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The Role of Immune Mediators in Airway Inflammation.

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A thesis submitted to the Faculty of Medicine, University of Glasgow,

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Summary

Asthma is a chronic inflammatory condition of the airways characterised by reversible airflow obstruction, airway hyper-responsiveness and inflammatory infiltrates in the airway walls containing eosinophils, T lymphocytes and mast cells. T helper (Th) lymphocyte subsets, defined by the cytokines they secrete, are thought to play a key role in the in the initiation and perpetuation of chronic airway inflammation. Th2 cells, producing interleukin (IL)-4, IL-5, IL-9 and IL-13, are thought to be of particular importance. In contrast, Th1 cells producing interferon (IFN)-γ may counteract the development of Th2 responses and so down-regulate the asthmatic response.

The prevalence of asthma is increasing but the reasons for this are not fully understood. In addition, some patients do not respond adequately to treatment with corticosteroids, currently the most effective anti-inflammatory agents used routinely in human asthma. There is therefore continual interest in developing new therapeutic agents for asthma. A greater understanding of the regulation of inflammatory responses in asthma will assist in the identification of potential targets for therapeutic intervention.

The aims of this thesis were **(i)** to assess the role of the cytokine IL-18 in allergic airway inflammation by determining IL-18 levels in induced sputum in asthmatic subjects in comparison to normal subjects, and by studies in a murine model of allergic asthma using IL-18 gene deficient mice and **(ii)** to assess the potential anti-inflammatory actions of simvastatin and thymosin beta 4 sulfoxide in the murine asthma model.

IL-18 is a pro-inflammatory cytokine which can promote IFN-γ secretion and, in association with IL-12, enhance the development of Th1 responses. However, in some circumstances it may also stimulate Th2 responses. IL-18 therefore has the potential to suppress or exacerbate allergic airway inflammation. The role of IL-18 in both clinical and experimental asthma remains unclear.

Statins are inhibitors of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, in cholesterol biosynthesis. As such they have been widely used as cholesterol lowering agents in clinical practice. They have previously been shown to have anti-inflammatory properties independent of their cholesterol-lowering ability in clinical studies of atherosclerotic disease and in animal models of Th1-mediated inflammation.

Thymosin beta 4 sulfoxide (Tβ4SO) is a 5 kDa peptide. Intracellularly its principal activity is to regulate actin polymerization. Corticosteroid treatment of monocytes *in vitro* induces the release of Tβ4SO extracellularly, where it can inhibit neutrophil chemotaxis. Exogenous administration of Tβ4SO has been shown to reduce neutrophilic inflammation in animal models.

In this study it is shown that IL-18 is detectable in induced sputum fluid and IL-18 mRNA is expressed in induced sputum cells from asthmatic and normal subjects. IL-18 protein levels in induced sputum, and IL-18 mRNA expression in induced sputum cells were not significantly different between these groups. IL-18 production was localised to sputum macrophages. However, cigarette smoking significantly reduced IL-18 levels in induced sputum fluid in both asthmatic and normal subjects. In

asthmatics, but not normal subjects, the reduction in IL-18 levels in sputum fluid was associated with reduced IL-18 mRNA expression in induced sputum cells.

A murine model of allergic asthma, using BALB/c mice sensitised and challenged with ovalbumin (OVA), was used to examine the role of IL-18 in allergic responses *in vivo*. IL-18 gene knockout (ko) had significantly reduced bronchoalveolar lavage (BAL) total cell count and eosinophilia compared to wild-type (WT) mice. IL-18 ko mice had reduced IL-4 expression in thoracic lymph nodes, as assessed by quantitative PCR, and significantly reduced OVA-specific IL-4 secretion from thoracic lymph node cultures assessed by ELISA. Serum OVA-specific IgG1, IgG2a and IgE and total IgE levels were not significantly different between IL-18 ko and WT mice.

The murine model of allergic asthma was also used to examine the anti-inflammatory activities of simvastatin and Tβ4SO in a Th2-mediated, eosinophilic condition. Simvastatin treatment, either orally or intraperitoneally, and Tβ4SO intraperitoneally reduced the total inflammatory cell infiltrate and eosinophilia in BAL fluid in response to inhaled OVA challenge. At higher doses of simvastatin intraperitoneally, a histological reduction in inflammatory infiltrates in the lungs was observed. Treatment with simvastatin intraperitoneally, but not orally, and Tβ4SO were also associated with a reduction in IL-4 and IL-5 levels in BAL fluid. OVA-induced IL-4 and IL-5 secretion was reduced in thoracic lymph node cultures from both simvastatin-treated and Tβ4SO-treated mice. Neither simvastatin nor Tβ4SO treatment altered serum total IgE or OVA-specific IgG1 and IgG2a levels.

The results described show that IL-18 can be detected in the induced sputum fluid of asthmatic and normal subjects and that cigarette smoking significantly reduces its levels. Studies in a murine model of allergic asthma suggest that IL-18 has a pro-inflammatory role in allergic airway inflammation, at least in part through its ability to induce IL-4 secretion. Both simvastatin and thymosin beta 4 sulfoxide had convincing anti-inflammatory properties in the murine model of asthma used, and these agents, or related compounds, may have therapeutic potential in human asthma.

List of Contents

Contents

List of Tables and Figures

Chapter 1 General introduction

Chapter 2 Materials and methods

Chapter 3 The effect of cigarette smoking on interleukin-18 levels in induced sputum from asthmatics and normal subjects

Chapter 4 The effect of IL-18 deficiency in a murine model of allergic asthma

Chapter 5 The anti-inflammatory actions of simvastatin in a murine model of allergic asthma

Chapter 6 The anti-inflammatory actions of thymosin beta 4 sulfoxide in a murine model of allergic asthma

Chapter 7 General discussion

Publications

Full publications

McKay, A., Leung, B. P., McInnes, I. B., Thomson, N. C. and Liew, F. Y. (2004) A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J Immunol.* **172**, 2903-8.

McKay, A. Komai-Koma, M., MacLeod, K.J., Campbell, C.C., Kitson, S.M., Chaudhuri, R., McSharry, C., Liew, F.Y. and Thomson, N.C. (2004) Interleukin-18 levels in induced sputum are reduced in asthmatic and normal smokers. *Clin Exp Allergy*. (Accepted for publication).

Abstracts

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McKay, A., Leung, B.P., McInnes, I.B., Culshaw, S., Thomson, N.C. and Liew, F.Y. (2002) Reduced severity and IL-4 response in a murine model of allergic asthma in IL-18 deficient mice. Poster presentation at the American Thoracic Society Conference, Atlanta, USA, May 2002.

McKay, A., Leung, B.P., McInnes, I.B., Young, J.D., Stevenson, R.D., Thomson, N.C. and Liew FY. (2002) A novel anti-inflammatory role for thymosin beta 4 sulfoxide in a murine model of allergic asthma. Oral presentation at the European Respiratory Society Conference, Stockholm, September 2002.

McKay, A., Leung, B.P., McInnes, I.B., Culshaw, S., Thomson, N.C. and Liew FY. (2002) Simvastatin has an anti-inflammatory role in a murine model of allergic asthma. *Thorax* **57**, (S3):iii37. Oral presentation at British Thoracic Society Meeting, London, December 2002.

McKay, A., Leung, B.P., McInnes, I.B., Culshaw, S., Thomson, N.C. and Liew FY. (2003) Simvastatin reduces airway eosinophilia in a murine model of allergic asthma. Accepted as poster presentation at the American Thoracic Society Conference, Seattle, USA, May 2003.

Abbreviations

ACD, allergic contact dermatitis

AcP, accessory protein

AP-1, activator protein-1

AHR, airway hyper-responsiveness

BAL, bronchoalveolar lavage

BSA, bovine serum albumin

CCR, chemokine receptor

CD, cluster of differentiation antigens

CIA, collagen-induced arthritis

Con A, Concanavalin-A

CTLA-4, cytotoxic T lymphocyte antigen 4

dATP, 2'-deoxyadenosine 5'-triphosphate

dCTP, 2'deoxycytosine 5'-triphosphate

dGTP, 2'deoxy guanosine 5'-triphosphate

d, day

DC, dendritic cell

DNA, deoxyribonucleic acid

DNase, deoxyribonuclease

dNTP, 2' deoxynucleotide 5'-triphosphate

DTT, 1,4 dithioreitol

dTTP, 2'deoxythymidine 5'-triphosphate

dUTP, 2'-deoxyuracil 5'-triphosphate

EAE, experimental allergic encephalomyelitis

EDTA, ethylene diamine tetra acetic acid

ELISA, enzyme-linked immunosorbance assay

eNOS, endothelial nitric oxide

erk, extracellular regulated kinase

FACS, fluorescence-activated cell sorter

FAM, 6-carboxy fluorescin

FCS, fetal calf serum

FITC, fluoroscein isothiocynate

GITR, glucocorticoid-induced TNF receptor

GM-CSF, granulocyte-macrophage colony stimulating factor

GRE, glucocorticoid response element

H&E, haemotoxylin and eosin

HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A

HPRT, hypoxanthine-guanine phosphoribosyltransferase

ICAM, intercellular adhesion molecule

ICSBP, IFN consensus sequence binding protein

IFN, interferon

Ig, immunoglobulin

i.p., intraperitoneal

i.n., intranasal

I-κB, inhibitor-κB

IKK, I-κB kinase

IL, interleukin

IL-1R, interleukin-1 receptor

IL-1Rrp, IL-1 receptor related peptide

IL-18R, interleukin-18 receptor

iNOS, inducible nitric oxide

IRAK, IL-1R-associated kinase

JAK, Janus kinase

JNK, c-Jun N terminal kinase

ko, (gene) knockout

LFA, leukocyte function antigen

LT, leukotriene

LPS, lipoplysaccharide

MAP, mitogen activated protein

MCP, monocyte chemotactic protein

MEK, MAP kinase kinase or MKK

MHC, major histocompatibility complex

MIP, macrophage inflammatory protein

mRNA, messenger RNA

MTB, *Mycobacterium tuberculosis*

NF-AT, nuclear factor of activated T cells

NF-κB, nuclear factor-κB

NIK, NF-κB inducing kinase

NK, natural killer

NO, nitric oxide

NOS, NO synthase

OD, optical density

OVA, ovalbumin

PBS, phosphate buffered saline

PBMC, peripheral blood monocyte

PE, phycoerythrin

PG, prostaglandin

PI-3K, phosphoinositde-3 kinase

PP, pyrophosphate

PPAR-γ, peroxisome proliferator-activated receptor gamma

RANTES, regulated on activation, normal T-cell expressed and secreted

RNA, ribonucleic acid

RT-PCR, reverse transcription polymerase chain reaction

SEM, standard error of the mean

SNP, single nucleotide polymorphisms

STAT, signal transducer and activator of transcription

TAB, TAK binding protein

TAK, transforming growth factor-β activating kinase

TAMRA, 6-carboxy-tetramethyl-rhodamine

Tβ4SO, thymosin beta 4 sulfoxide

TCR, T cell receptor

TGF-β, transforming growth factor β

Th, T helper (lymphocyte)

TIR, Toll/IL-1 receptor

TLR, Toll-like receptor

TNF, tumour necrosis factor

TRAF, TNF-R-associated factor

Tris, Tris (hydroxymethyl) methylamine

U, units

UTR, untranslated region

WT, wild-type

M, molar

mM, milimolar

mg, milligram

ml, millilitre

μg, microgram

μl, micro litre

μM, micromolar

ng, nanogram

nM, nanomolar

pg, picogram

pM, picomolar

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Declaration

This study represents original work carried out by the author, and has not been submitted in any form to any other University. Where use has been made of materials provided by others, due acknowledgement has been made.

Anne McKay

May 2004

Chapter 1

General Introduction

1.1 Asthma

1.1.1 Introduction

Asthma is a chronic inflammatory condition of the airways characterised by reversible airflow obstruction, airway hyper-responsiveness (AHR) and inflammatory infiltrates in the airway walls containing eosinophils, T lymphocytes and mast cells. Atopic asthma is associated with raised serum IgE levels. T helper (Th) lymphocyte subsets, defined by the cytokines they secrete are thought to play a key role in the in the initiation and perpetuation of this inflammation. In particular, Th2 cells, producing the cytokines interleukin (IL)-4, IL-5 and IL-13, are thought to be of particular importance. However a complex network of cell types and inflammatory mediators (reviewed by Barnes et al., 1998 and Larché et al., 2003) contribute to the development of the inflammatory response (Figure 1.1).

