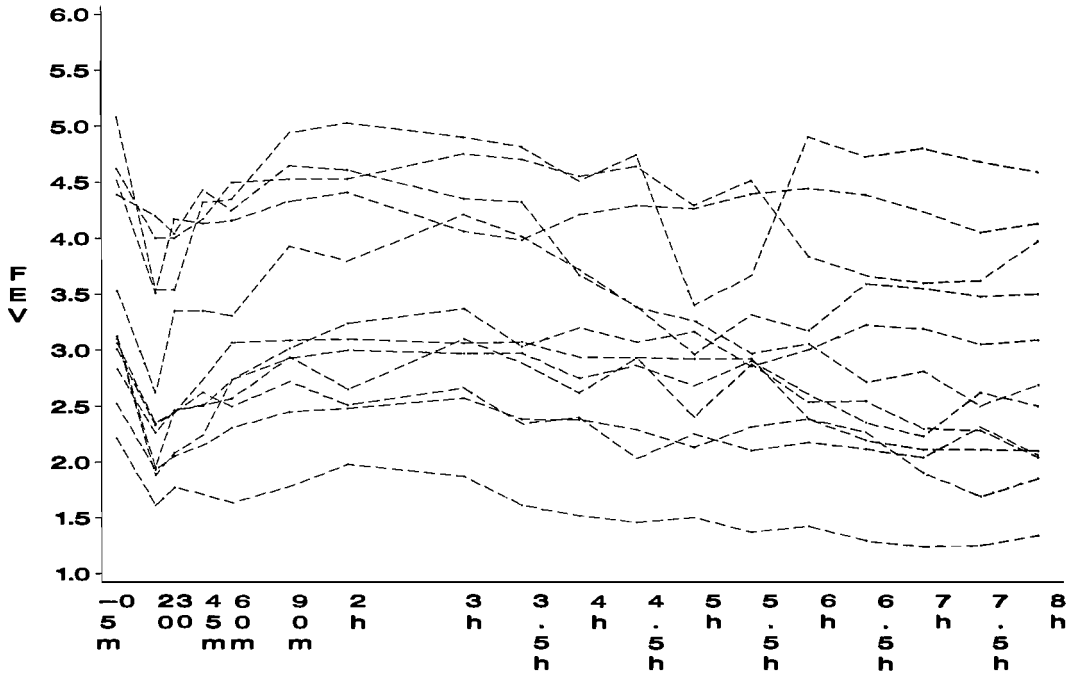
Study SR – AL – 02

Figure 4: Plot of % Fall in FEV values during Allergen Challenge
Active Group



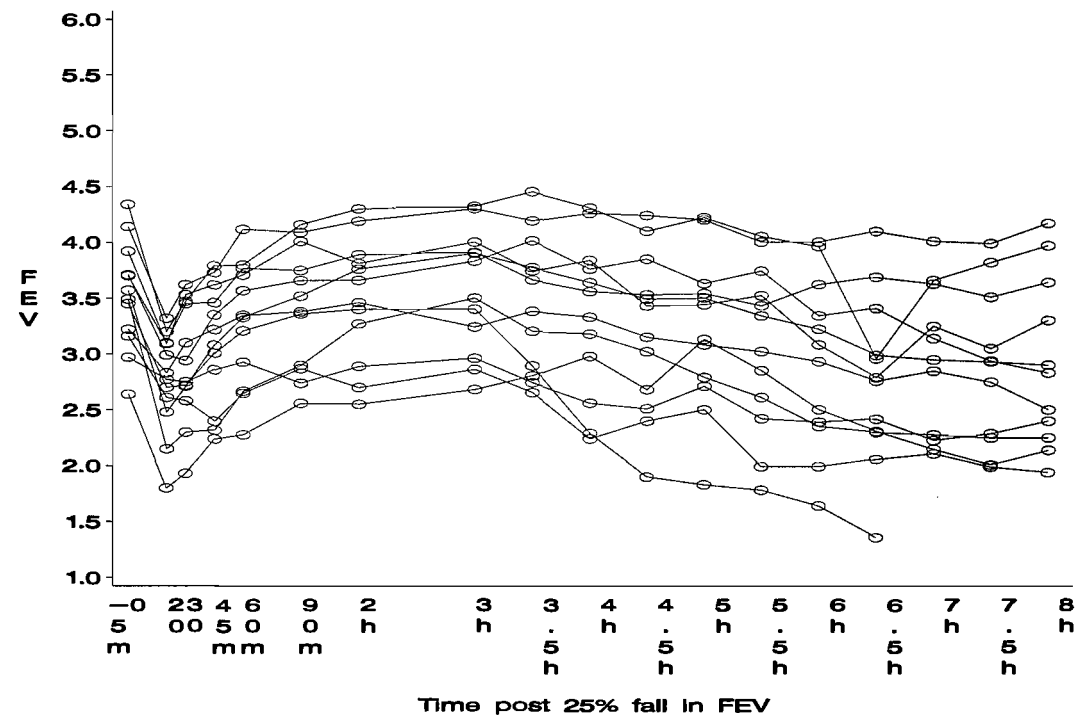
Study SR — AL — 02

Figure 5: Patient profiles of FEV values during Allergen Challenge (Visit 7)
Placebo Group



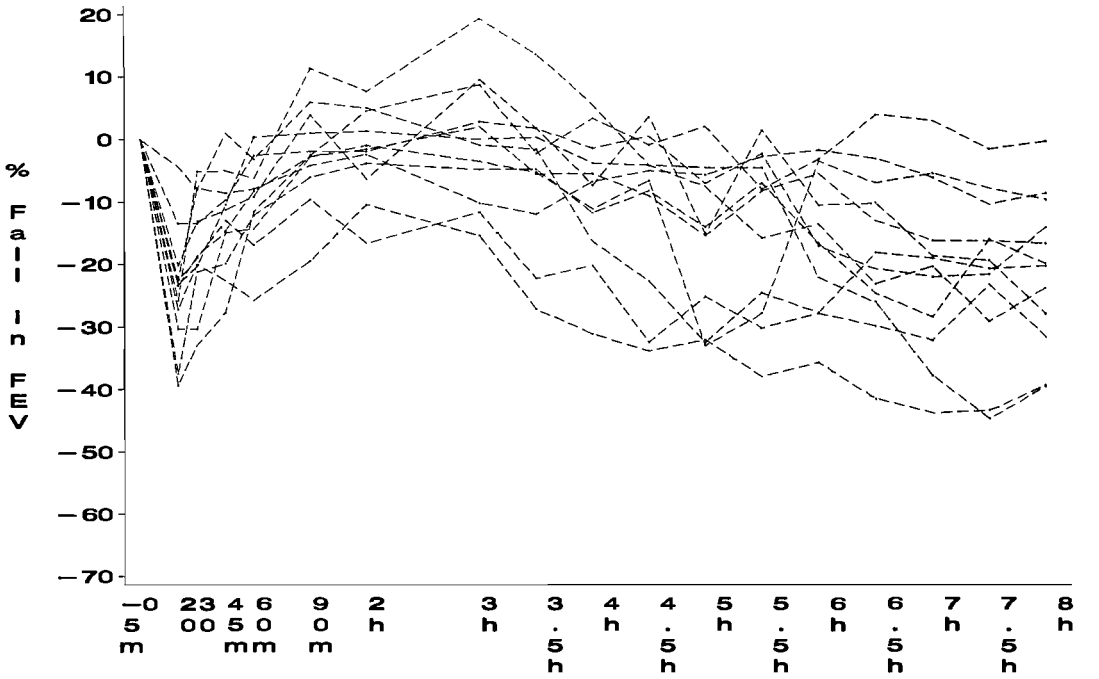
Study SR — AL — 02

Figure 6: Patient profiles of FEV values during Allergen Challenge (Visit 7)
Active Group



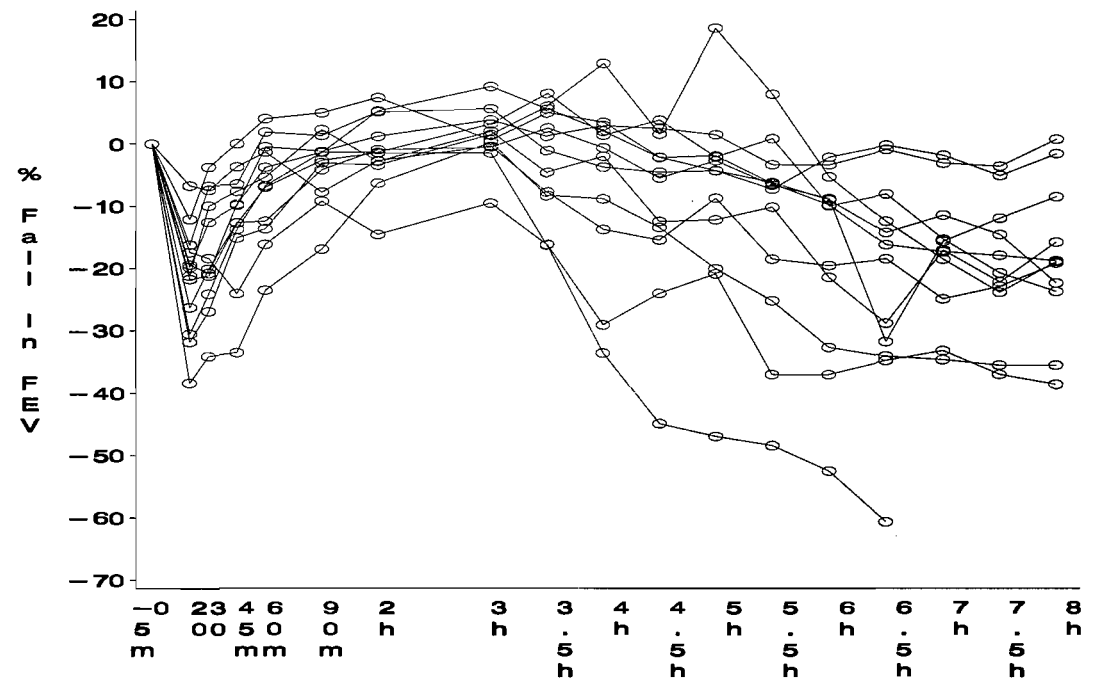
Study SR—AL—02

Figure 7: Patient profiles of % Fall in FEV values during Allergen Challenge (Visit 7) Placebo Group