The functions of the major cell types involved in the development of airway inflammation in asthma are discussed below. The actions of specific cytokines which have a significant role in the control of allergic responses are also highlighted.

1.1.2 Cellular inflammation in asthma

1.1.2.1 T helper (Th) lymphocytes

After antigen is presented to naïve $CD4^+$ T cells via peptide presentation in the groove of the class II major histocompatibilty complex (MHC-II) on antigen presenting cells,

such T helper lymphocytes can develop into either Th1 cells, secreting IFN-γ or Th2 cells which produce IL-4, IL-5, IL-9 and IL-13 (Abbas et al., 1996). In the presence of IL-12, which signals intracellularly through STAT-4-dependent pathways, the differentiation of Th1 cells is induced. Th1 cells specifically express the transcription factor T-bet (Szabo et al., 2000). In contrast, the development of Th2 cells is reliant on the presence of IL-4 and STAT-6-dependent signalling pathways which induce the activity of the Th2-specific transcription factor GATA-3 (Zhang et al., 1997).

Th1 lymphocytes selectively express the IL-18 receptor alpha (IL-18Rα) chain on their cell surface (Xu et al., 1998a) while Th2 cells express the orphan receptor T1/ST2 (Lohning et al., 1998, Xu et al., 1998b). Both these molecules are required for the production of effective Th1 or Th2 responses, since mice deficient in IL-18Rα have impaired Th1 responses (Hoshino et al., 1999a), and T1/ST2 deficient mice have reduced Th2 effector functions (Townsend et al., 2000).

As stated above, asthma is characterised by a predominance of Th2 lymphocytes and their pattern of cytokine secretion (Robinson et al., 1992). Bronchial biopsies from atopic and non-atopic asthmatics show elevated levels of IL-4, IL-5 and IL-13 (Humbert et al., 1996) and increased expression of GATA-3 and STAT-6 have also been observed (Christodoulopoulos et al., 2001). Murine models of asthma have confirmed the importance of these cytokines in the development of allergic lung inflammation; mice deficient in IL-4 have reduced airway inflammation (Hogan et al., 1997) whereas transgenic mice over-expressing IL-5 (Lee et al., 1997) or IL-13 (Zhu et al., 1999) have enhanced inflammatory responses.

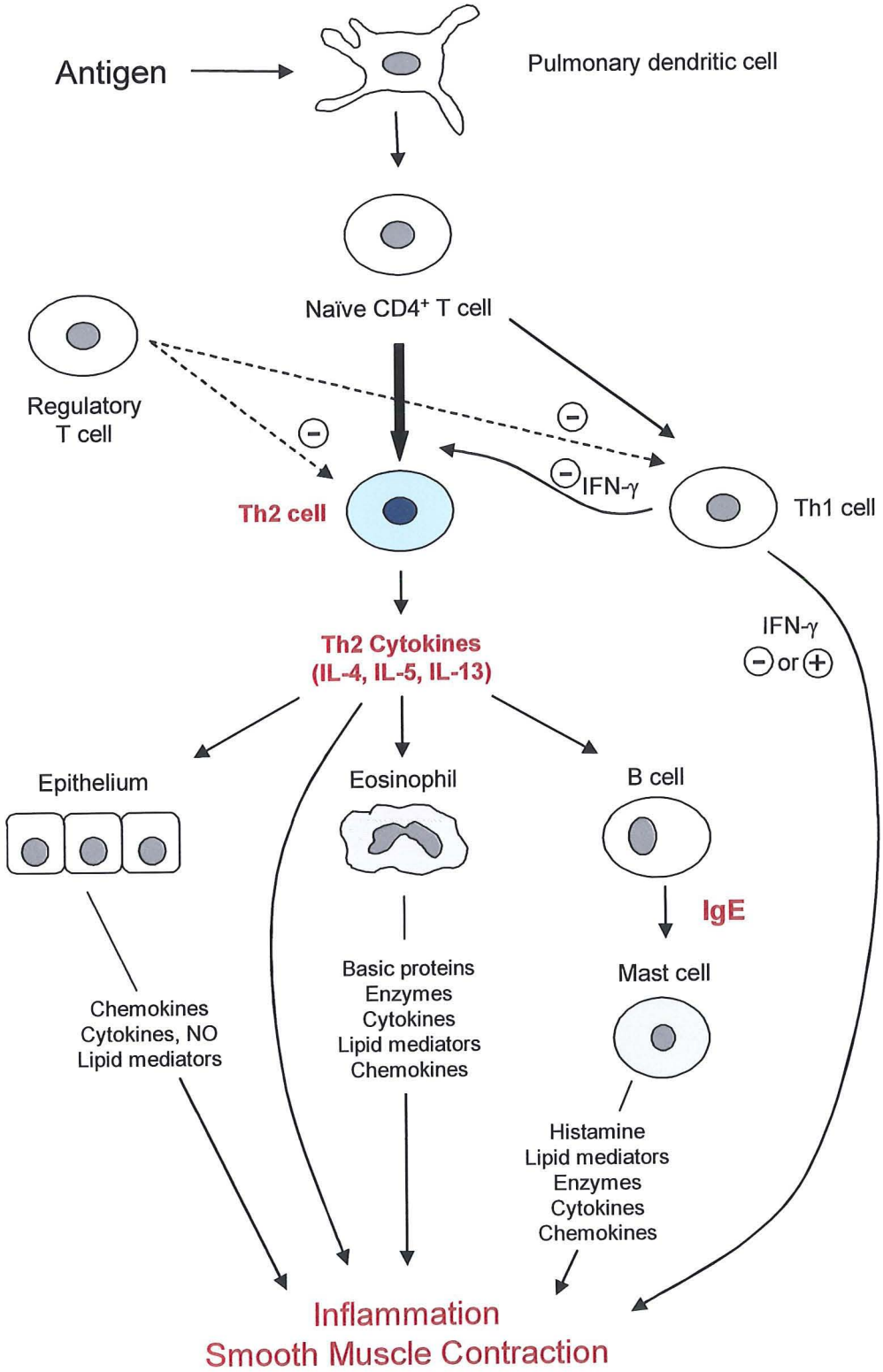


Figure 1.1. Cell types involved in the pathogenesis of asthma.

Key cell types involved in the development of airway inflammation in asthma. Th2 lymphocytes play an important role. (Adapted from Lewis, 2002).

Th1 lymphocyte responses were originally proposed to be protective in asthma since immunotherapy for specific allergies resulted in skewing of the immune response towards a Th1 profile (McHugh et al., 1995). In addition, antigen-induced IFN-γ production was reduced in peripheral blood monocytes (PBMC) from chronic asthmatics when compared to normal controls (Smart et al., 2002) and the presence of IFN-γ was shown to be necessary for the inhibition of the development of airway eosinophilia by Th1 cells in a murine model of asthma (Cohn et al., 1999). However, IFN-γ is produced by bronchoalveolar lavage lymphocytes from asthmatics (Krug et al., 1996) and may enhance some aspects of airway inflammation (Ford et al., 2001). In a murine model of asthma, the transfer of Th1 cells into mice did not reduce airway inflammation, but enhanced it (Hansen et al., 1999, Randolph et al., 1999a, Randolph et al., 1999b). Therefore, although Th2 responses are more important in the development of asthmatic airway inflammation, Th1 responses may also contribute.

The specific effects of the cytokines discussed above, and additional cytokines important in allergic responses, are summarised in Table 1.1.

1.1.2.2 Regulatory T cells

Since the Th1/Th2 paradigm cannot fully explain the regulation of the activity of T lymphocytes in asthma it is now thought that regulatory T cells, which actively control or suppress the function of other cells, might be involved. Four types of these cells have now be identified - $CD4^+CD25^+$ lymphocytes, TR cells, Th3 cells and natural killer T (NKT) cells. The properties of each of these cell types are discussed in this section.

Cytokine	Actions
IL-3	Differentiation and activation of eosinophils, mast cells basophils and neutrophils
IL-4	B-cell switch to IgE synthesis Enhances Th2 development Mast cell development Eosinophil and basophil activation Mucus hypersecretion
IL-5	Eosinophil and basophil differentiation, maturation and activation
IL-9	Mast cell and eosinophil development, AHR, mucus secretion
IL-10	Suppresses Th1/Th2 activation Favours regulatory T cell production
IL-13	Mast cell development B-cell switch to IgE, AHR Eosinophilia, mucus hypersecretion
GM-CSF	Differentiation and activation of esoinophils and mast cells
IFN-γ	Inhibits IgE synthesis and Th2 induction Activates eosinophils and macrophages
IL-12	Favours Th1 development Inhibits IgE synthesis

Table 1.1. Cytokines involved in the regulation of the asthmatic response.

The cytokines shown have important regulatory roles in asthma. This list has been chosen to highlight cytokines which will be discussed with regard to the actions of IL-18. GM-CSF – granulocyte-macrophage colony stimulating factor.

(Adapted from Larché et al., 2003).

$CD4^+CD25^+$ cells comprise 10% of all $CD4^+$ cells. They specifically express the cell surface marker glucocorticoid-induced TNF receptor (GITR) (Shimizu et al., 2002) and the transcription factor *Foxp3* (Hori et al., 2003). They are thought to suppress immune responses by the secretion of transforming growth factor (TGF)-β and through cell-cell contact mechanisms involving cell-surface bound TGF-β and the interaction of cytotoxic T lymphocyte antigen 4 (CTLA-4) with its ligand (Nakamura et al., 2001). In murine models of asthma, they have been shown to suppress the development of airway inflammation (Suto et al., 2001) but do not suppress AHR (Hadeiba and Locksley, 2003). $CD4^+CD25^+$ cells from atopic donors have suppressive activity on antigen-induced Th2 cytokine production *in vitro* (Bellinghausen et al., 2003a, Bellinghausen et al., 2003b), but the suppressive activity of such cells is less than that from non-atopic donors (Ling et al., 2004), implying that disordered activity of this subset of regulatory T cells may allow the development of allergic responses.

Th3 cells produce TGF-β and have been shown to suppress the development of experimental autoimmune encephalitis (Chen et al., 1996). TR1 are $CD4^+$ cells which secrete high levels of IL-10, and can suppress experimental colitis (Groux et al., 1997) and Th2 responses *in vitro* (Cottrez et al., 2000). Although no direct role for these cells has been demonstrated in asthma, since T cells engineered to produce TGF-β (Hansen et al., 2000) or IL-10 (Oh et al., 2002) have a suppressive role in experimental models, it is possible that these cells are involved in the down-regulation of the asthmatic response.

In contrast NKT cells can enhance Th2 responses. These cells are either $CD4^+$ or $CD4^-$ $CD8^-$ cells which have an invariant Vα14 T cell receptor chain and recognise glycolipid antigens in association with CD1d, an MHC class I-like protein. They have been shown to have a regulatory role in autoimmune disease (reviewed by Wilson and Delovitch,

2003). They are dependent on IL-15 for survival (Matsuda et al., 2002). When activated, these cells can produce IL-4, IL-13 and IFN-γ. Although NKT cells are not essential for the development of Th2 responses (Brown et al., 1996), they are required for the development of AHR and allergen-induced airway inflammation (Akbari et al., 2003, Lisbonne et al., 2003) in murine models of asthma. NKT cells may therefore have pro-inflammatory activity in human asthma.

1.1.2.3 Dendritic cells

Dendritic cells are the principal antigen presenting cell in the airway wall, and as such, they are able to influence the development of both Th2 lymphocytes and regulatory T cells. Dendritic cells from the respiratory mucosal wall preferentially induce Th2 cytokine responses (Stumbles et al., 1998) and have been shown to be required for the development of eosinophilia and AHR in a murine model of asthma (Lambrecht et al., 1998). The exact mechanisms by which dendritic cells induce Th2 responses remain unclear. When exposed to allergens *in vitro,* dendritic cells from atopic donors secrete IL-13 which will enhance Th2 responses (Bellinghausen et al., 2003a). In addition, the Th2-promoting activity of dendritic cells may involve the actions of specific co-stimulatory molecules such as inducible costimulator (ICOS), which has been implicated in the development of Th2-mediated inflammatory responses (Tesciuba, et al. 2001). Pulmonary dendritic cells producing IL-10 have been implicated in the induction of tolerance to inhaled allergen a murine asthma model (Akbari et al., 2001).