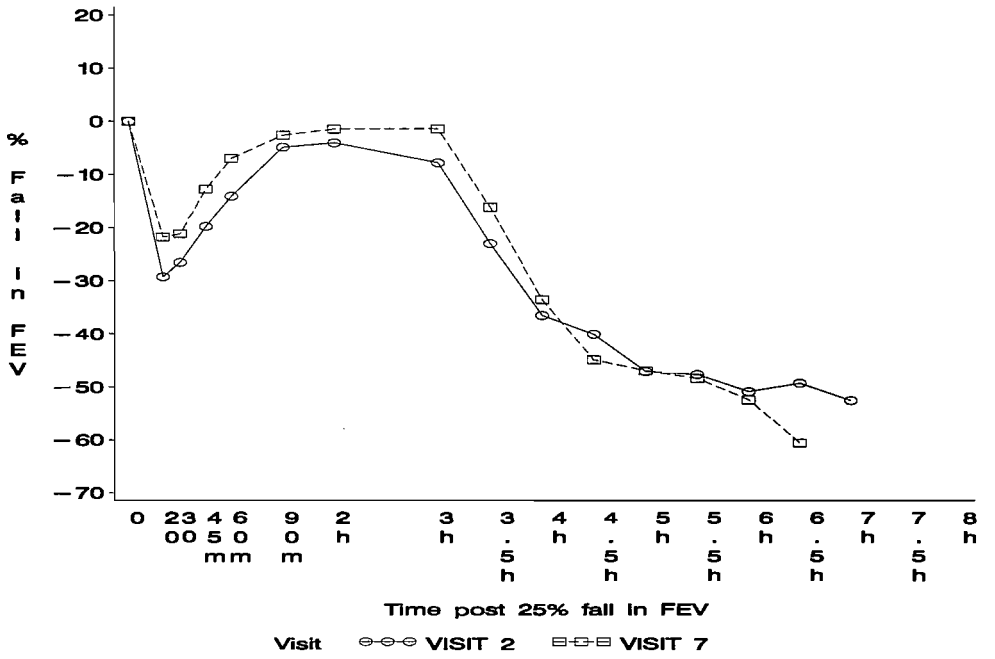
Study SR—AL—02

Figure 8: Patient profiles of % Fall in FEV values during Allergen Challenge (Visit 7) Active Group



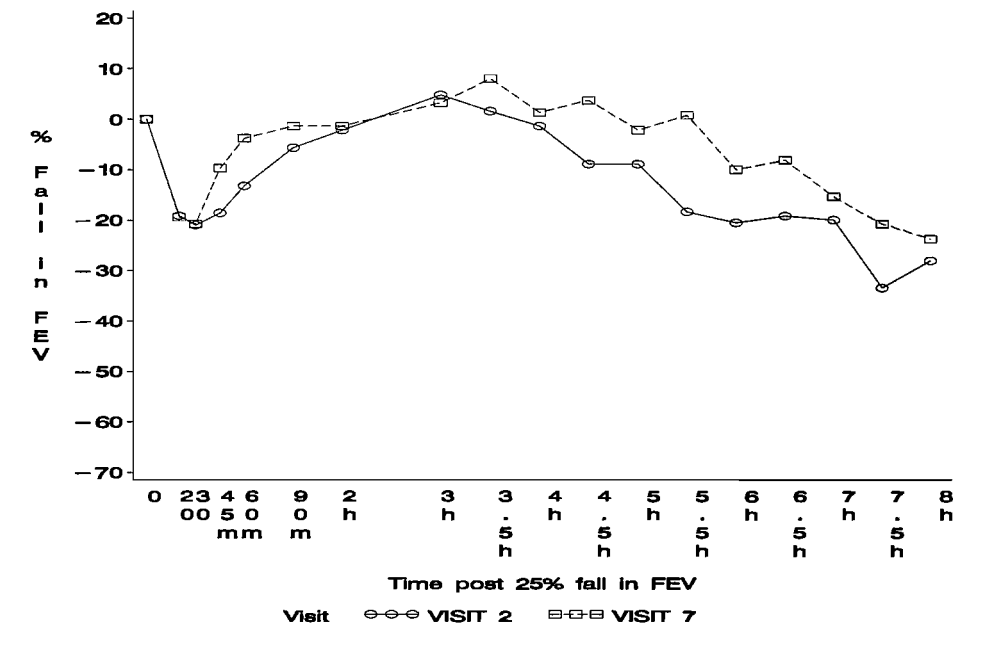
Study SR – AL – 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 24



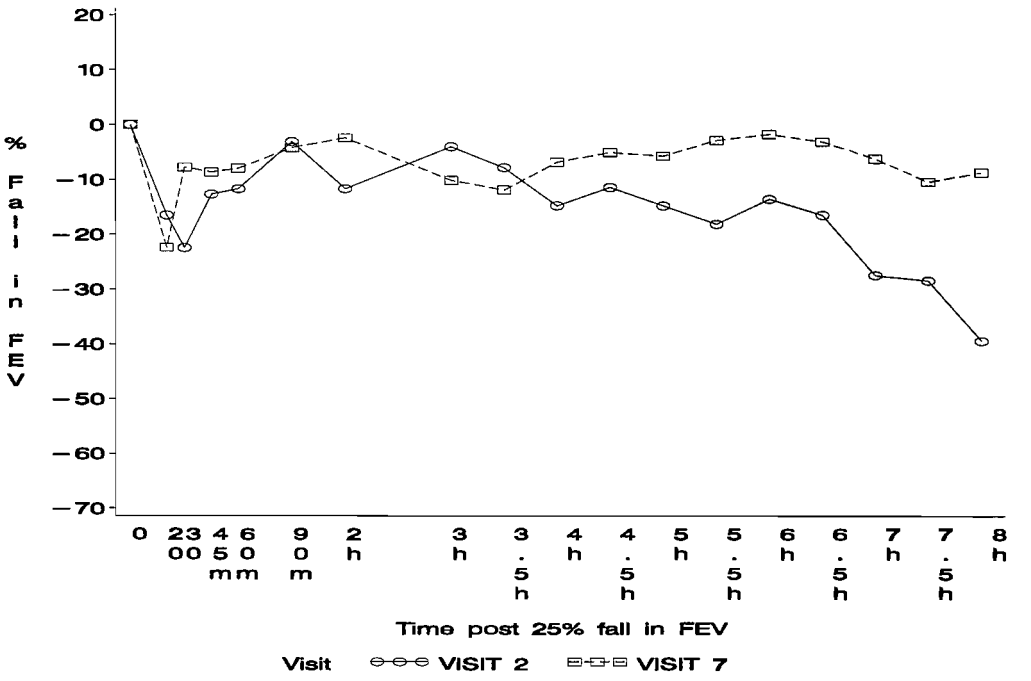
Study SR – AL – 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 23



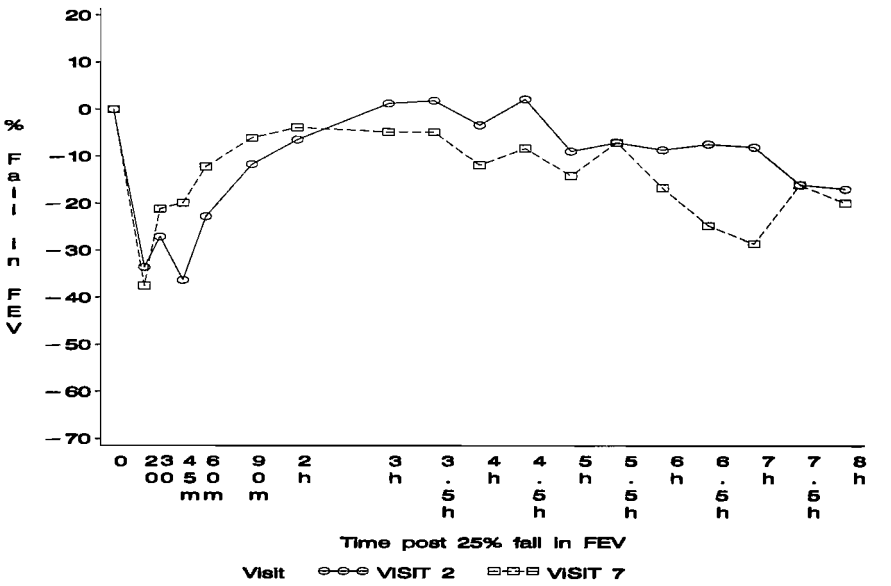
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 20



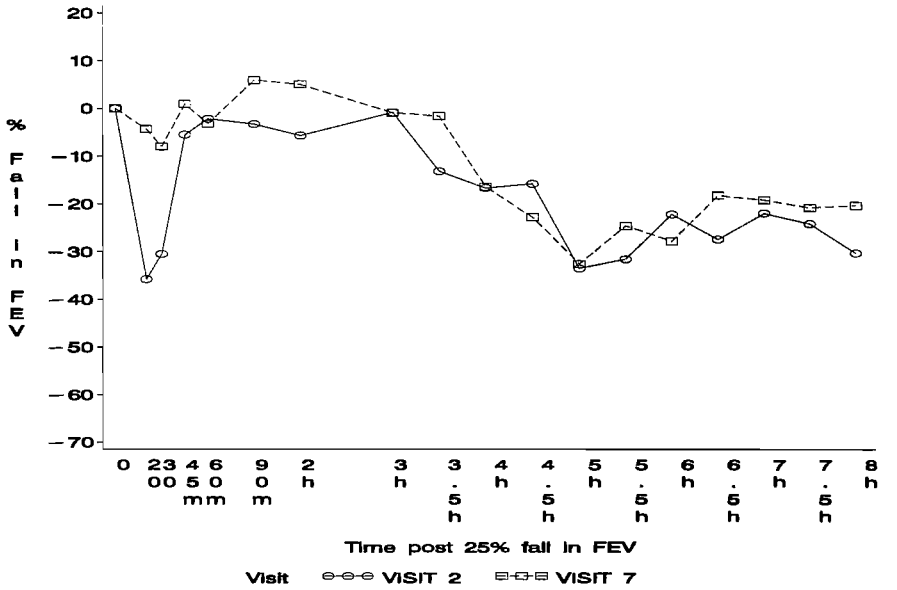
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 18



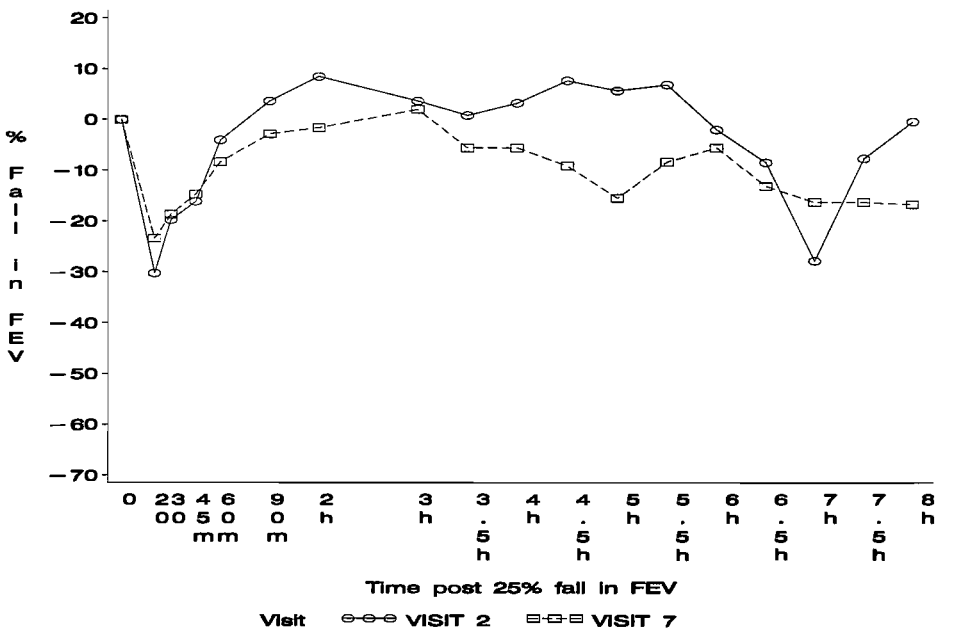
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 16



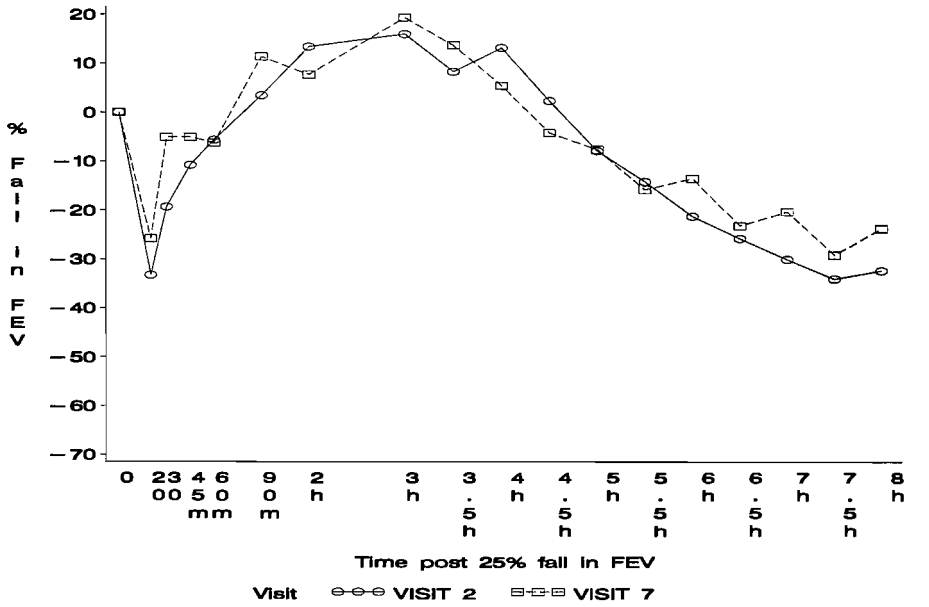
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 22



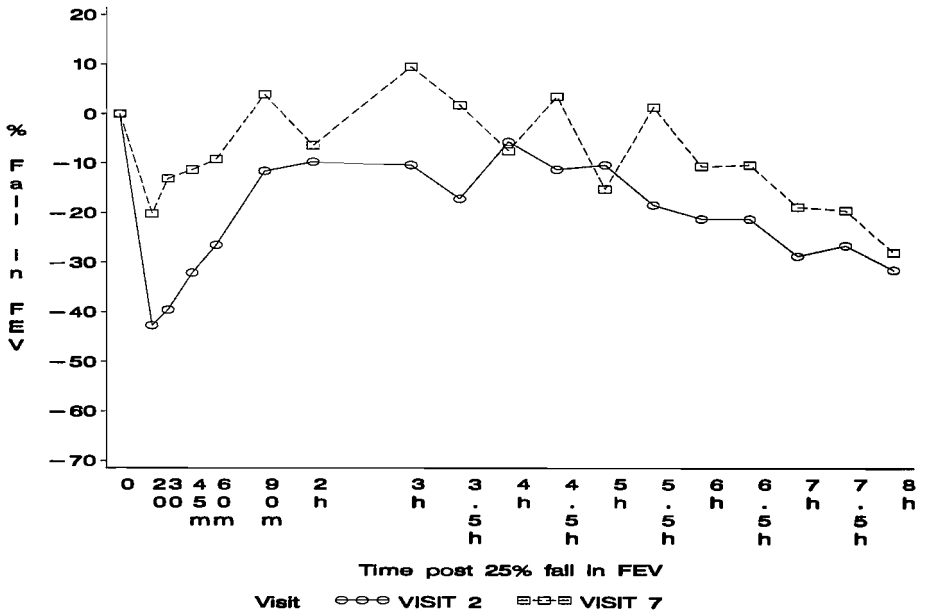
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 14



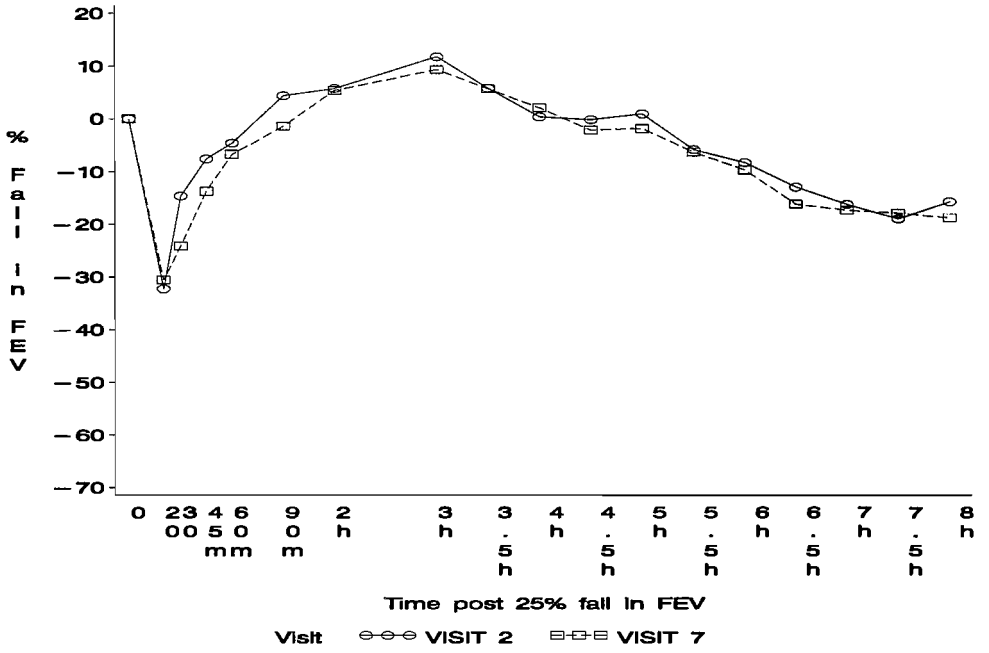
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 21



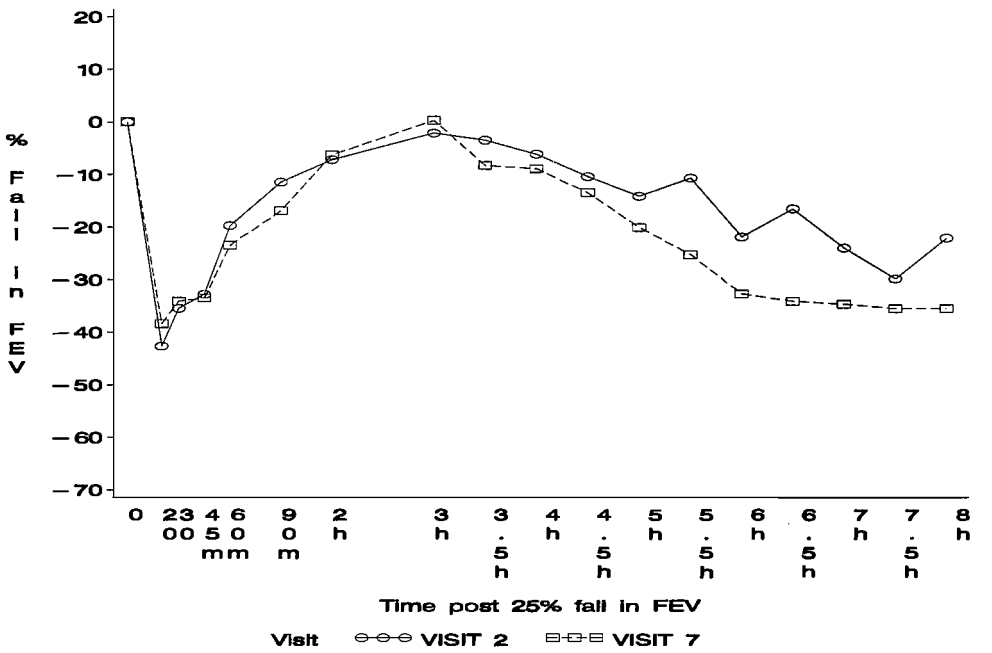
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 10