1.1.2.4 Eosinophils

While Th2 lymphocytes and regulatory T cells are modulators of the inflammatory response in asthma, eosinophils, mast cells and basophils are often considered as effector cells in the allergic response because of the inflammatory mediators they produce.

Elevated levels of eosinophils are present in the airways of both atopic (Bentley et al., 1993) and non-atopic asthmatics (Gaga et al., 2000). They are important in the development of bronchoconstriction in the late asthmatic response (De Monchy, et al. 1985). Eosinophil numbers in the BAL fluid of asthmatics correlate with the severity of human asthma (Louis R, et al.1997). IL-5 is essential for the maturation of eosinophils and their migration to the lungs. Mice deficient in IL-5 do not develop airway eosinophilia and AHR after antigen challenge (Pope et al., 2001). Granulocyte-macrophage colony stimulating factor (GM-CSF) is also important in eosinophil maturation and survival (Esnault and Malter, 2001). Chemokines are important in trafficking of eosinophils to the lungs, and blocking the chemokine receptor CCR3, which binds several chemokines including eotaxin, with a monoclonal antibody, has been shown to specifically inhibit pulmonary eosinophilia and AHR in a murine model of asthma (Justice et al., 2003).

Eosinophils express surface receptors for IgE (Rajakulasingam et al., 1998) and this may enhance eosinophil migration to the lungs during allergic inflammation (Coyle et al., 1996). The binding of IgE to its cell surface receptors, induces degranulation of eosinophils. Other molecules including the complement component C3b and cytokines, such as IL-5 and GM-CSF, also enhance degranulation (Carlson, et al.1993).

Eosinophil granules contain many inflammatory mediators which promote the influx of other inflammatory cells into the airways. These include eosinophil cationic protein (ECP), myelin basic protein (MBP), eosinophil peroxidase and reactive oxygen species such as H_2O_2. Lipid mediators such as the prostaglandin (PG)E_2 and the leukotriene (LT)C_4 are also produced (reviewed by Lacy and Moqbel, 2001).

In addition to their role as effector cells in asthmatic inflammation, eosinophils have the potential to modulate T lymphocyte responses. Eosinophils can produce a wide range of cyokines including IL-4 (Bandeira-Melo et al., 2002) and IL-13 (Woerly et al., 2002). Eosinophils also have the ability to present antigen to T lymphocytes and enhance the release of IL-4 and IL-13 from them (Shi et al., 2000).

1.1.2.5 Mast cells and basophils

Whereas eosinophils are important in the late asthmatic response, mast cells, and the inflammatory mediators they secrete, are responsible for the immediate bronchoconstrictor response to inhaled antigen. Both mast cells and basophils are seen in bronchial biopsies in asthmatic subjects (Koshino et al., 1996), although mast cells, and not basophils, are permanently present in the airways. Both cell types express the high affinity IgE (FcεRI) receptor on their surface, the presence of which can be up-regulated on the surface of mast cells by IgE (Yamaguchi et al., 1997). Allergen-induced cross-linking of these receptors by IgE, causes the release of mediators from cell granules resulting in acute bronchoconstriction. Studies in mast cell "knockin" mice have shown that mast cells are involved in the development of AHR (Kobayashi et al., 2000) and also in the development of chronic inflammatory changes in the lung

(Williams and Galli, 2000). Both mast cells and basophils release histamine and LTC_4 and also produce chemokines and cytokines, including IL-4, IL-5 and TNF-α (Bradding et al., 1994), so contributing to the influx of inflammatory cells, including lymphocytes and eosinophils, in the late-phase of the asthmatic response. Mast cells have also been shown to act as antigen presenting cells (Mecheri and David, 1997).

1.1.2.6 Other cells involved in the asthmatic response

The cell types described above are thought to be particularly important in the development of airway inflammation in asthma. However, many other cells are present in the airways which are able to secrete pro-inflammatory molecules. Increasingly, airway epithelial cells are now thought to be an important source of inflammatory mediators, including the eosinophil-attracting chemokines regulated on activation, normal T-cell expressed and secreted (RANTES) (Berkman et al., 1996) and eotaxin (Ying et al., 1997). Neutrophils are thought to secrete many pro-inflammatory molecules and are present in increased amounts in severe asthmatics and in those with steroid-resistant disease (Wenzel et al., 1997). Macrophages can also secrete pro-inflammatory cytokines such as IL-6 and TNF-α (Gosset et al., 1991) but they also have been shown to have anti-inflammatory actions (Strickland et al., 1996). These additional cell types may all contribute to allergic airway inflammation.

1.2 Interleukin-18

1.2.1 Introduction

Many cytokines, including IL-4, IL-5 and IL-13, have a well-characterised role in the development of allergic inflammation, as described in the previous section. More recently discovered cytokines may also be involved in the control of airway inflammation in asthma, but their actions are less well established. Interleukin-18 is one such cytokine which was originally identified as interferon-gamma-inducing factor, with a key role in the development of endotoxic shock in mice pre-treated with *Propionibacterium acnes* and then challenged with LPS. In these initial studies IL-18 was a potent stimulator of IFN-γ secretion from Th1 cells and augmented the cytolytic activity of NK cells.(Okamura et al., 1995). Although IL-18 was first proposed to be a promoter of Th1 lymphocyte responses, and hence have a possible anti-inflammatory action in asthma, the actions of IL-18 are now thought to be more extensive, including a pro-inflammatory role in Th2 responses (reviewed by Nakanshi et al., 2001).

In this section, the structure of the IL-18 gene and protein will be discussed, as will factors regulating their activity. IL-18 acts by binding to a specific cell surface receptor, and the characteristics of this receptor are also described below. The role of IL-18 in innate and cell mediated immunity, including its ability to promote both Th1 and Th2 responses, will be discussed in later sections.

1.2.2 The interleukin IL-18 gene

The gene for murine IL-18 is found on chromosome 9. It contains 7 exons, the first two of which are non-coding. Two promoters for this gene have been identified both of which lack TATA sequences. IL-18 mRNA lacks destabilising sequences found commonly in other cytokine genes. The promoter upstream of Exon 2 is constitutively activated while the promoter upstream of exon 1 can be upregulated by LPS (Tone et al., 1997). This latter promoter contains a site for binding of an IFN consensus sequence binding protein (ICSBP) which is critical for its activity, whereas the promoter before exon 2 includes an essential PU.1 binding site (Kim et al., 1999). The activity of these transcription factors is increased by IFN-γ (Weisz et al., 1994) and LPS (Shackelford et al., 1995). In murine macrophages, the up-regulation of IL-18 gene expression by IFN-γ also involves transcription factor activator protein (AP)-1 (Kim et al., 2000). A nuclear factor (NF)-κB binding-sequence is also present in the promoter region of IL-18; TNFα and hydrogen peroxide have been shown to increase IL-18 mRNA expression via NF-κB-dependent mechanisms in rat cardiac myocytes (Chandrasekar et al., 2003). In contrast, the transcription factor Bc16, acts as a repressor of IL-18 expression in murine macrophges (Takeda et al., 2003).

The human IL-18 gene is found on chromosome 11 and consists of 6 exons and five introns. The first 92 bp of the 5' flanking region are essential for the constitutive expression of IL-18 in monocytic cells (Kalina et al., 2000a). There is 75% homology in the 5' flanking regions of the human and murine IL-18 genes, which suggests that there is conservation of regulatory mechanisms between the two species (el Kares et al., 2000). Polymorphisms at -137bp and -167bp in the promoter regions of the human gene have been linked to the susceptibility to post-injury sepsis (Stassen et al., 2003) and a polymorphism at 105 bp in the coding region of the gene has been associated with the

pathogenesis of asthma (Higa et al., 2003a). Additional single nucleotide polymorphisms (SNP) at the human IL-18 gene locus have been suggested as markers of susceptibility to allergic rhinitis and to the development of specific IgE to common allergens (Kruse et al., 2003).

IL-18 mRNA was first found in liver macrophages and Kupffer cells (Okamura et al., 1995) but has since been detected in a wide range of cell types including T and B lymphocytes, dendritic cells, osteoblasts, microglia, astrocytes and keratinocytes (Nakanishi et al., 2001). IL-18 therefore has the potential to be involved in the regulation of immune responses in several organs.

1.2.3 IL-18 protein structure

Murine IL-18 is initially produced as the 192 amino acid peptide pro-IL-18 with a molecular weight of 24kDa peptide (Okamura et al., 1995). Human pro-IL-18 is 193 amino acids long and has 65% homology with murine IL-18 (Ushio et al., 1996). A homodimer of human IL-18 has been found in serum (Shida et al., 2001). Neither murine or human pro-IL-18 has a conventional hydrophobic signal peptide at the N-terminus but both contain an unusual leader sequence in this region. Mature, active IL-18 is produced by the cleavage of 35 amino acids from the N-terminus of the precursor peptide. Interleukin-1β converting enzyme (ICE or caspase-1) is the most important enzyme responsible for the conversion of pro-IL-18 into mature IL-18 (Ghayur et al., 1997, Gu et al., 1997). Proteinase-3 can also cleave IL-18 into its active form (Sugawara et al., 2001), whereas caspase-3 cleaves active IL-18 into inactive peptides (Akita et al., 1997). It is thought that the processing of IL-18 by these proteases is more important

than alterations in gene expression in the control of the biological activity of IL-18, since levels of IL-18 mRNA are often relatively stable in many cell types.

In both its peptide sequence and secondary and tertiary protein structure, IL-18 bears similarity to IL-1β. Both these cytokines fold into β-pleated sheets with a tertiary beta-trefoil barrel configuration (Kato et al., 2003). Mutational analysis has shown that the glutamate and lysine residues, at positions 42 and 89 respectively, are important for the binding of IL-18 to its receptor and its serum binding protein (Kim et al., 2001a).

In addition to modulation of the activity of IL-18 by intracellular proteolytic processing and regulation of gene activity, the extracellular actions of IL-18 can be inhibited by its binding to specific serum IL-18 binding proteins (IL-18 BP). Isoforms of IL-18 BP have been found in both humans and mice (Aizawa et al., 1999, Novick et al., 1999). These proteins contain a single extracellular immunoglobulin domain but do not contain a transmembrane region. They are not related to the IL-1 or IL-18 receptor family but do show homology with pox virus proteins, which are able to inhibit the actions of IL-18 *in vitro* (Xiang and Moss, 1999, Calderara et al., 2001) and *in vivo* (Born et al., 2000). The expression of IL-18BP is increased by IFN-γ in human keratinocytes, colonic carcinoma cell lines and renal mesangial cells (Muhl et al., 2000) and peripheral blood monocyte cultures (Veenstra et al., 2002), so providing a negative-feedback loop for the actions of IL-18. Serum levels of IL-18 BP, and IL-18, are elevated in the serum of patients with several inflammatory conditions, including rheumatoid arthritis (Bresnihan et al., 2002), where the raised levels of IL-18BP are proposed to counter-regulate the effects of increased IL-18 and therefore raised IFN-γ levels. Consistent with this proposal, the administration of exogeneous IL-18BP has been shown to have anti-inflammatory

effects in collagen-induced arthritis in mice, an animal model of rheumatoid arthritis (Plater-Zyberk et al., 2001, Banda et al., 2003, Smeets et al., 2003).

The actions of IL-18 may also be antagonised by the presence of IL-1H, an IL-1-receptor agonist (IL-1ra)- related cytokine which is able to bind to the IL-18 receptor (Pan et al., 2001).

1.2.4 The IL-18 receptor and its intracellular signalling

The interleukin-18 receptor is a heterodimer of two proteins chains, both of which are members of the IL-1 receptor (IL-1R) subfamily of the Toll/interleukin-1 receptor (TIR) family. These subunits are now termed IL-18 receptor α and β. In common with other members of this family the extracellular portions of the IL-18 receptor chains have three immunoglobulin (Ig)-like domains while the intracellular regions contain a TIR domain which is essential for the initiation of intracellular signalling. A schematic diagram of the IL-18 receptor complex and its signalling pathways is shown in Figure 1.2. There is significant homology between the human and mouse IL-18Rα and IL-18Rβ subunits (Parnet et al., 1996).

IL-18 receptor α (IL-18Rα) was originally identified as IL-1Rrp (Torigoe et al., 1997). It was shown to be required for the actions of IL-18, since splenocytes from mice deficient in IL-1Rrp had defective IFN-γ production and cytolytic activity and did not activate NF-κB in response to IL-18 (Hoshino et al., 1999a). The human IL-18 receptor α gene is found within the IL-1R gene cluster on chromosome 2q12 (Dale and Nicklin, 1999). IL-18 initially binds to the IL-18Rα chain and the ligated receptor is then

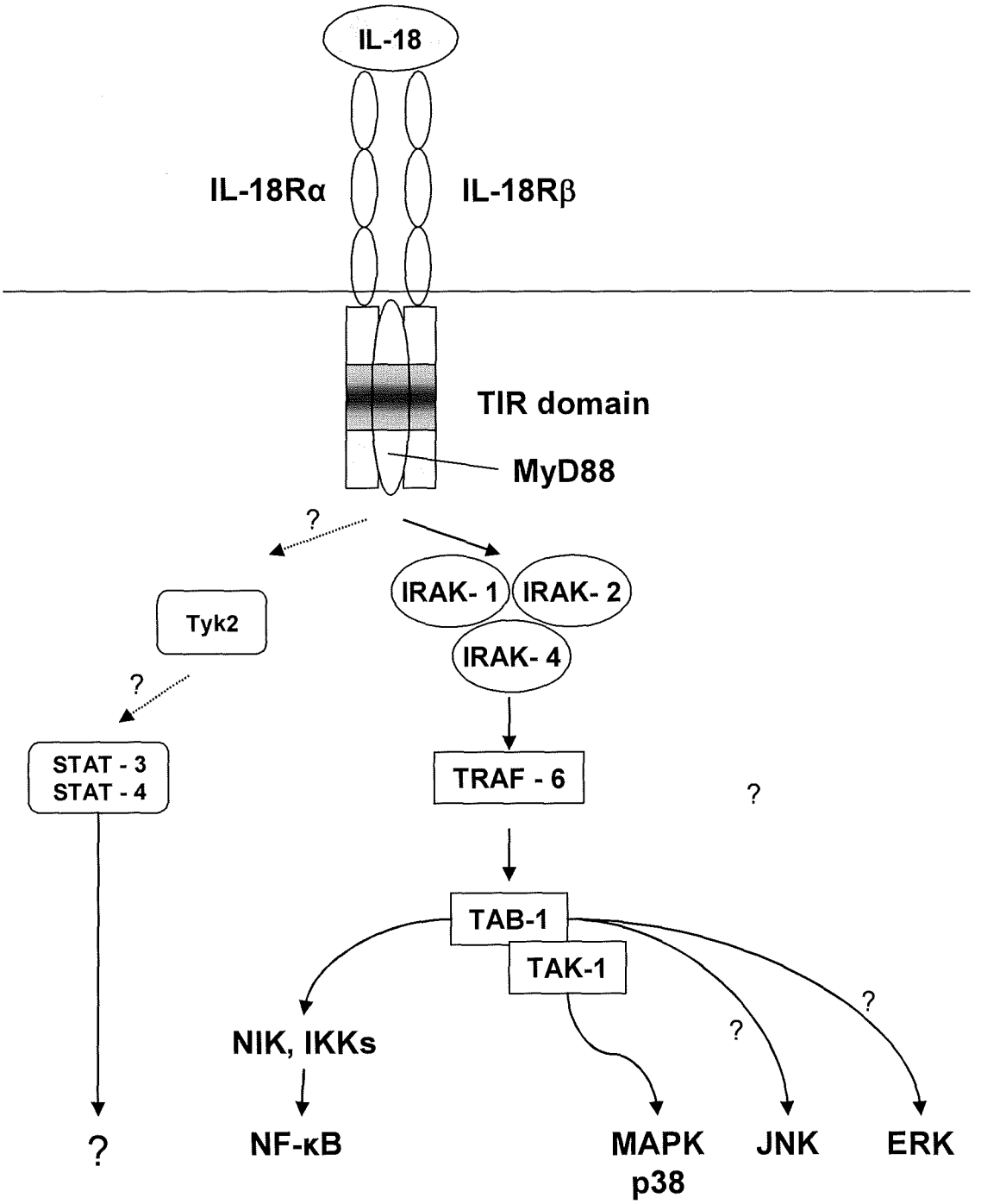


Figure 1.2. The IL-18 receptor complex and its intracellular signalling pathways.

IL-18 principally triggers intracellular signalling via the TIR domain, recruiting MyD88, IRAKs and TRAF-6. The signalling mechanisms mediated by Tyk2 and STAT-3/STAT-4 are less well defined. (Adapted from O'Neill and Dinarello, 2000).

recognised by the IL-18Rβ chain (originally called IL-1R-accessory protein (AcP)-like protein, AcPL) to form a heterodimeric transmembrane receptor. Both receptor chains are needed for signal transduction (Debets et al., 2000). IL-18Rα alone has a relatively low affinity for IL-18 (dissociation constant (K_d) ~ 20 nM) but this increases significantly on association with the β subunit of the receptor (K_d ~ 0.3nM) (Born et al., 1998). The increased affinity for IL-18 is thought to be due to conformational changes which occur in IL-18Rα on binding to IL-18Rβ subunit (Wu et al., 2003). The third of the extracellular domain closest to the cell membrane is thought to be important in the formation of the complex of IL-18 with both parts of its receptor complex (Azam et al., 2003).

Intracellularly, the TIR domain of the IL-18 receptor initiates intracellular signalling through MyD88 and interleukin-1 receptor associated kinase (IRAK) in a similar way to IL-1, resulting in the activation of p38 mitogen-activated protein (MAP) kinases (Thomassen et al., 1998), NF-κB and c-Jun N-terminal kinase (JNK) (Azam et al., 2003). Mice deficient in MyD88 (Adachi et al., 1998) and IRAK (Kanakaraj et al., 1999) have decreased NFκB activation, IFN-γ production and NK cell cytotoxicity in response to IL-18. In addition to IRAK-1 and 2, IRAK-4 has been shown to be essential for IL-18 induced responses in T helper lymphocytes and NK cells (Suzuki et al., 2003).

Although the IL-18 receptor signals through IRAK/MyD88 in lymphocytes to produce nuclear translocation of NF-κB, in macrophages STAT-4 activation is required to induce IFN-γ production by IL-12 and IL-18 (Schindler et al., 2001). IL-18 also activates the transcription factors STAT-3 and the MAP $p44^{erk-1}$ and $p\text{-}42^{erk-2}$ in NK cell lines (Kalina et al., 2000b). Absence of the Janus associated kinase (JAK)

signalling molecule Tyk2 also resulted in reduced cytolytic activity and IFN-γ production in NK cells after stimulation with IL-18 (Shimoda et al., 2002).

As stated above, the IL-18Rα chain has been shown to be selectively expressed on Th1 lymphocytes, but not Th2 cells (Xu et al., 1998a). IL-12 up-regulates IL-18Rα expression on Th1 cells and subsequently signalling through this receptor by IL-18 maintains IL-12Rβ expression, an essential process for continuing Th1 cell development (Yoshimoto et al., 1998a). Naïve murine $CD4^+$ T cells do express low levels of IL-18Rα, but this can be down-regulated by the presence of IL-4 after antigen stimulation, so helping skew the immune response towards Th1 cell development (Smeltz et al., 2001). The suppression of IL-18Rα expression by IL-4 can be inhibited by the presence of IFN-γ (Smeltz et al., 2002). IFN-α also promotes the expression of IL-18Rα and β in NK and T cells (Sareneva et al., 2000) while TNF-α up-regulates IL-18 Rα expression in myeloid and monocytoid cell lines (Nakamura et al., 2000).

Non-lymphoid cell types including neutrophils (Leung et al., 2001), eosinophils (Wang et al., 2001) and fibroblast-like synoviocytes (Moller et al., 2002a) also express the IL-18Rα chain. Messenger RNA for the IL-18 receptor is present in several organs, including the lung, liver and heart (Parnet et al., 1996). Therefore IL-18 is likely to have a broad range of actions, extending beyond its ability to modulate T helper lymphocyte responses. These activities will be discussed in the following section.

1.3 The role of IL-18 in cellular immunity

1.3.1 Th1 lymphocyte responses

IL-18 was initially discovered as a cytokine which induced IFN-γ production from Th1 lymphocytes, both alone and synergistically with IL-12, in both murine (Okamura et al., 1995) and human T cells (Micallef et al., 1996). Similarly, IL-18 also enhanced antigen-induced IFN-γ production in murine Th1 cell lines (Kohno et al., 1997). Although the effect of IL-18 on IFN-γ production is similar to that of IL-12, these two cytokines may use different mechanisms to mediate this response since IL-18 can directly activate the human IFN-γ promoter in $CD4^+$ cells whereas IL-12 requires additional stimulation through T-cell receptor signalling (Barbulescu et al., 1998). The synergism of IL-18 with IL-12 on IFN-γ production is thought to be, at least in part, due to the ability of IL-12 to up-regulate expression of the IL-18Rα chain on the surface of T lymphocytes, as discussed above. IL-18 will only potentiate development of Th1 cells in the presence of IL-12 and is unable to do this alone (Robinson et al., 1997). In addition to its ability to stimulate IFN-γ release from Th1 cells, IL-18 enhanced the Fas ligand-mediated cytolytic activity of a murine Th1 clone (Dao et al., 1996). Studies in IL-18 deficient mice have shown impaired Th1 responses, and NK cell activity, to *Proprionibacterium acnes* and BCG (Takeda et al., 1998). IL-18 deficiency also conferred increased susceptibility to *Leishmania major* infection and increased the severity of septic arthritis in a mouse model of this disease (Wei et al., 1999). IL-18 can directly promote the polarisation and migration of Th1 cells *in vitro* (Komai-Koma et al., 2003).

Although IL-18 has a direct effect on the development and activity of Th1 lymphocytes it may also enhance the development of a Th1 response through the activation of

dendritic cells. Monocyte-derived human dendritic cells (DC1) *in vitro* express the IL-18Rα, secrete IFN-γ and migrate in response to IL-18 (Gutzmer et al., 2003). IL-18 is released constitutively from such cells. Plasmacytoid-derived dendritic cells (DC2) also express the IL-18R and can polarize allogeneic T helper lymphocytes towards a Th1 response *in vitro* (Kaser et al., 2003).

Through its ability to enhance Th1 development and increase IFN-γ production, IL-18 will indirectly augment the innate immune response, through the activation of macrophages. However, IL-18 will also directly promote the activity of cells of the innate immune system, such as NK cells, macrophages and neutrophils. The actions of IL-18 on NK cells were noted at the time of its discovery (Okamura et al., 1995). Subsequently, IL-18 has been shown to stimulate murine bone-marrow derived macrophages to produce IFN-γ (Munder et al., 1998) and peritoneal macrophages to secrete TNF-α, IL-1β, IL-6 and IL-8 (Netea et al., 2000). IL-18-induced TNF-α secretion and nitric oxide (NO) production is increased from murine macrophages after infection with *Listeria monocytogenes* (Neighbors et al., 2001) and human $CD14^+$ mononuclear cells produce IL-8 and IL-1β after stimulation with IL-18 (Puren et al., 1998). Neutrophil activity is enhanced by IL-18 (Wyman et al., 2002) and IL-18 will promote neutrophil accumulation at sites of inflammation (Leung et al., 2001).

Because of its Th1-promoting properties, IL-18 has been suggested to have a pro-inflammatory action in several inflammatory conditions. However, the ability of IL-18 to enhance the innate immune response alongside specific Th1 cell mediated immunity is likely to be important in host defence against infection and tumour development. Since IL-18 is expressed in the respiratory epithelium (Cameron et al., 1999) and is produced by human and murine alveolar macrophages (Shigehara et al., 2001, Jordan et

al., 2001, respectively), IL-18 has the potential to modulate immune responses in the lung. The effects of IL-18 in infectious and inflammatory disease will be discussed below, using lung disease to provide examples of specific functions. Table 1.2 highlights the proposed role of IL-18 in several extra-pulmonary conditions.