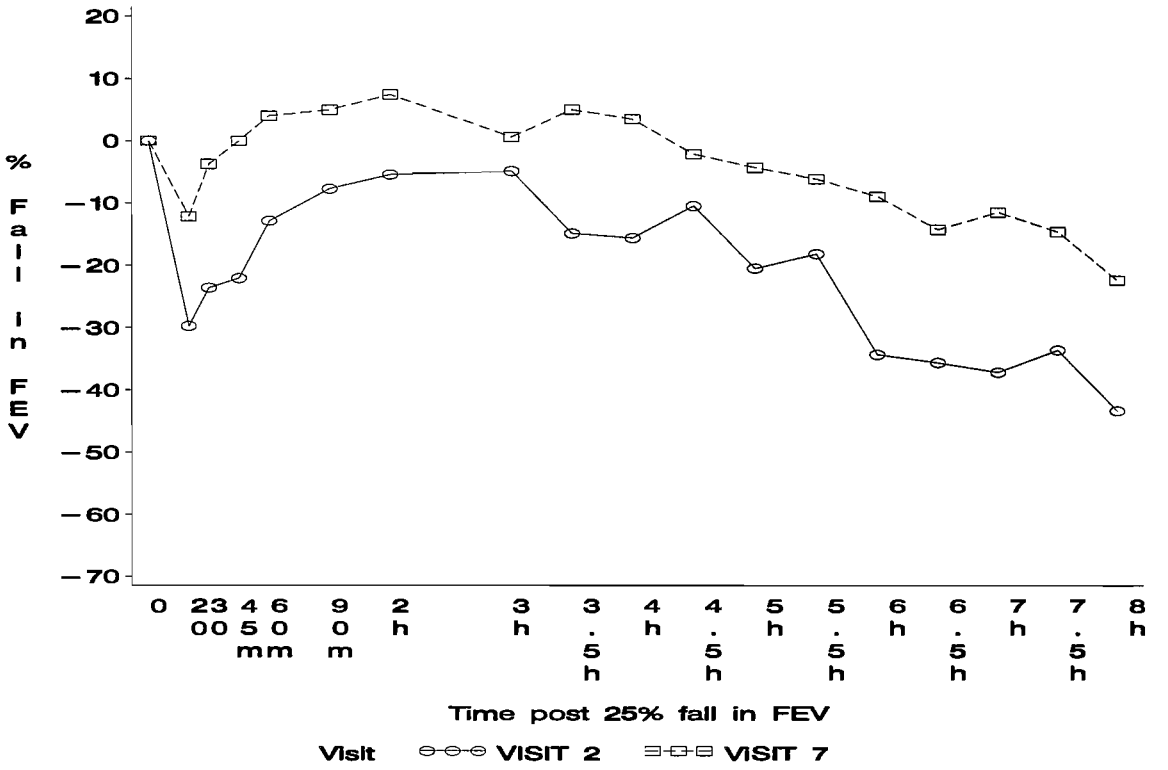
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 4



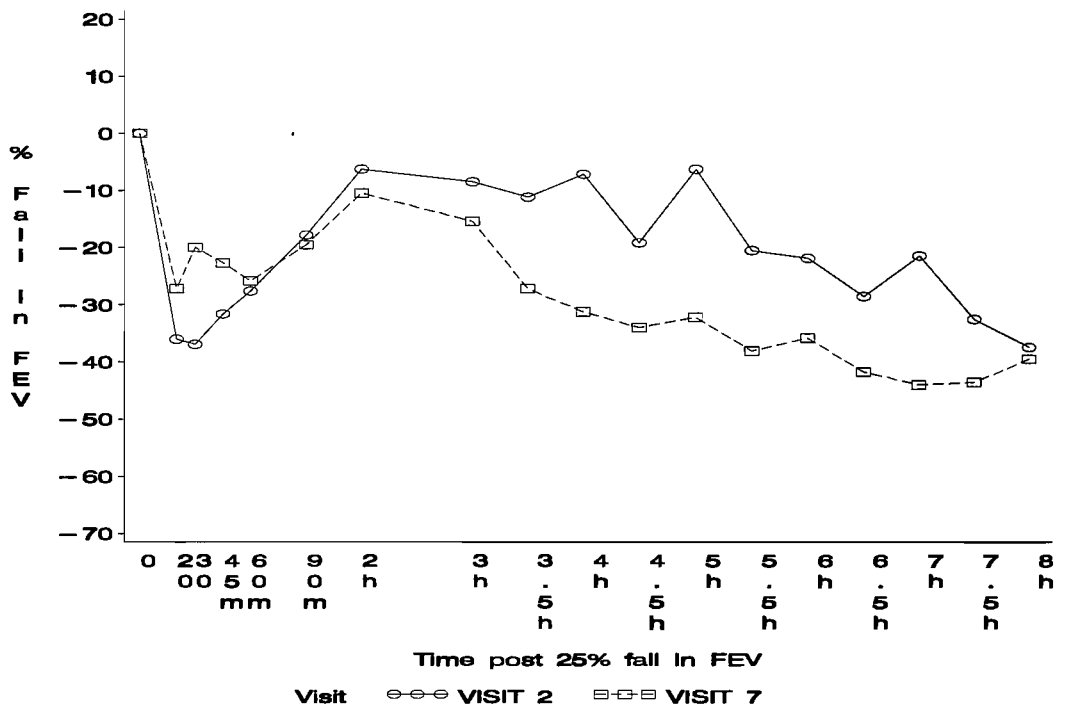
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 15



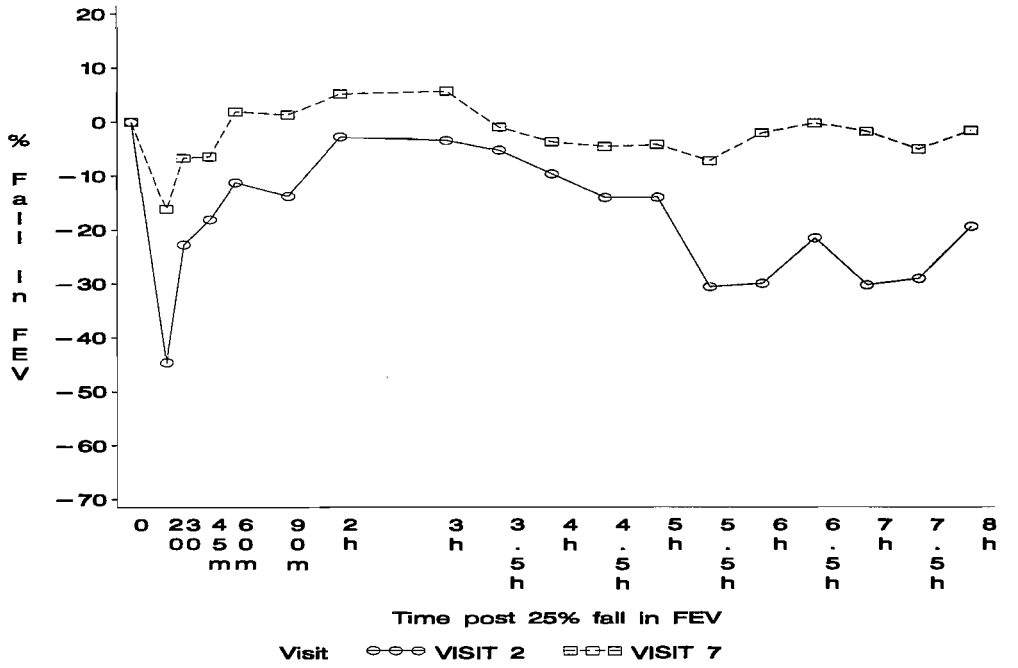
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 11



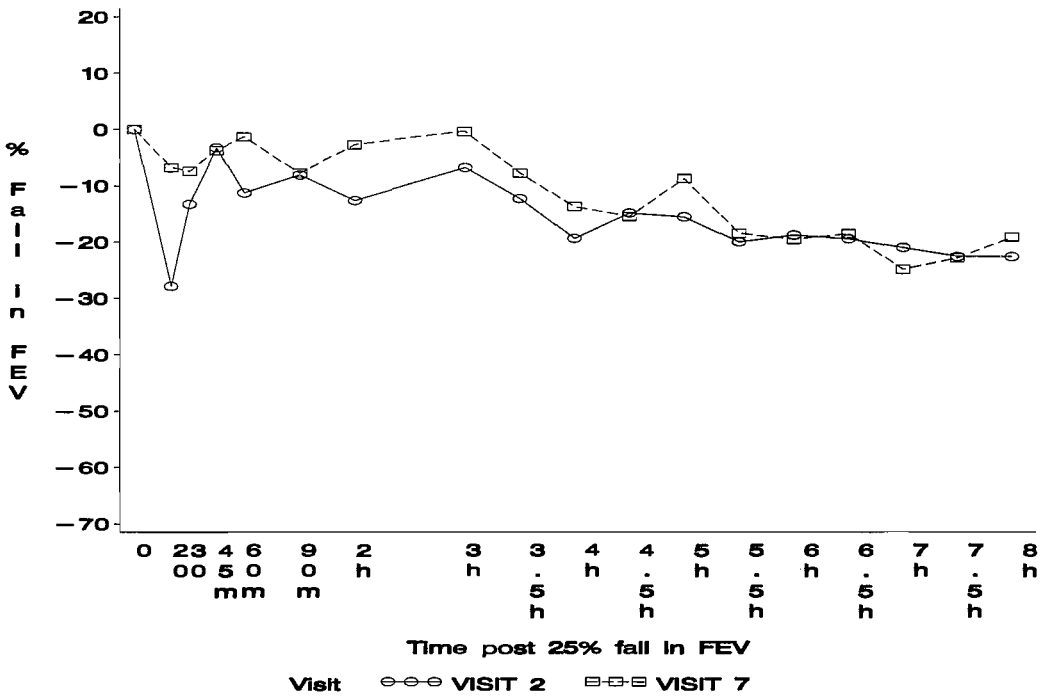
Study SR – AL – 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 13



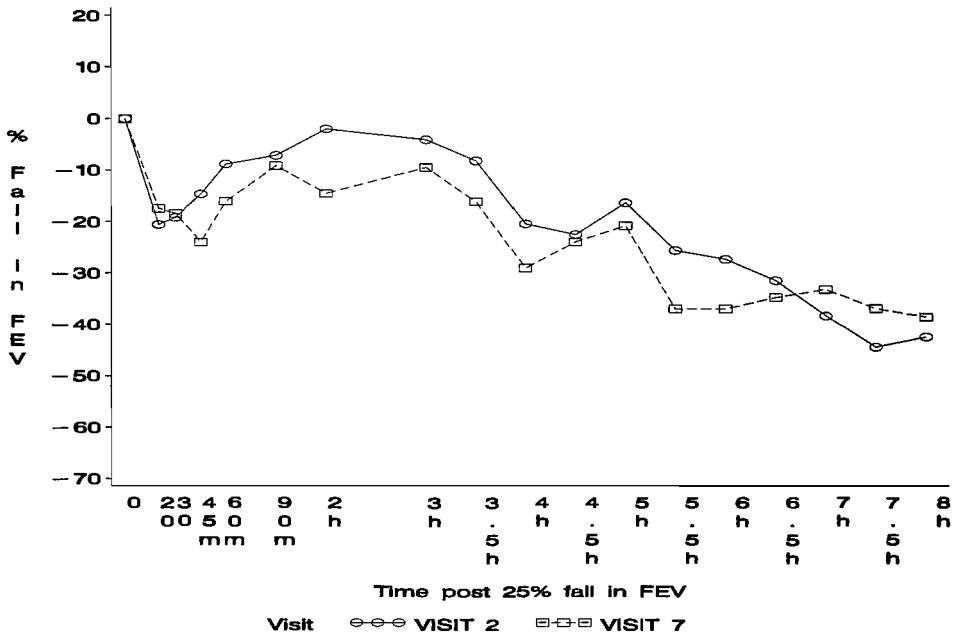
Study SR – AL – 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 17