1.3.2 Infectious disease

1.3.2.1 Bacterial infection

1.3.2.1.1 Intracellular bacteria

Mycobacterium tuberculosis (MTB) causes infection characterised by the formation of granulomata in the lungs. In human tuberculosis, Th1 responses and IFN-γ production in the lung are thought to be important in defence against MTB but conversely there may be suppressed responses in the periphery, depending on the stage of disease. Levels of circulating IL-18 were elevated in patients with severe active tuberculosis (Yamada et al., 2000) and correlated with disease activity in this study. IL-18 levels have been shown to be elevated in the bronchoalveolar fluid (Morosini et al., 2003) and in the pleural fluid (Song et al., 2002) of sufferers of MTB. Monocytes and alveolar macrophages exposed to MTB show increased levels of IL-18 secretion when cultured with T cells or IFN-γ (Vankayalapati et al., 2000). However, consistent with the presence of peripheral anergy in some MTB patients, mycobacterium-stimulated

Proposed Role of IL-18	
Pathogenic	
Autoimmune Disease	
Rheumatoid arthritis	(Gracie et al., 1999)
Insulin dependent diabetes	(Rothe et al., 1997)
Multiple sclerosis	(Wildbaum et al., 1998)
Inflammatory disease	
Crohn's disease	(Pizarro et al., 1999)
Atherogenesis	(Gerdes et al., 2002)
Graft versus host disease	(Fujimori et al., 2000)
Infectious disease	
HIV	(Shapiro et al., 1998)
Mycobacterium leprae	(Garcia et al., 1999)
Protective	
Infectious disease	
Mycobacterium leprae	(Kobayashi et al., 1998)
Salmonella typhinurium	(Mastroeni et al., 1999)
Shigella flexneri	(Sansonetti et al., 2000)
Vaccinia	(Tanaka-Kataoka et al., 1999)
Leishmania major	(Wei et al., 1999)
Trypanosma cruzi	(Meyer Zum Buschenfelde et al., 1997)
Plasmodium	(Singh et al., 2002)

Table 1.2. The role of interleukin in extra-pulmonary disease.

This highlights specific conditions in which IL-18 is thought to play an important role.

(After Gracie et al., 2003).

PBMC from MTB patients produced less IL-18 and IFN-γ than those from healthy volunteers (Vankayalapati et al., 2003). In animal models of mycobacterial infection, IL-18 knockout mice have increased susceptibility to infection with MTB resulting in increased numbers of live bacteria in their lungs and large pulmonary and splenic granulomata; in these mice, the administration of exogeneous IL-18 reduced the severity of disease (Sugawara et al., 1999). Similarly, increased systemic expression of IL-18, produced by the intramuscular injection of a viral vector encoding the IL-18 gene, reduced bacterial load in the lungs of mice infected with *Mycobacterium avium* (Kim et al., 2001b, Shiratsuchi and Ellner, 2001). In consequence, the production of IL-18 in the lung was thought to be important in immunity against mycobacterial infection.

The intracellular pathogen *Chlamydia trachomatis,* can induce a pneumonitis in mice. Although lung inflammation was slightly increased in IL-18 knockout mice infected with this organism, the absence of IL-18, unlike that of IL-12, did not affect bacterial clearance. In contrast to mycobacterial infection, IL-18 was therefore not thought to be essential for the resolution of this intracellular infection (Lu et al., 2000).

1.3.2.1.2 Extracellular bacteria

Streptococcus pneumoniae is a common pathogen in community acquired pneumonia in humans. In mice infected with *S. pneumoniae,* an increase in IL-18 gene expression and protein levels in the lung was observed. There was an increase in bacterial load in the lung and increased incidence of systemic infection in IL-18 deficient mice, indicating that IL-18 was required for efficient clearance of this bacterial infection. The influence of IL-18 in the immune response to *S. pneumoniae* was thought to be independent from

that of IL-12 (Lauw et al., 2002). Likewise, mice infected with *Legionella pneumophilia* also had increased levels of IL-18 in the lung during infection, but inhibition of the activity of IL-18 with a monoclonal antibody, although reducing IFN-γ production, did not significantly alter the resolution of lung infection. However, inhibition of the activity of IL-12 decreased the clearance of *L. pneumophilia* from the lung (Brieland et al., 2000).

In contrast to its action in pulmonary infection with *S. pneumoniae* and *L. pneumophilia,* IL-18 was found to impair the clearance of the gram negative extracellular bacteria *Pseudomonas aeruginosa* from the lungs of infected mice (Schultz et al., 2003). This organism is often a cause of hospital acquired pneumonia. Exotoxin A from *Ps. Aeruginosa* has been shown to reduce IL-18 mRNA expression in infected murine lungs (Wieland et al., 2002) and impair host defence to this organism (Schultz et al., 2001a). Other pro-inflammatory cytokines, such as IFN-γ, have also been shown to inhibit the clearance of *Ps. Aeruginosa* (Schultz et al., 2001b). The inhibitory effect of IL-18 on resolution of *Pseudomonas* infection in the lung is in contrast to its actions in other Gram negative infections, such as *Salmonella typhimurium*, where it contributes to resolution of infection (Mastroeni et al., 1999).

The small extracellular bacterium *Mycoplasma pneumoniae* can also cause pneumonia in humans. Serum levels of IL-18 were raised in patients suffering from *M. pneumoniae* and there was a correlation with disease severity (Tanaka et al., 2002). IL-18 levels were also elevated in the pleural fluid of children with *M. pneumoniae* with pleural effusions (Narita et al., 2000). As yet, there is no information from animal models on how IL-18 may influence the course of this infection.

1.3.2.2 Yeast and fungal and infection

In clinical practice, persistent fungal infection usually only occurs in the immunocompromised. The yeast-like organism *Crypotoccus neoformans* can cause disseminated disease, including meningio-encephalitis, but initial infection is usually by inhalation of the organism. Th1 driven cell-mediated immunity is required to prevent dissemination of this organism from the lung. In a murine model of this infection, clearance of *C. neoformans* was enhanced, and dissemination of infection prevented, by exogeneous administration of IL-18 (Kawakami et al., 1997). Consistent with this was the finding that clearance of *C. neoformans* was impaired in IL-18 deficient mice, but dissemination to the brain was not reduced (Kawakami et al., 2000a). In the absence of IL-12, the ability of IL-18 to induce IFN-γ from NK cells is important in enhancing host resistance to infection (Kawakami et al., 2000b).

Candida albicans is a yeast which can also cause systemic infection in the immunocompromised. Mice deficient in caspase-1, and hence decreased mature IL-18, did not have increased susceptibility to primary infection but had impaired Th1 responses on re-infection with this organism (Mencacci et al., 2000). Mice deficient in IL-18, but not IL-12, had increased mortalility in disseminated candidal infection and decreased monocyte infiltration at sites of infection (Netea et al., 2003). Again, the induction of IFN-γ by IL-18 was thought to be vital in the development of an effective response to infection.

The fungus *Aspergillus fumigatus* may colonise the lung and produce invasive disease. IL-18 was shown to protect against pulmonary infection in a murine model of this infection. Inhalation of *A fumigatus* resulted in an increase in IL-18, IFN-γ, TNFα and

IL-12 in BAL fluid. Neutralisation of IL-18 activity, by the use of a monoclonal antibody against its receptor, reduced IFN-γ secretion while neutralisation of both IL-12 and IL-18 resulted in increased growth of *A fumigatus* from lung tissue *in vitro* (Brieland et al., 2001).

1.3.2.3 Viral infection

IL-18 can promote effective innate and adaptive immunity to viral infection by enhancing the activity of NK cells and cytotoxic $CD8^+$ T lymphocytes respectively. In NK cells, IL-18 can up-regulate both perforin-mediated activity (Hyodo et al., 1999) and FasL-mediated cell cytotoxicity (Tsutsui et al., 1996) against virally infected cells. It can also directly stimulate the development of $CD8^+$ cells in an IL-12-independent manner (Okamoto et al., 1999). The role of IL-18 has been studied in a number of viral respiratory tract infections.

Infection of human macrophages by influenza A induces IL-18 secretion (Sareneva et al., 1998), an effect dependent on proteolytic processing of IL-18 by caspase 1 (Pirhonen et al., 1999). Studies in IL-18 deficient mice demonstrated that IL-18 is able to augment early pulmonary immune responses to influenza A infection, by enhancing IFN-γ production and NK cell activity, implying that IL-18 has a protective role in this infection (Liu et al., 2004). Adenoviral infection in humans causes symptoms similar to the common cold. IL-18 and IL-12 levels were increased in the BAL fluid in adenovirally-infected mice (Berclaz et al., 2002). However, mice treated with an anti-IL-18 antibody had decreased inflammatory lung lymphocytic infiltrates and neither IL-18 nor IL-12 was absolutely required for the development of Th1 lymphocytes during

the immune response to adenoviral infection (Xing et al., 2000). Respiratory syncitial virus infection (RSV) is an important cause of bronchiolitis in children. In a murine model of this condition a predominantly Th1 cytokine response was produced. However, the absence of IL-18 did not affect the inflammatory response, unlike the absence of IFN-γ (Boelen et al., 2002). Therefore, although IL-18 may have a protective role in Influenza A infection, the importance of its actions in other viral respiratory infections, such as RSV and adenovirus, are less clear.

1.3.3 Interstitial lung disease

IL-18 has been demonstrated to have a pro-inflammatory role in autoimmune conditions such as rheumatoid arthritis (Gracie et al., 1999). Although such conditions may involve the lung, this is a rare occurrence and so evidence is lacking for the actions of IL-18 in these cases. However, some information is available on the effects of IL-18 in a range of inflammatory interstitial lung diseases in which T cell mediated immunity is important. In all the conditions described below, IL-18 has been implicated as having a pro-inflammatory action.

Sarcoidosis is a granulomatous disease of unknown aetiology which predominantly involves the lung. As such, Th1 lymphocyte responses are believed to be important in its pathogenesis. Patients with sarcoidosis have been shown to have elevated IL-12 and IL-18 levels in BAL fluid. IL-18 has been localised to granulomata in sarcoid lung, (Shigehara et al., 2001) and alveolar macrophages from sarcoid patients secrete increased amounts of IL-18 in response to LPS compared to those from healthy controls (Ho et al., 2002). Pulmonary $CD4^+$ lymphocytes from sarcoid subjects, have increased

activation of the transcription factors AP-1 and NF-κB; IL-18 has been implicated in contributing to the up-regulation of these pro-inflammatory transcription factors *in vitro* (Greene et al., 2000). A polymorphism (CA/AA) at -607 bp in the IL-18 promoter has been linked to the development of sarcoidosis. (Takada et al., 2002). IL-18 levels have also been found to be elevated in the BAL fluid of people with berylliosis, another granulomatous lung disease (Barna et al., 2002). IL-18 mRNA expression has also been detected in lymph node cells and lung macrophages from animal models of silicosis, but consistent pattern of expression has been established (Garn et al., 2000, Davis et al., 2001).

The repeated administration of IL-2 and IL-18 to mice induced a fatal interstitial lymphocytic pneumonia (Okamoto et al., 2002). Increased IL-18 mRNA expression has been found in patients with lymphoid interstial pneumonia (LIP) (Teruya-Feldstein et al., 2001). Elevated concentrations of IL-18 have been observed the BAL fluid of patients with bronchiolitis obliterans organising pneumonia (BOOP), in which a lymphocytic alveolitis occurs, and also in the fibrotic lung disease, usual interstitial pneumonia (UIP) (Forlani et al., 2002).

In animal models of acute lung injury, IL-18 mRNA expression and protein levels were increased when lung injury was induced by either immune complex deposition (Jordan et al., 2001) or endotoxaemia (Arndt et al., 2000). In the latter study, lung haemorrhage also induced IL-18 mRNA expression but did not alter IL-18 protein levels.

1.3.4 Tumour immunity

In addition to its effects in the immune reponse to bacterial infection and inflammatory conditions, IL-18 has been shown to have anti-tumour activity. This anti-tumour effect is partially mediated by the ability of IL-18 to enhance the cytotoxicity of NK cells (Osaki et al., 1998) and $CD8^+$ cytotoxic T lymphocytes (Kohyama et al., 1998). However, the induction of T helper lymphocytes responses by IL-18 is also contributes to its anti-tumour acitivity (Xiang et al., 2001). IL-18 may also inhibit angiogenesis in tumours, so preventing their growth (Cao et al., 1999). Local production of IL-18 could be derived from tumour-associated macrophages (Wahl and Kleinman, 1998). Dendritic cell vaccines genetically engineered to over-express IL-18 have shown anti-tumour effects in experimental models (Tatsumi et al., 2003). Exogeneous administration of IL-18 prevented the development of metastases in an animal model of lung cancer (Iwasaki et al., 2002). In clinical disease, elevated serum levels of IL-18 were observed in patients with metastatic cancer (Lissoni et al., 2000). Lymphocytes isolated from malignant pleural effusions produced IFN-γ in response to IL-18 and IL-12 but this was not associated with increased cell cytotoxicity (Yao et al., 2002).

1.4. The role of IL-18 in allergic disease and humoral immunity

1.4.1 Th2 lymphocyte responses

From the above discussion it is clear that IL-18 has an important role in infectious and inflammatory disease associated with its capacity to enhance the development of Th1 responses and augment IFN-γ production from both Th1 lymphocytes and NK cells. However, it has become apparent that it also has a role in the development of Th2 responses. Low levels of IL-18 Rα are found on the surface of naïve T cells allowing them to respond to IL-18. Yoshimoto et al. (2000) found that $CD4^+$ T cells stimulated with IL-18 and IL-2 produce low levels of IL-4, and, when subsequently stimulated with αCD3 can develop into Th2 cells in an IL-4 dependent manner. However, IL-18 can also stimulate Th2 cell development after antigen stimulation without the presence of IL-4 (Xu et al., 2000). In the latter study, the ability of IL-18 to induce Th2 differentiation varied with the strain of mouse from which the T cells were isolated and was inhibited by IL-12, indicating that genetic background, and therefore probably other cytokines, are important in influencing this effect. Transgenic mice over-expressing IL-18 had higher levels of both Th2 and Th1 cytokines in their serum and in spleen cell cultures (Hoshino et al., 2001). Th1 cells stimulated with antigen, IL-18 and IL-2 *in vitro* have been shown to secrete cytokines conventionally considered to be Th2 cytokines, such as IL-9, IL-13 and GM-CSF, and Th1 type memory cells also enhanced airway inflammation in a murine model of asthma (Sugimoto et al., 2004). This suggests that IL-18 may act directly on Th1 cells as well Th2 lymphocytes to promote allergic responses. Levels of the IL-18Rα chain have been shown to be increased on pulmonary lymphocytes and macrophages after antigen challenge in a murine asthma model (Kuribayashi et al., 2002), implying that IL-18 can act on these cells to regulate inflammation in the airways during allergic responses.

In association with IL-2, IL-18 can induce the production of IL-13 and GM-CSF in NK cells and T cells (Hoshino et al., 1999b). NKT cells can also be stimulated to secret IL-4, IL-9 and IL-13 by combined treatment with IL-18 and IL-2 (Yoshimoto et al., 2003). Basophils and mast cells cultured with IL-3 also express IL-18Rα and secrete IL-13 and IL-4 after stimulation with IL-18 and IL-3 (Yoshimoto et al., 1999). Exogeneous IL-18 treatment in mice induces the activity of the histamine-producing enzyme, histamine decarboxylase, in the lung, liver, spleen and bone (Yamaguchi et al., 2000). Histamine can stimulate IL-18 production in PBMC cultures (Kohka et al., 2000) and this may represent a positive feedback mechanism on IL-18 secretion in allergic responses, where large amounts of histamine are released from mast cells. IL-18 may also have a direct action on eosinophils; these cells have been found to express the IL-18Rα chain and secrete IL-8 in response to IL-18 (Wang et al., 2001).

1.4.2 Allergic disease

Because of its ability to promote Th2 lymphocyte differentiation and stimulate Th2 cytokine secretion *in vitro*, there has been interest in the role of IL-18 in allergic conditions such as asthma and atopic dermatitis.

Polymorphisms in the coding and promoter regions of the human IL-18 gene (Higa et al., 2003a) and IL-18Rα gene (Watanabe et al., 2002) have been associated with asthma. Serum IL-18 levels were elevated in patients with acute asthma exacerbations (Tanaka et al., 2001a) and also in stable asthmatics (El-Mezzein et al., 2001) and those suffering from the related allergic conditions, atopic dermatitis (El-Mezzein et al., 2001) and allergic rhinitis (Verhaeghe et al., 2002). In contrast other studies have shown that IL-18 levels are reduced in bronchoalveolar lavage (BAL) fluid from asthmatics (Ho et al.,

2002) and that IL-18 mRNA expression in the airway epithelium of asthmatics was decreased (Cameron et al., 1999) compared to normal controls and patients with sarcoidosis.

There is also conflicting evidence for the role of IL-18 in murine models of asthma. At the time of antigen challenge IL-18 has anti-allergic properties when administered with IL-12 (Hofstra et al., 1998) or when expressed in an adenoviral vector (Walter et al., 2001); in the latter case the anti-inflammatory effect of IL-18 was dependent on the presence of IL-12 and IFN-γ. Similarly, IL-18 given after sensitisation, but before the time of antigen challenge, decreased eosinophilic inflammation in an RSV-induced mouse asthma model (Zhang et al., 2003). The use of a neutralising antibody to IL-18 resulted in an increase in airway inflammation in a murine model of fungal asthma (Blease et al., 2001) and mice deficient in IL-18 had an increased inflammatory response in the airways (Kodama et al., 2000), supporting the hypothesis that IL-18 has anti-inflammatory activity in allergic airway inflammation. However, IL-18 can increase IL-4 and IL-5 production and airway eosinophilia if given at the time of allergic sensitisation (Wild et al., 2000), and can also enhance eosinophil influx to the airways (Kumano et al., 1999), at least in part by stimulating eotaxin production (Campbell et al., 2000).

IL-18 has a clearer pro-inflammatory action in atopic dermatitis. Several studies have shown raised levels of IL-18 in the serum of atopic dermatitis patients (including Shida et al., 2001, Tanaka et al., 2001b, El-Mezzein et al., 2001). In one study, serum IL-18 levels correlated with blood eosinophil levels (Yoshizawa et al., 2002).

In an experimental model of atopic dermatitis in NC/Nga mice, IL-18 levels were increased in the serum (Tanaka et al., 2001b) and skin (Xu et al., 1998c) of affected

mice. In particular, cutaneous Langerhans cells have been identified as an important source of IL-18 (Wang et al., 2002). Transgenic mice over-expressing IL-18 spontaneously developed dermatitis and elevated levels of Th2 cytokines and histamine even in the absence of IgE and STAT-6 (Konishi et al., 2002); contact hypersensitivity in mice was also increased in a similar study (Kawase et al., 2003). Inhibition of the activity of IL-18 by the administration of IL-18 BP reduced skin inflammation in a mouse model of allergic contact dermatitis (Plitz et al., 2003).

Conversely, administration of an anti-IL-18 antibody failed to inhibit the development of cutaneous allergic inflammation in NC/Nga mice (Higa et al., 2003b). One clinical study has shown that the amount of IL-18mRNA and IL-18 production from the monocytes of atopic dermatitis sufferers was lower than that of healthy controls (Higashi et al., 2001).

1.4.3 The role of IL-18 in IgE production.

Atopic disease is characterised by increased levels of serum IgE. B cells which have been activated with anti-CD40, can be stimulated to produce IFN-γ by combined treatment with IL-18 and IL-12. This inhibits IL-4-induced production of IgE and IgG1 and enhances IgG2a production (Yoshimoto et al., 1997). IL-18-secreting macrophages were shown to have a role in suppressing IgE production *in vivo* in SJL mice, which characteristically have low serum IgE responses to helminth infections (Yoshimoto et al., 1998b). In a murine model of asthma, when mice were treated with IL-12 and IL-18 decreassed serum IgE levels accompanied the reduction in airway inflammation (Hofstra et al., 1998).

In the absence of IL-12, IL-18 can enhance IgE production. Serum IgE levels were increased in transgenic mice over-expressing IL-18 either systemically (Hoshino et al., 2001) or cutaneously (Konishi et al., 2002) and also when IL-18 was given alone at the time of antigen sensitisation in a murine model of asthma (Wild et al., 2000). The increase in IgE production in response to IL-18 is augmented by IL-18-induced up-regulation of CD40L on the surface of $CD4^+$ T cells (Hoshino et al., 2000). It is dependent on IL-4/IL-4Rα signalling, but not IL-13, and requires activation of the transcription factor STAT-6 (Yoshimoto et al., 2000). $CD4^+$ T cells and NKT cells are also essential for this response (Yoshimoto et al., 2003).

IL-18 was initially proposed to have an anti-inflammatory action in asthma because of its action in Th1 lymphocyte development. However, since Th1 responses may enhance the development of airway inflammation in asthma, and IL-18 can also directly stimulate Th2 responses and cytokine production, altering the actions of IL-18 for therapeutic benefit in asthma may be complicated. Further assessment of the role of IL-18 in allergic inflammation is needed in order to decide if this would be worthwhile.

Since the direct manipulation of the activity of specific cytokines to produce an anti-inflammatory response in asthma may be difficult, agents which generally suppress the allergic response could be used to treat asthma. At present, corticosteroids are the most commonly used drugs for this purpose. However, there is continual interest in developing new therapeutic agents. A summary of the actions of corticosteroids in asthma will be discussed in the following section, as does a discussion of the properties of two potential anti-inflammatory agents simvastatin and thymosin beta 4 sulfoxide.

1.5 Anti-inflammatory molecules

Asthma is now considered to be an inflammatory condition of the airways. Modern treatment is therefore aimed at suppressing airway inflammation. Corticosteroids are currently the most commonly used drugs for this purpose. New therapeutic strategies include the targeting of the activities of specific pro-inflammatory cytokines, but novel agents are also being developed. The actions of corticosteroids and those of two compounds with potential anti-inflammatory actions in asthma, simvastatin and thymosin beta 4 sulfoxide, are discussed below.

1.5.1 Corticosteroids

Corticosteroids are the most effective anti-inflammatory agents used in the treatment of asthma. Their anti-inflammatory action is principally mediated by binding to cytosolic glucocorticoid receptors. These receptors then translocate to the nucleus and dimerise before binding to glucocorticoid response elements (GRE) in the DNA to inhibit gene activity. Corticosteroids probably mediate most of their effects by suppressing the expression of the transcription factors AP-1, NF-κB and nuclear factor of activated T cells (NF-AT), and the subsequent down-regulation of the expression of many other pro-inflammatory genes (Barnes and Adcock, 2003). However, corticosteroids can also destabilise the mRNA of some pro-inflammatory cytokines (Amano et al., 1993) and alter protein production by other post transcriptional mechanisms (Mozo et al., 1998), although these mechanisms are probably of lesser importance for their anti-inflammatory actions.

In asthma corticosteroid treatment can effectively suppress the expression of IL-4 and IL-5 in BAL fluid (Robinson et al., 1993) and IL-13 expression in bronchial biopsies (Naseer et al., 1997); the reduction in the levels of these Th2 cytokines was accompanied by an increase in IFN-γ and IL-12 mRNA. Also, increased production of the anti-inflammatory cytokine IL-10 was observed from the alveolar macrophages of steroid-treated asthmatic patients (John et al., 1998). *In vitro*, steroids inhibit the allergen-induced production of IL-3, IL-5 and GM-CSF from PBMC cultures from asthmatics (Powell et al., 2001). In a murine model of asthma, the corticosteroid dexamethasone reduced airway inflammation and IL-5, IL-13 and eotaxin secretion (Eum et al., 2003). In addition, corticosteroids directly induce the apoptosis of lymphocytes (Caron-Leslie et al., 1991) and eosinophils (Adachi et al., 1996), and this is thought to be relevant to the resolution of airway inflammation after exacerbations of asthma (Woolley et al., 1996).

Conversely, corticocosteroids have been shown to have Th2 promoting activity *in vitro,* including enhancement of IL-4-induced IgE production (Jabara et al., 1991), but *in vivo* these activities are thought to be overridden by their anti-inflammatory properties (Barnes, 2001).