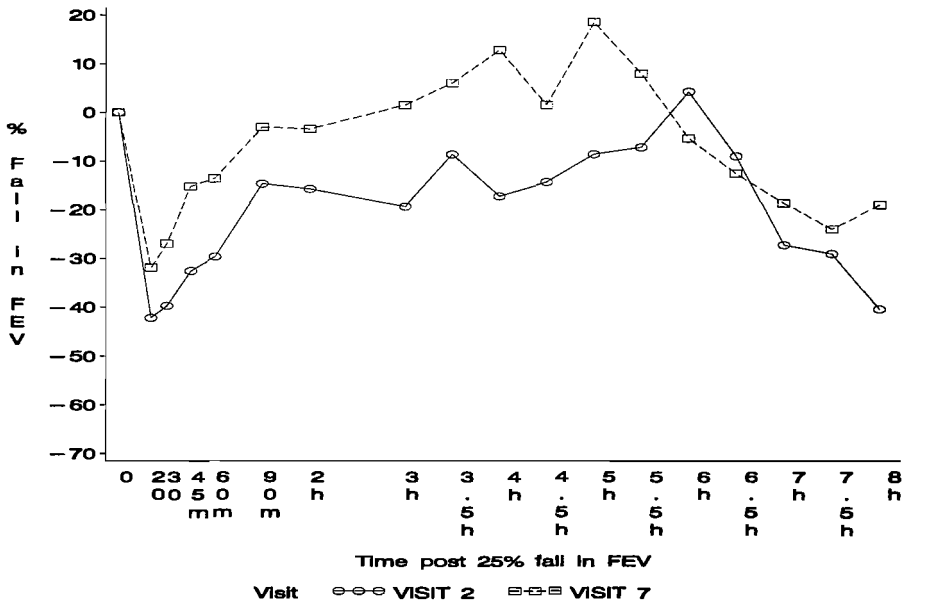
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 19



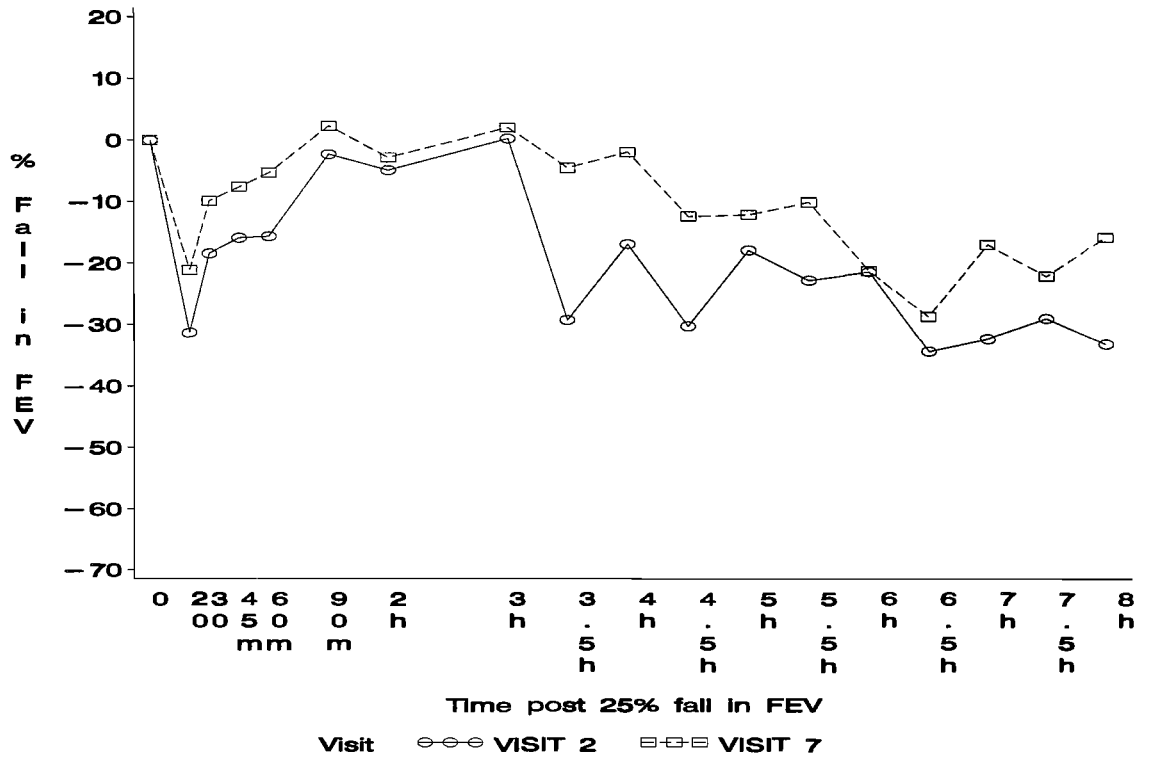
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 12



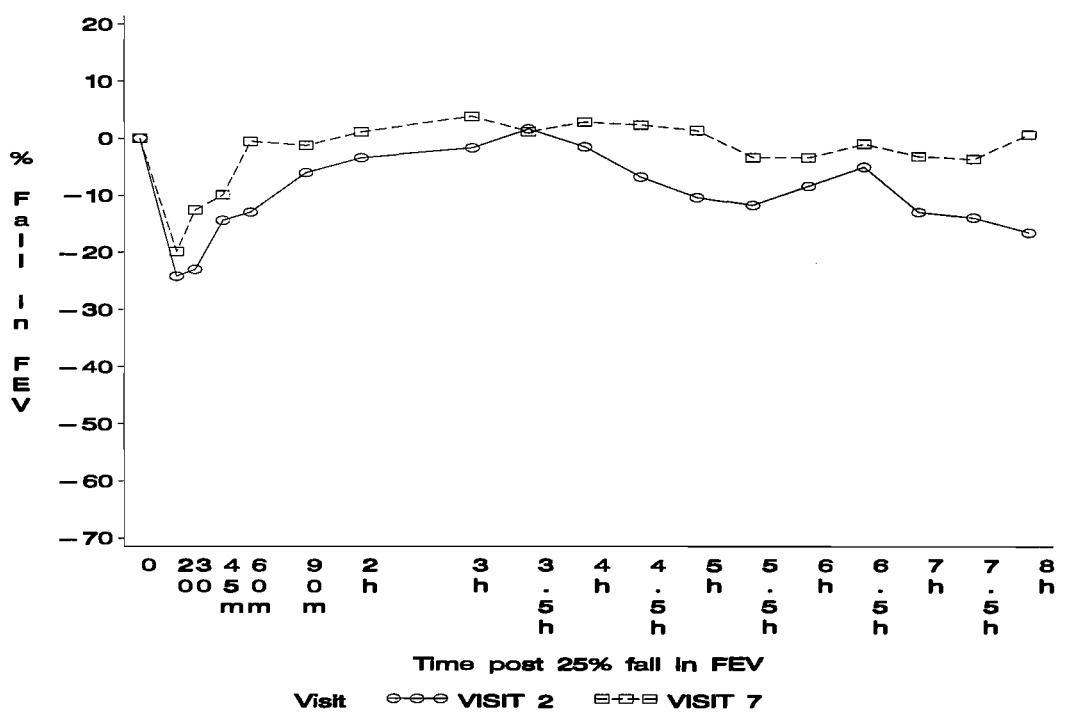
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 6



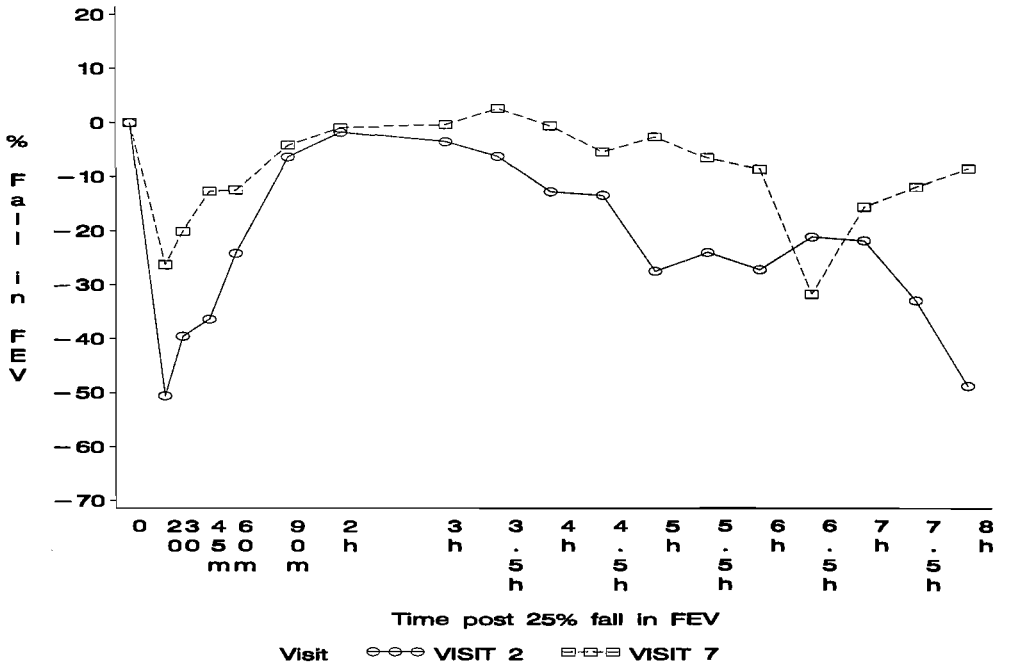
Study SR — AL — 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 7