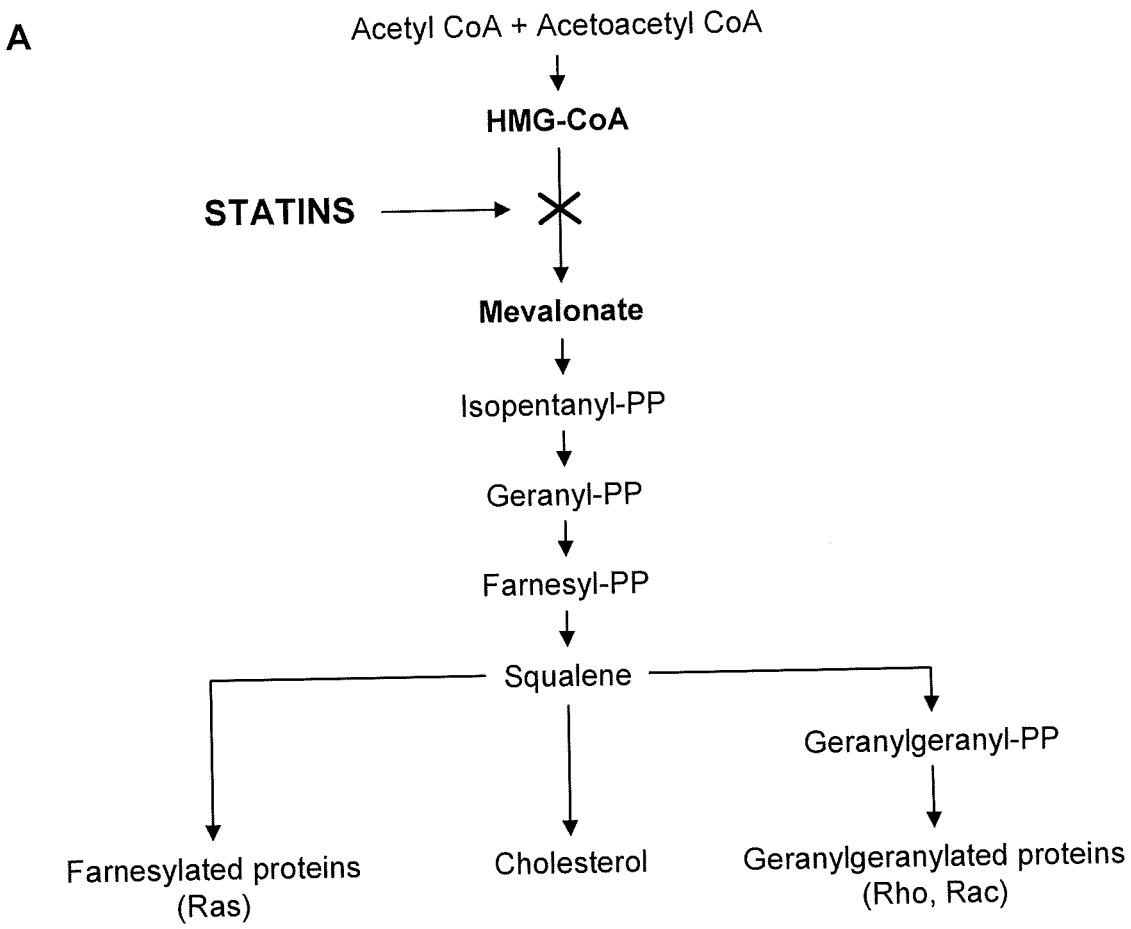
Although corticosteroids are effective treatment for most asthmatics, some patients have relatively steroid-resistant disease. The exact mechanisms of steroid resistance are unclear, but proposed mechanisms include intrinsic insensitivity of T lymphocytes to corticoteroid treatment (Syed et al., 1998), alterations in the relative levels of expression of the glucocorticoid receptor α and β isoforms (Sousa et al., 2000) and increased infiltration of neutrophils, which are inherently steroid-insensitive (Green et al., 2002). Cigarette smoking induces a neutrophilia in the airways of asthmatics (Chalmers et al.,

2001) and reduces the effectiveness of steroid treatment in asthma, both by inhalation (Chalmers et al., 2002) and oral administration (Chaudhuri et al., 2003). This effect may at least be partly due to the compounds in cigarette smoke inhibiting the action of the enzyme histone deacetylase (Ito et al., 2001), which is thought to be involved in mediating the DNA binding activity of the glucocorticoid-glucocorticoid receptor complex (Kagoshima et al., 2001).

Despite being a valuable treatment for the majority of asthmatics, corticosteroids have significant side effects at high doses. Because of these side effects, and the occurrence of steroid-resistance in some patients, there is interest in the development of new anti-inflammatory agents for the treatment of asthma. Two potential agents are described below.

1.5.2 Simvastatin

Statins are inhibitors of the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. A schematic representation of cholesterol biosynthesis is shown in Figure 1.3*A*. Statins are widely used as cholesterol-lowering agents in clinical practice. Simvastatin, the chemical structure of which is illustrated in Figure 1.3*B*, is a naturally occurring member of this class of drugs. Simvastatin treatment is known to decrease morbidity and mortality from coronary artery disease (Scandinavian Simvastatin Survival Study (4S), 1994). An additional ant-inflammatory role for statins, independent of their cholesterol-lowering activity, was suggested by clinical studies which demonstrated that statin treatment after cardiac



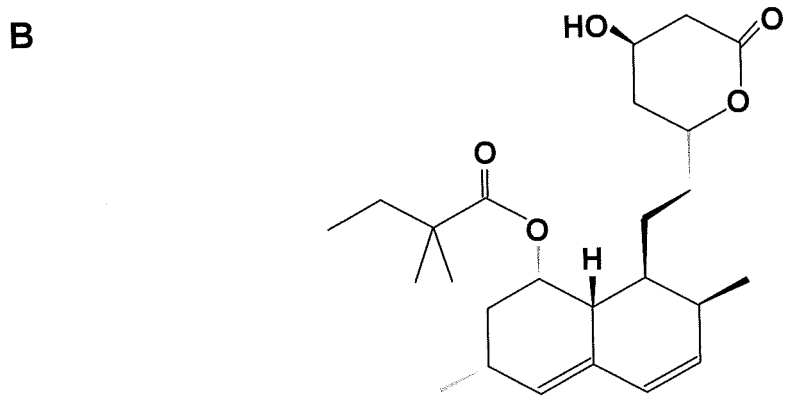


Figure 1.3. The cholesterol biosynthetic pathway and structure of simvastatin.

(A) A schematic diagram of the cholesterol biosynthetic pathway, indicating the site of action of statins. **(B)** The chemical structure of simvastatin.

HMG-CoA - 3-hydroxy-3-methylglutaryl-coenzyme A; PP – pyrophosphate

transplantation could reduce organ rejection (Kobashigawa et al., 1995) and coronary graft vessel disease (Wenke et al., 1997). Decreased serum inflammatory markers, such as C-reactive protein, were observed in statin-treated atherosclerosis patients (Albert et al., 2001) and *ex vivo* there was reduced inflammation in atherosclerotic plaques from subjects who had received statin treatment (Crisby et al., 2001). Statins have also been shown to have anti-inflammatory and anti-atherosclerotic activity in animal models of these conditions (Sparrow et al., 2001).

The immunomodulatory effects of statins are thought to be mediated through various mechanisms (Takemoto and Liao, 2001). Although these are not yet fully understood, they are thought to include suppression of cell migration to sites of inflammation by inhibiting the expression of cellular adhesion molecules such as the integrin CD11b on monocytes (Weber et al., 1997), lymphocyte function-associated antigen (LFA)-1 on leukocytes and intercellular adhesion molecule (ICAM)-1 on the vascular endothelium (Niwa et al., 1996). In addition, statins have been shown to allosterically inhibit the interaction between the cellular adhesion molecules LFA-1 and intercellular adhesion molecule ICAM-1 (Weitz-Schmidt et al., 2001). Stains also reduce IFN-γ-induced expression of major histocompatibility complex (MHC)-II on antigen presenting cells (Kwak et al., 2000).

By inhibiting the production of L-mevalonic acid and its metabolites, statins prevent the isoprenylation, and hence trafficking to the cell surface membrane of the signalling molecules such as Ras and Rho, which are involved in the activation of lymphocytes (Czyzyk et al., 2003, Greenwood et al., 2003). Statins up-regulate the acivity of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) (Grip et al., 2002) leading to down-regulation of pro-inflammatory molecules, including NF-κB

(Zelvyte et al., 2002). Both these mechanisms may contribute to the inhibition of the production of pro-inflammatory cytokines and chemokines. Statins can also inhibit the production of cytokines, such as IL-6, and chemokines, including monocyte chemotactic protein (MCP)-1 and RANTES (Diomede et al., 2001), so further reducing influx of inflammatory cells. In relation to atherogenesis, statins augment the activity of the endothelial nitric oxide synthase (eNOS), which is thought to be protective against the development of atherosclerosis (Ni et al., 2001), but decrease activity of pro-inflammatory inducible nitric oxidse synthase (iNOS) (Wagner et al., 2002).

Atherosclerotic plaques contain a predominance of Th1 lymphoctes (Frostegard et al., 1999). Since statins inhibit inflammation in plaques, recent studies have focussed on the potential ability of statins to modulate Th1-predominant disease, such as rheumatoid arthritis (Dolhain et al., 1996) and multiple sclerosis (Lassmann et al., 2001). In collagen-induced arthritis (CIA), an animal model of rheumatoid arthritis, simvastatin treatment reduced the development of arthritis and also decreased the severity of established disease (Leung et al., 2003). Similarly, in experimental allergic encephalitis, a model of multiple sclerosis, treatment with atorvastatin prevented the onset and reversed the severity of relapsing disease (Youssef et al., 2002, Aktas et al., 2003).

Statins, or related compounds, therefore have the potential to be effective immunosuppressive agents in clinical inflammatory disease beyond their established role in the treatment of atherosclerosis. Studies in rats have shown that simvastatin is taken up and metabolised in the lungs (Nezasa et al., 2002) and can attenuate the development of pulmonary hypertension when given intraperitoneally (Girgis et al., 2003) or orally (Nishimura et al., 2002). These studies suggest that simvastatin is active in the lung and could potentially have anti-inflammatory activity in pulmonary disease.

1.5.3 Thymosin beta 4 and its sulfoxide

Although small molecular pharmacological agents may be used as anti-inflammatory agents, peptide molecules with immunomodulatory activity exist which may also have therapeutic potential in asthma.

The beta thymosins are a family of 5 kDa acidic peptides which are highly conserved in mammalian cells. Their main intracellular function is to sequester monomeric G-actin and prevent its polymerisation into filamentous F-actin (reviewed by Huff et al, 2001). As such they have an important role in the control of actin-based cytoskeletal remodelling.

Thymosin beta 4 (β4) comprises 70-80% of all beta thymosins and is ubiquitously expressed in cells. It is a 43 amino acid peptide, the sequence of which is shown in Figure 1.4. The peptide is thought to fold into two α-helices, comprising residues 5 to 19 and 30 to 40, on either side of the G-actin binding region. The gene for murine thymosin β4 is found on the X chromosome and has three exons and two introns (Li et al., 1996). A splice variant of this gene is present in murine spleen and thymic cells (Rudin et al., 1990). Seven genes homologous to rodent thymosin β4 have been identified in the human genome, but some of these may be pseudogenes (Clauss et al., 1991).

A role for thymosin β4 in modulation of the immune response was initially suggested when thymosin β4 was found induce terminal terminal deoxynucleotidyl transferase in thymocytes (Low et al., 1981). Subsequently, the expression of thymosin β4 was shown to be down-regulated in a similar way to the class II human leukocyte antigen, Fc

receptor, and complement receptor in a myeloma cell line. Increased expression of the thymosin β4 gene was also present in increased amounts in mature granulocytes (Gondo et al., 1987). In addition, thymosin β4 mRNA levels were up-regulated by IFN-α in a lymphoblastoid cell line (McMahon et al., 1986). Levels of thymosin β4 expression have also been shown to be elevated during the S phase of the cell cycle in thymocytes (Schobitz et al., 1991).

Although thymosin β4 is present in high concentrations in blood leukocytes (Hannapel et al, 1993), it is only present at low concentrations in plasma since the peptide lacks an appropriate signal sequence for secretion from cells. However, thymosin β4 can be released extracellularly at sites of tissue damage and inflammation (Frohm et al, 1996), where it can have additional actions beyond its intracellular role.