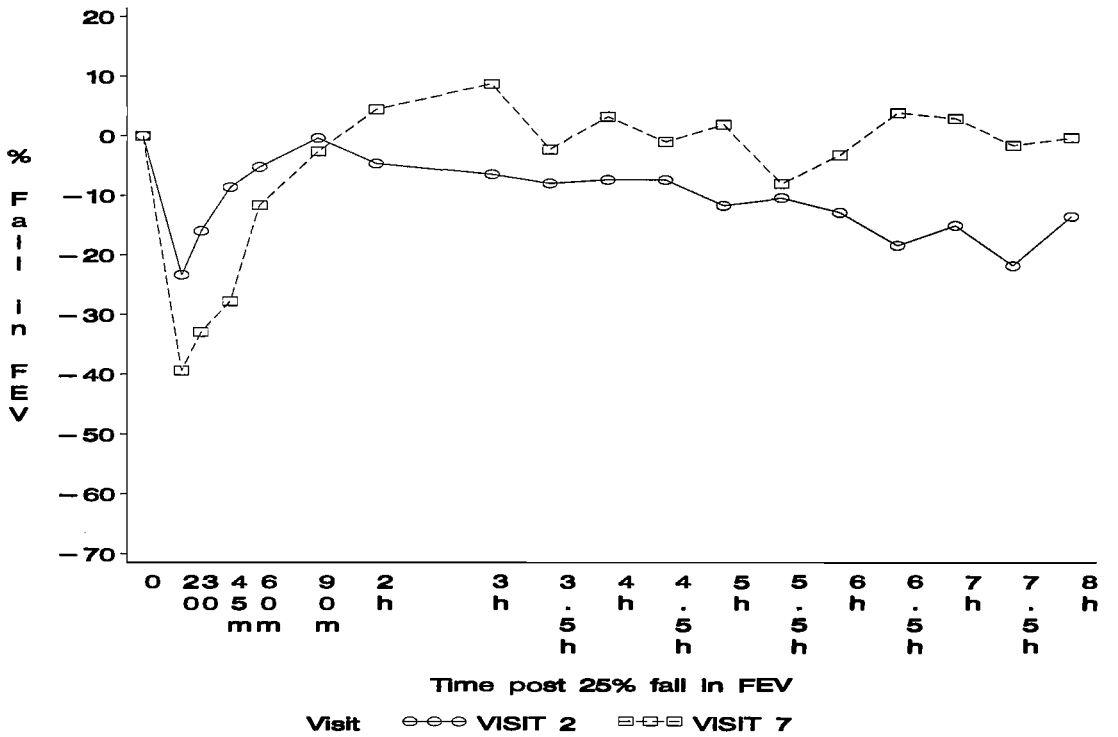
Study SR – AL – 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Active Group Patient Number = 3



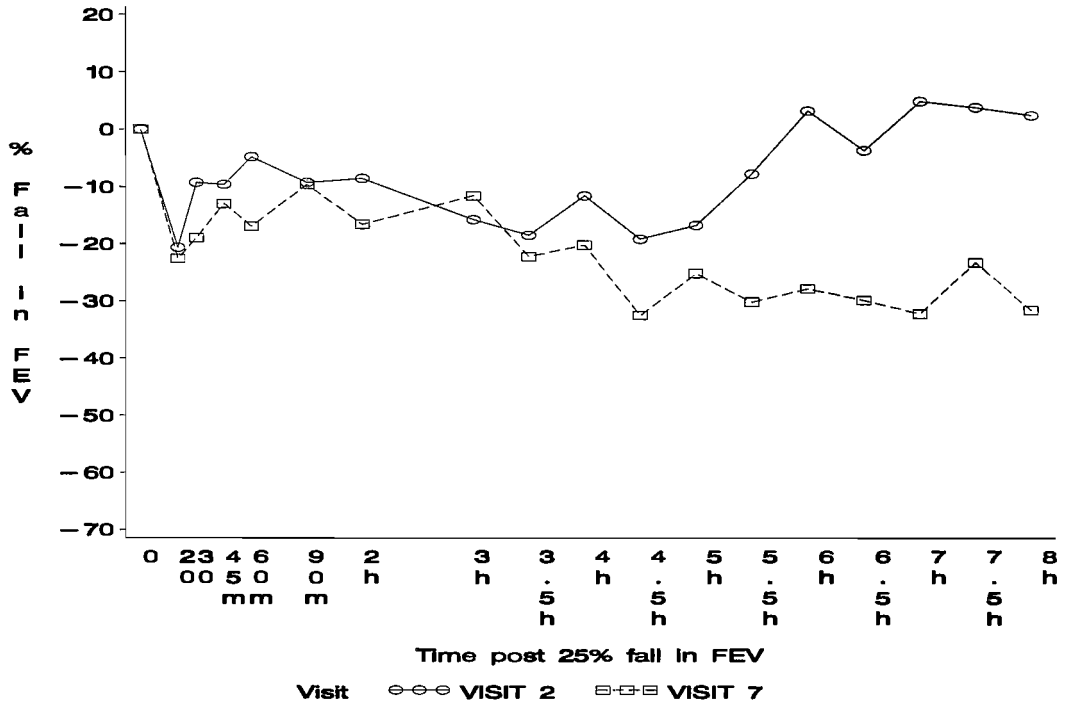
Study SR – AL – 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 8



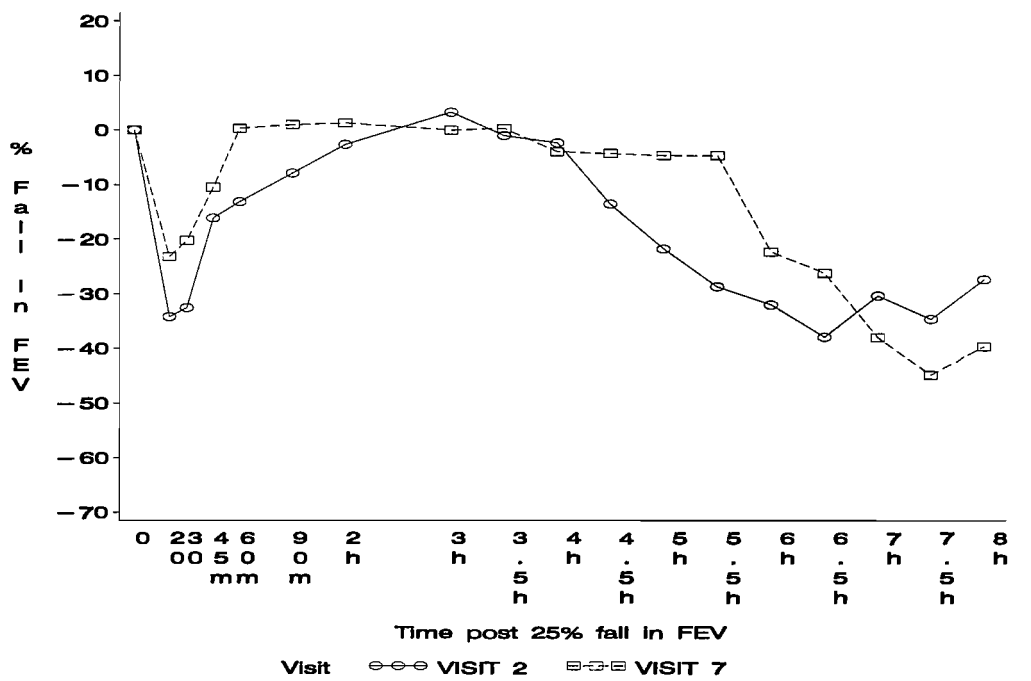
Study SR—AL—02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number=5



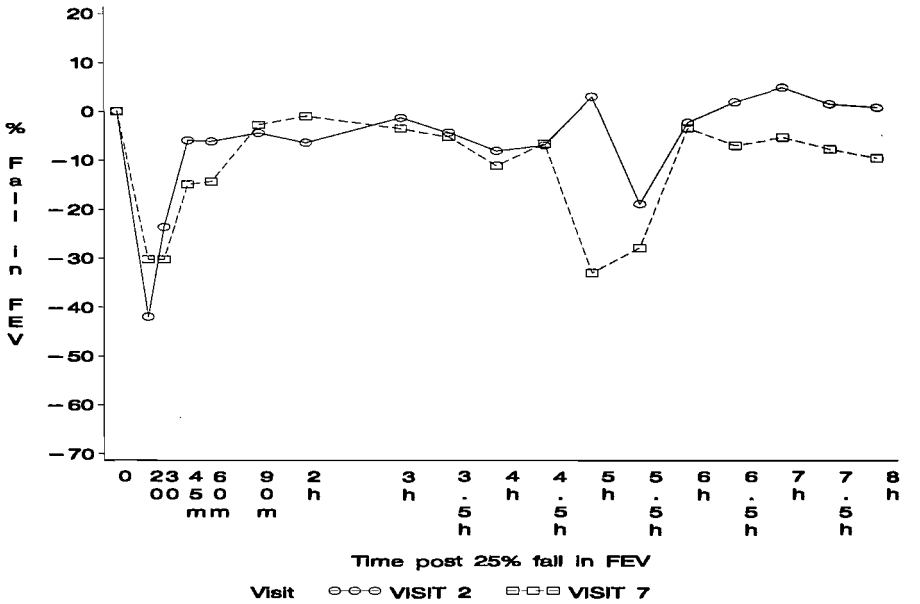
Study SR—AL—02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number=9



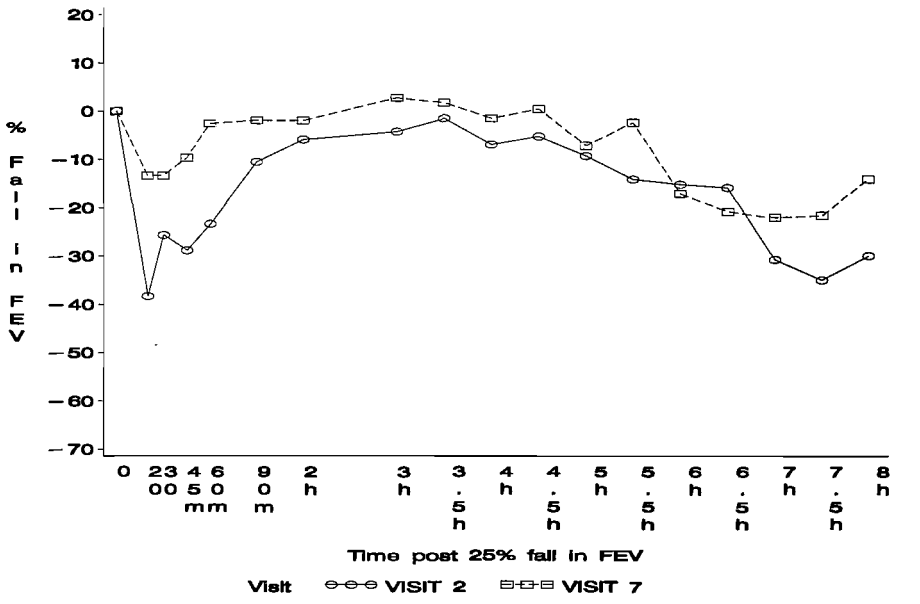
Study SR – AL – 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 2



Study SR – AL – 02

Figure 9: Individual Patient profiles of % change in FEV values during Allergen Challenge
Placebo Group Patient Number = 1



APPENDIX 3

Adverse Effects

Table 1: Number of Patients with each Adverse Event - Before Treatment

Body System	Adverse Event	Treatment Group					
		Active		Placebo		Overall	
BODY AS A WHOLE	HEADACHE	4	(33.3 %)	2	(16.7 %)	6	(25.0 %)
RESPIRATORY SYSTEM	ASTHMA	1	(8.3 %)	2	(16.7 %)	3	(12.5 %)
BODY AS A WHOLE	CHILLS	1	(8.3 %)	1	(8.3 %)	2	(8.3 %)
BODY AS A WHOLE	ALLERGY REACT			1	(8.3 %)	1	(4.2 %)
	FLU SYND			1	(8.3 %)	1	(4.2 %)
	INJURY ACCID			1	(8.3 %)	1	(4.2 %)
	PAIN	1	(8.3 %)			1	(4.2 %)
RESPIRATORY SYSTEM	SINUSITIS	1	(8.3 %)			1	(4.2 %)
SKIN AND APPENDAGES	RASH			1	(8.3 %)	1	(4.2 %)
	SKIN DIS			1	(8.3 %)	1	(4.2 %)

Table 2 : Number of Patients with each Adverse Event - After Treatment

Body System	Adverse Event	Treatment Group					
		Active		Placebo		Overall	
BODY AS A WHOLE	CHILLS	4	(33.3 %)	3	(25.0 %)	7	(29.2 %)
BODY AS A WHOLE	HEADACHE	2	(16.7 %)	3	(25.0 %)	5	(20.8 %)
RESPIRATORY SYSTEM	ASTHMA	1	(8.3 %)	4	(33.3 %)	5	(20.8 %)
BODY AS A WHOLE	CYST			1	(8.3 %)	1	(4.2 %)
	FEVER			1	(8.3 %)	1	(4.2 %)
	FLU SYND			1	(8.3 %)	1	(4.2 %)
	INJURY ACCID			1	(8.3 %)	1	(4.2 %)
	PAIN	1	(8.3 %)			1	(4.2 %)
	PAIN ABDO			1	(8.3 %)	1	(4.2 %)
	PAIN BACK			1	(8.3 %)	1	(4.2 %)
CARDIOVASCULAR SYSTEM	SYNCOPE	1	(8.3 %)			1	(4.2 %)
DIGESTIVE SYSTEM	NAUSEA	1	(8.3 %)			1	(4.2 %)
	TONGUE DIS	1	(8.3 %)			1	(4.2 %)
MUSCULOSKELETAL SYSTEM	TENDON DIS	1	(8.3 %)			1	(4.2 %)
NERVOUS SYSTEM	HYPERTONIA	1	(8.3 %)			1	(4.2 %)
RESPIRATORY SYSTEM	PHARYNGITIS			1	(8.3 %)	1	(4.2 %)
SPECIAL SENSES	PAIN EAR			1	(8.3 %)	1	(4.2 %)

Table 3: Number of Reports of each Adverse Event - Before Treatment

Body System	Adverse Event	Treatment Group: Active		Treatment Group: Placebo		Overall	
BODY AS A WHOLE	HEADACHE	4	(21.1 %)	2	(10.5 %)	6	(31.6 %)
BODY AS A WHOLE	CHILLS	2	(10.5 %)	1	(5.3 %)	3	(15.8 %)
RESPIRATORY SYSTEM	ASTHMA	1	(5.3 %)	2	(10.5 %)	3	(15.8 %)
BODY AS A WHOLE	ALLERGY REACT			1	(5.3 %)	1	(5.3 %)
	FLU SYND			1	(5.3 %)	1	(5.3 %)
	INJURY ACCID			1	(5.3 %)	1	(5.3 %)
	PAIN	1	(5.3 %)			1	(5.3 %)
RESPIRATORY SYSTEM	SINUSITIS	1	(5.3 %)			1	(5.3 %)
SKIN AND APPENDAGES	RASH			1	(5.3 %)	1	(5.3 %)
	SKIN DIS			1	(5.3 %)	1	(5.3 %)

Note: Each occurrence of adverse event is counted.
Note: Total number of adverse events reported used as denominator for percentages.

Table 4: Number of Reports of Adverse Event - After Treatment

Body System	Adverse Event	Treatment Group: Active		Treatment Group: Placebo		Overall	
BODY AS A WHOLE	HEADACHE	4	(9.3 %)	10	(23.3 %)	14	(32.6 %)
BODY AS A WHOLE	CHILLS	6	(14.0 %)	3	(7.0 %)	9	(20.9 %)
RESPIRATORY SYSTEM	ASTHMA	1	(2.3 %)	5	(11.6 %)	6	(14.0 %)
BODY AS A WHOLE	CYST			1	(2.3 %)	1	(2.3 %)
	FEVER			1	(2.3 %)	1	(2.3 %)
	FLU SYND			1	(2.3 %)	1	(2.3 %)
	INJURY ACCID			1	(2.3 %)	1	(2.3 %)
	PAIN	1	(2.3 %)			1	(2.3 %)
	PAIN ABDO			1	(2.3 %)	1	(2.3 %)
	PAIN BACK			1	(2.3 %)	1	(2.3 %)
CARDIOVASCULAR SYSTEM	SYNCOPE	1	(2.3 %)			1	(2.3 %)
DIGESTIVE SYSTEM	NAUSEA	1	(2.3 %)			1	(2.3 %)
	TONGUE DIS	1	(2.3 %)			1	(2.3 %)
MUSCULOSKELETAL SYSTEM	TENDON DIS	1	(2.3 %)			1	(2.3 %)
NERVOUS SYSTEM	HYPERTONIA	1	(2.3 %)			1	(2.3 %)
RESPIRATORY SYSTEM	PHARYNGITIS			1	(2.3 %)	1	(2.3 %)
SPECIAL SENSES	PAIN EAR			1	(2.3 %)	1	(2.3 %)

Note: Each occurrence of adverse event is counted.
Note: Total number of adverse events reported used as denominator for percentages.

Table 5: Number of Patients with each Adverse Event by Maximum Intensity - Before Treatment

		Active				Placebo				Overall			
Body System	Adverse Event	Mild	Moderate	Severe	Missing	Mild	Moderate	Severe	Missing	Mild	Moderate	Severe	Missing
BODY AS A WHOLE	ALLERGY REACT						1 (8.3 %)				1 (4.2 %)		
	CHILLS	1 (8.3 %)					1 (8.3 %)			1 (4.2 %)	1 (4.2 %)		
	FLU SYND						1 (8.3 %)				1 (4.2 %)		
	HEADACHE	2 (16.7%)	1 (8.3 %)	1 (8.3		2 (16.7%)				4 (16.7%)	1 (4.2 %)	1 (4.2 %)	
	INJURY ACCID						1 (8.3 %)				1 (4.2 %)		
	PAIN		1 (8.3 %)								1 (4.2 %)		
RESPIRATORY SYSTEM	ASTHMA	1 (8.3 %)				1 (8.3 %)		1 (8.3 %)		2 (8.3 %)		1 (4.2 %)	
	SINUSITIS		1 (8.3 %)								1 (4.2 %)		
SKIN AND APPENDAGES	RASH					1 (8.3 %)				1 (4.2 %)			
	SKIN DIS								1 (8.3				1 (4.2 %)

Table 6: Number of Patients with each Adverse Event by Maximum Intensity - After Treatment

		Active				Placebo				Overall			
Body System	Adverse Event	Mild	Moderate	Severe	Missing	Mild	Moderate	Severe	Missing	Mild	Moderate	Severe	Missing
BODY AS A WHOLE	CHILLS	2 (16.7%)	2 (16.7%)			1 (8.3 %)	2 (16.7%)			3 (12.5%)	4 (16.7%)		
	CYST						1 (8.3 %)				1 (4.2 %)		
	FEVER							1 (8.3 %)				1 (4.2 %)	
	FLU SYND						1 (8.3 %)				1 (4.2 %)		
	HEADACHE	1 (8.3 %)	1 (8.3 %)			1 (8.3 %)	2 (16.7%)			2 (8.3 %)	3 (12.5%)		
	INJURY ACCID						1 (8.3 %)				1 (4.2 %)		
	PAIN		1 (8.3 %)								1 (4.2 %)		
	PAIN ABDO							1 (8.3 %)				1 (4.2 %)	
	PAIN BACK							1 (8.3 %)				1 (4.2 %)	
CARDIOVASCULAR SYSTEM	SYNCOPE		1 (8.3 %)								1 (4.2 %)		
DIGESTIVE SYSTEM	NAUSEA		1 (8.3 %)								1 (4.2 %)		
	TONGUE DIS	1 (8.3 %)								1 (4.2 %)			
MUSCULOSKELETAL SYSTEM	TENDON DIS				1 (8.3 %)								1(4.2 %)
NERVOUS SYSTEM	HYPERTONIA		1 (8.3 %)								1 (4.2 %)		
RESPIRATORY SYSTEM	ASTHMA				1 (8.3 %)	1 (8.3 %)	2 (16.7%)	1 (8.3 %)		1 (4.2 %)	2 (8.3 %)	1 (4.2 %)	1(4.2 %)
	PHARYNGITIS					1 (8.3 %)				1 (4.2 %)			
SPECIAL SENSES	PAIN EAR					1 (8.3 %)				1 (4.2 %)			

Table 7: Number of Patients with each Adverse Event by Relationship - Before Treatment

		Active				Placebo				Overall			
Body System	Adverse Event	None	Unlikely	Possible	Probable	None	Unlikely	Possible	Probable	None	Unlikely	Possible	Probable
BODY AS A WHOLE	ALLERGY REACT					1 (8.3 %)				1 (4.2 %)			
	CHILLS	1 (8.3 %)				1 (8.3 %)				2 (8.3 %)			
	FLU SYND					1 (8.3 %)				1 (4.2 %)			
	HEADACHE	3 (25.0%)	1 (8.3 %)			1 (8.3 %)	1 (8.3 %)			4 (16.7%)	2 (8.3 %)		
	INJURY ACCID					1 (8.3 %)				1 (4.2 %)			
	PAIN	1 (8.3 %)								1 (4.2 %)			
RESPIRATORY SYSTEM	ASTHMA	1 (8.3 %)				1 (8.3 %)		1 (8.3 %)		2 (8.3 %)		1 (4.2 %)	
	SINUSITIS	1 (8.3 %)								1 (4.2 %)			
SKIN AND APPENDAGES	RASH							1 (8.3 %)				1 (4.2 %)	
	SKIN DIS					1 (8.3 %)				1 (4.2 %)			

Table 8: Number of Patients with each Adverse Event by Relationship - After Treatment

		Active				Placebo				Overall			
Body System	Adverse Event	None	Unlikely	Possible	Probable	None	Unlikely	Possible	Probable	None	Unlikely	Possible	Probable
BODY AS A WHOLE	CHILLS	2 (16.7%)	2 (16.7%)			1 (8.3 %)	2 (16.7%)			3 (12.5%)	4 (16.7%)		
	CYST						1 (8.3 %)				1 (4.2 %)		
	FEVER						1 (8.3 %)				1 (4.2 %)		
	FLU SYND						1 (8.3 %)				1 (4.2 %)		
	HEADACHE	1 (8.3 %)	1 (8.3 %)			2 (16.7%)	1 (8.3 %)			3 (12.5%)	2 (8.3 %)		
	INJURY ACCID					1 (8.3 %)				1 (4.2 %)			
	PAIN	1 (8.3 %)								1 (4.2 %)			
	PAIN ABDO						1 (8.3 %)				1 (4.2 %)		
	PAIN BACK						1 (8.3 %)				1 (4.2 %)		
CARDIOVASCULAR SYSTEM	SYNCOPE	1 (8.3 %)								1 (4.2 %)			
DIGESTIVE SYSTEM	NAUSEA				1 (8.3 %)								1 (4.2 %)
	TONGUE DIS	1 (8.3 %)								1 (4.2 %)			
MUSCULOSKELETAL SYSTEM	TENDON DIS	1 (8.3 %)								1 (4.2 %)			
NERVOUS SYSTEM	HYPERTONIA	1 (8.3 %)								1 (4.2 %)			
RESPIRATORY SYSTEM	ASTHMA		1 (8.3 %)			1 (8.3 %)	1 (8.3 %)	2 (16.7%)		1 (4.2 %)	2 (8.3 %)	2 (8.3 %)	
	PHARYNGITIS						1 (8.3 %)				1 (4.2 %)		
SPECIAL SENSES	PAIN EAR						1 (8.3 %)				1 (4.2 %)		

Table 9: Number of Patients with each serious Adverse Event - Before Treatment

Body System	Adverse Event	Treatment Group		
		Active	Placebo	Overall
RESPIRATORY SYSTEM	ASTHMA		1 (8.3 %)	1 (4.2 %)

Table 10: Number of Patients with each serious Adverse Event - After Treatment

Body System	Adverse Event	Treatment Group		
		Active	Placebo	Overall
BODY AS A WHOLE	FEVER	1 (8.3 %)	1 (4.2 %)	
	PAIN ABDO	1 (8.3 %)	1 (4.2 %)	
	PAIN BACK	1 (8.3 %)	1 (4.2 %)	
RESPIRATORY SYSTEM	ASTHMA	1 (8.3 %)	1 (4.2 %)	

APPENDIX 4

Laboratory tests

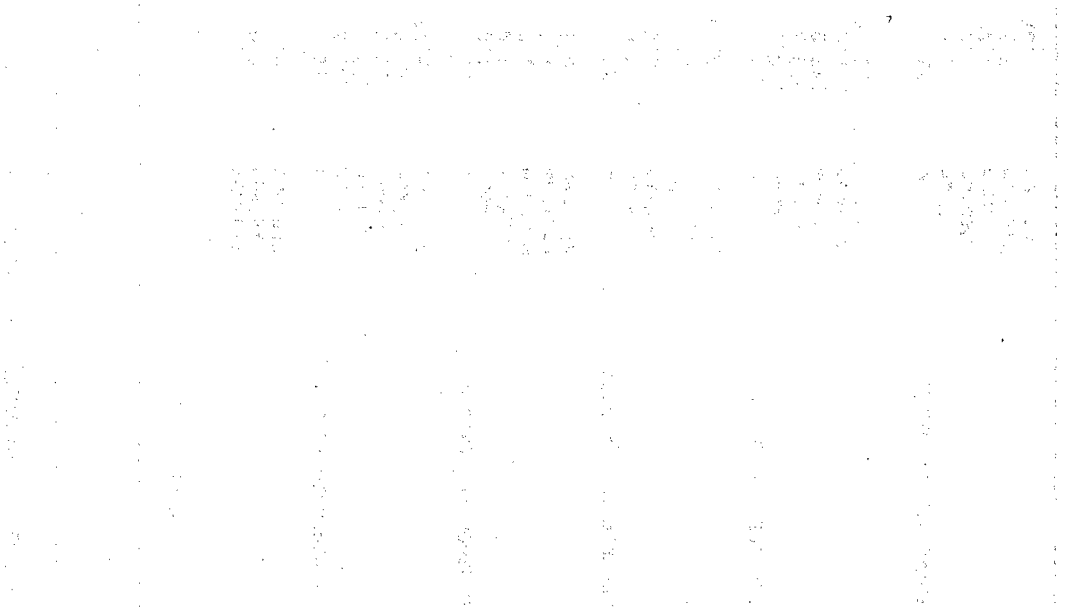


Table 1: Summary of Laboratory Data - Haematology

Parameter		Active		Placebo	
		Visit 1	Visit 9	Visit 1	Visit 9
EOSINOPHILS (X10/9/L)	N	12	11	12	12
	Mean	0.3	0.3	0.3	0.2
	Median	0.3	0.3	0.3	0.3
	Minimum	0.2	0.1	0.1	0.0
	Maximum	0.6	0.4	0.6	0.4
	Std.Dev	0.13	0.08	0.15	0.12
HAEMOGLOBIN (G/DL)	N	12	11	12	12
	Mean	150.8	150.4	148.8	146.8
	Median	150.0	153.0	154.5	151.0
	Minimum	139.0	130.0	117.0	118.0
	Maximum	163.0	163.0	168.0	161.0
	Std.Dev	8.77	10.66	15.81	14.47
LYMPHOCYTES (X10/9/L)	N	12	11	12	12
	Mean	1.8	1.7	1.9	1.7
	Median	1.9	1.6	1.9	1.5
	Minimum	1.0	1.0	1.1	1.1
	Maximum	2.7	2.8	3.1	2.6
	Std.Dev	0.52	0.48	0.58	0.55
NEUTROPHILS (X10/9/L)	N	12	11	12	12
	Mean	3.0	2.6	3.8	3.6
	Median	2.6	2.5	3.7	3.4
	Minimum	2.0	1.7	2.0	1.8
	Maximum	5.0	3.8	6.9	5.9
	Std.Dev	0.89	0.70	1.42	1.00
PLATELETS (X10/9/L)	N	12	11	12	12
	Mean	209.2	213.8	258.8	261.3
	Median	209.5	225.0	259.0	275.5
	Minimum	137.0	148.0	189.0	180.0
	Maximum	257.0	261.0	340.0	317.0
	Std.Dev	35.74	36.85	46.44	43.33
TOTAL WBC (X10/9/L)	N	12	11	12	12
	Mean	5.6	5.0	6.5	6.1
	Median	5.5	4.6	6.4	6.2
	Minimum	3.8	3.8	3.9	3.7
	Maximum	7.3	6.7	9.9	7.6
	Std.Dev	1.07	0.99	1.72	1.21

Table 2: Summary of Laboratory Data - Biochemistry

Parameter		Active		Placebo	
		Visit 1	Visit 9	Visit 1	Visit 9
ALBUMIN (G/L)	N	12	12	12	12
	Mean	41.4	40.9	40.8	40.3
	Median	42.0	40.5	42.0	40.5
	Minimum	39.0	34.0	36.0	37.0
	Maximum	44.0	47.0	46.0	43.0
	Std.Dev	1.62	3.96	3.10	1.97
ALK PHOS (IU/L)	N	12	12	12	12
	Mean	145.3	162.5	155.8	164.6
	Median	137.5	155.5	152.0	153.0
	Minimum	83.0	78.0	86.0	96.0
	Maximum	239.0	257.0	266.0	279.0
	Std.Dev	47.23	57.16	51.35	55.18
ALT (IU/L)	N	12	12	12	12
	Mean	25.4	76.1	27.3	25.4
	Median	27.0	20.5	28.5	25.5
	Minimum	14.0	9.0	7.0	12.0
	Maximum	33.0	682.0	46.0	36.0
	Std.Dev	7.03	191.00	11.88	7.99
CALCIUM (MMOL/L)	N	12	12	12	12
	Mean	2.4	2.3	2.3	2.3
	Median	2.4	2.3	2.3	2.4
	Minimum	2.3	2.2	2.2	2.2
	Maximum	2.5	2.6	2.5	2.4
	Std.Dev	0.07	0.09	0.10	0.08

Table 3: Summary of Laboratory Data - Biochemistry

Parameter		Active		Placebo	
		Visit 1	Visit 9	Visit 1	Visit 9
CREATININE (UMOL/L)	N	12	12	12	12
	Mean	87.1	89.4	90.3	86.8
	Median	86.5	87.0	91.0	86.5
	Minimum	74.0	75.0	74.0	65.0
	Maximum	108.0	110.0	107.0	113.0
	Std.Dev	9.28	11.16	10.62	12.88
GLOBULIN (G/L)	N	12	12	12	12
	Mean	29.6	29.4	30.0	30.1
	Median	29.0	29.5	29.5	31.0
	Minimum	24.0	24.0	24.0	24.0
	Maximum	34.0	35.0	36.0	34.0
	Std.Dev	2.81	3.26	3.41	3.00
POTASSIUM (MMOL/L)	N	12	12	12	12
	Mean	3.9	3.8	4.0	3.8
	Median	3.9	3.9	3.9	3.9
	Minimum	3.7	3.3	3.4	3.4
	Maximum	4.2	4.1	4.7	4.3
	Std.Dev	0.17	0.21	0.35	0.25
SODIUM (MMOL/L)	N	12	12	12	12
	Mean	140.2	140.8	140.0	139.8
	Median	140.5	140.5	140.0	140.0
	Minimum	137.0	137.0	136.0	137.0
	Maximum	143.0	144.0	142.0	142.0
	Std.Dev	1.59	2.14	1.71	1.59
TOTAL BILIRUBIN (UMOL/L)	N	12	12	12	12
	Mean	15.4	16.2	11.5	15.0
	Median	9.0	11.5	8.0	11.0
	Minimum	2.0	6.0	4.0	6.0
	Maximum	57.0	48.0	37.0	44.0
	Std.Dev	15.47	12.75	8.88	12.74
TOTAL PROTEIN (G/L)	N	12	12	12	12
	Mean	71.0	70.3	70.8	70.4
	Median	71.5	69.5	70.5	71.0
	Minimum	64.0	63.0	67.0	65.0
	Maximum	75.0	78.0	76.0	75.0
	Std.Dev	3.25	4.74	3.30	3.00
UREA (MMOL/L)	N	10	11	12	10
	Mean	5.1	5.2	4.7	5.2
	Median	5.1	4.8	4.8	5.1
	Minimum	3.9	4.1	3.0	3.7
	Maximum	7.1	8.3	7.3	6.7
	Std.Dev	0.85	1.25	1.21	1.07