Extracellular thymosin β4 is able to promote angiogenesis (Malinda et al, 1998) and it also has anti-inflammatory properties. Thymosin β4 enhances wound healing (Malinda et al, 1999; Philp et al 2003), with an associated decrease in polymorphonuclear cells infiltration and inflammatory cytokine expression (Sosne et al, 2001). Given intraperitoneally, thymsosin β4 reduced lethality and down-regulated IL-1α production in a rat model of endotoxin-induced septic shock (Badamchian et al, 2003).

Thymosin β4 contains a methionine at amino acid position 6 which can be oxidised to form a sulfoxide residue, forming the peptide thymosin β4 sulfoxide (Tβ4SO). This oxidation decreases the affinity of thymosin β4 (Heintz et al, 1994) for G-actin and may promote its extracellular release. Tβ4SO was identified as the factor reducing fMLP-induced neutrophil chemotaxis in supernatants derived from steroid-treated monocytes

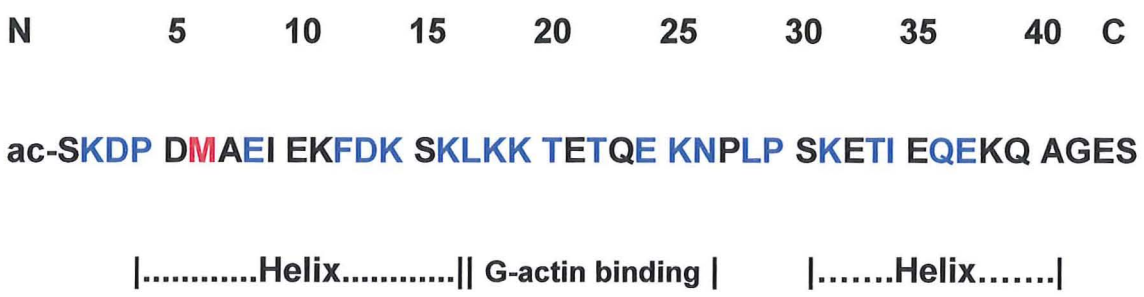


Figure 1.4. The peptide sequence of thymosin beta 4.

The methionine residue (in red) is oxidised to form thymosin beta 4 sulfoxide. The residues in blue are conserved between species. The peptide folds to from two α-helices on either side of the G-actin binding sequence. The first four amino acids can be cleaved from the N terminus, to form seraspenide (ac-SKDP) which has independent biological activity.

(Chettibi et al, 1994; Young et al, 1999). Tβ4SO was shown to promote wound healing in an *in vitro* model at ten times the potency of the equivalent dose of thymosin β4. Tβ4SO also decreased footpad swelling in carrageenin-induced inflammation in mice, whereas thymosin β4 did not (Young et al, 1999). A splice variant of thymosin β4 derived from lymphocytes also had anti-inflammatory activity in murine models of the T lymphocyte mediated-conditions, irritant contact dermatitis and allergic contact dermatitis (Girardi et al, 2003).

In addition to the direct anti-inflammatory actions of thymsosin β4 and Tβ4SO, thymosin β4 may have an indirect immunosuppressive effect by acting as precursor of the peptide acSDKP, also called seraspenide. This peptide, which can be enzymatically cleaved from the N-terminus of thymosin β4, has been shown to inhibit the proliferation of haemopoeitic stem cells (Lenfant et al., 1989, Grillon et al., 1990).

The gene for thymosin β4 is highly expressed in the lung (Gomez-Marquez et al., 1989). It is therefore possible that thymosin β4 may be oxidised to Tβ4SO and released extracellulary to exert its immunomodulatory effects in inflammatory lung disease.

1.6 The aims of the thesis.

IL-18 is a cytokine with the ability to induce both Th1 and Th2 mediated immune responses. As such it may have an important immunomodulatory role in asthma. As yet, there are conflicting reports of the activity of IL-18 in both human asthma and murine models of this disease. Further investigation of the actions of IL-18 is needed to assess its potential as therapeutic target for the treatment of clinical asthma.

This thesis will aim to elucidate further the role of IL-18 in asthma by:

- Establishing the presence of IL-18 in induced sputum from asthmatic and non-asthmatic subjects and comparing the levels of IL-18 between these groups, by both ELISA and quantitative polymerase chain reaction.
- Assessing if inhaled steroid therapy and cigarette smoking alter the levels of IL-18 detected in induced sputum samples.
- Determining which cell types in induced sputum produce IL-18 by immunocytochemistry.
- Using ovalbumin-sensitised BALB/c mice and IL-18 gene knockout (ko) mice to directly define the role of IL-18 *in vivo* in the development of an asthmatic response to a specific antigen.

Inhaled and oral corticosteroid treatment are the most effective anti-inflammatory agents used in the treatment of human asthma. Although most patients respond to this therapy, there are some patients who do not. There is therefore continual interest in the development of new anti-inflammatory agents for the treatment of this condition.

The statins are a group of drugs which are commonly used in clinical practice as cholesterol lowering agents. They are now thought to have anti-inflammatory activity independent of their lipid lowering activity, both in the development of clinical atherosclerosis and in animal models of inflammatory diseases, such as multiple sclerosis. Similarly, thymosin beta 4 sulfoxide has been shown to have anti-inflammatory properties. Neither of these compounds as been shown to have an immunomodulatory effects in conditions such as asthma, where eosinophils and Th2 lymphocytes are important mediators of disease activity.

An additional aim of this thesis will therefore be:

- Establishing any potential anti-inflammatory effects of simvastatin and thymosin beta 4 sulfoxide in allergic inflammation by the use of an ovalbumin-sensitised murine model of asthma.

Chapter 2

Materials and Methods

2.1 Reagents and buffers

The source of reagents either purchased or donated is given in the text or tables. Details of preparations of individual buffers and reagents are given within the text.

2.2 Cell culture

All culture media and supplements were obtained from Invitrogen, Paisley, UK. Dulbecco's modification of Eagle's medium (Dulbecco's MEM) supplemented with 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin ("complete Dulbecco's MEM") was used for sputum cell isolation. Supplements were stored in 5ml aliquots (x100 concentration) at –20°C then thawed and added to the medium prior to culture. RPMI 1640 supplemented with glutamine, penicillin and streptomycin ("complete RPMI") as above was used for murine lymphocyte isolation. Cells were cultured in the appropriate complete medium supplemented with 10% foetal calf serum (FCS) which had been *mycoplasma* screened by the manufacturer, heat inactivated at 56°C for 30 minutes in a circulating water bath, then stored in 50 ml aliquots at –20°C. Aliquots were thawed immediately prior to addition to the culture medium. Cell culture was performed at 37°C in a humidified incubator with 5% CO_2. Cells were counted directly using an improved Neubauer haemocytometer (VWR International, Leicester, UK) on a Nikon Labphot microscope. During cell purification and washing, cells were centrifuged at 400 x *g* at 4°C unless otherwise stated.

2.3 Ethical considerations

Sputum and peripheral blood samples from asthmatic patients and normal volunteers were collected after informed consent was obtained from all subjects. Approval from the Ethical Committee in the West Glasgow Hospitals NHS Trust was given for the clinical study (The Role of Interleukin-18 in Asthma, Protocol No. 99/81; Professor N.C. Thomson, Gartnavel General Hospital). All animal experimentation was performed under project licence following UK Home Office review, Project Licence 60/2217 procedure 11 for the murine model of allergic asthma.

2.4 Patients and clinical samples

Induced sputum samples were obtained from asthmatic patients and normal subjects attending the Asthma Research Unit, Gartnavel General Hospital, Glasgow. All asthmatic subjects met the American Thoracic Society Criteria (1987) for the diagnosis of asthma and had had no acute exacerbations of their asthma in the six weeks prior to providing a sample. Samples were collected from 20 normal smokers, 20 normal non-smokers, 35 asthmatic non-smokers (17 treated with β_2-agonist alone and 18 treated with β_2-agonist and inhaled corticosteroid) and 31 asthmatic smokers (17 treated with β_2-agonist alone and 18 treated with β_2-agonist and inhaled corticosteroid).

2.4.1 Induction of induced sputum samples

Sputum induction was performed as previously described (Pin et al., 1992). Briefly, volunteers were given salbutamol 200 μg by metered-dose inhaler and spirometry

checked. Sputum induction was then commenced using hypertonic (3%) saline administered via an ultrasonic nebuliser (Sonix 2000, Medix Ltd, Harlow, UK) over a period of 20 minutes. The subjects were encouraged to expectorate at any time throughout the procedure. In addition, inhalation was stopped every 5 minutes to permit expectoration, and to allow for spirometry to be carried out. The sample was collected in a sterile container and processed within 2 hours. The protocol dictated that the procedure would be discontinued if the forced expiratory volume in one second (FEV_1) fell by more than 20%; however, this did not occur in any patients.

2.4.2 Sputum cell preparation

All samples were processed without knowledge of the clinical information relating to the individual subject. Sputum samples were transferred to a petri dish and the volume and macroscopic characteristics of the whole sample recorded. Sputum plugs were separated from contaminating saliva using sterile forceps. The plugs were placed in a pre-weighed tube and the weight recorded. The sputum was then mixed with 2.5 times (w/v) of freshly prepared 0.05% dithiothreitol solution made by mixing dithiothreitol powder (Sigma Chemical Co., Poole, UK) with Dulbecco's MEM medium (Invitrogen) supplemented with 10% FCS. This was vortexed briefly and incubated at 37°C for 15 minutes with constant agitation. The suspension was filtered through 'Nytex' 50μm monofilament nylon filter cloth (Cadish Precision Meshes Ltd, London, UK) to remove mucus and then centrifuged at 300 x *g* for 10 minutes. The cell pellet was washed four times in Dulbecco's MEM medium before analysis. The supernatants were further centrifuged at 600 x *g* for 10 minutes to remove debris and stored at –70°C for future analysis.

Cell viability was determined during cell counting by trypan blue exclusion (0.1% (w/v) trypan blue (Sigma), 0.1% acetic acid (v/v) (VWR International)). Dead cells and epithelial cells were excluded from the total cell count estimation.

Differential cell counts in sputum cell preparations were estimated from cytospin preparations made using a cytocentrifuge (Shandon, Pittsburg, USA). The sputum cell samples were suspended at 1.0×10^6 cells/ml in sterile phosphate buffered saline (1 mM KH_2PO_4, 155 mM NaCl, 3 mM Na_2HPO_4, pH 7.4, LPS-free; Invitrogen) and then centrifuged at 400 rpm for 6 minutes. Cytopreps were fixed using 50% (v/v) acetone (VWR International) in absolute ethanol and then stained for 5 minutes with May-Grunwald-Giemsa stain (VWR International). Differential cell counts were performed by counting 300 cells per slide and expressed as percentages.

Sputum cell cytospin preparations to be used for immunocytochemistry were made on polylysine coated slides (VWR International) and fixed with ice cold dried acetone (VWR International), air dried, wrapped in Parafilm M® (SPI Supplies, West Chester, USA) to exclude moisture and then stored at –70°C until analysis.

After an aliquot of cells had been taken for cytospin analysis, as described above, the remaining cells were pelleted by centrifugation for future RNA purification. The cell pellet was then resuspended in cell lysis buffer (buffer RA1 from the NucleoSpin RNA II column system (Macherey-Nagel, Düren, Germany), described in 2.11.1) then stored at –70°C.

2.5 Murine model of allergic asthma

Murine models of allergic asthma are widely used (reviewed by Lloyd et al., 2001). Mice are sensitised to protein allergens with adjuvant and subsequently re-challenged repeatedly with allergen into the airways. This leads to the development of airway eosinophilia and airway hyper-responsiveness. Female BALB/c mice (6 - 8 weeks old, Harlan-Olac, Bicester, UK) were used except in studies involving IL-18 gene knockout (ko) mice, as detailed below. Mice were quarantined for at least 7 - 10 days before being used in experiments.

BALB/c IL-18 ko mice were produced and bred by Dr. X.Q. Wei, Department of Immunology, University of Glasgow as described by Wei et al., 1999. Both male and female mice (age 8 – 16 weeks) were used in experiments. Age and sex matched wild-type (WT) litter mates were used as controls.

All animals were housed in the Joint Animal Facility, University of Glasgow, in accordance with UK Home Office animal care guidelines.

Avertin (Augustin and Sim, 1997) was used as an anaesthetic during all experiments. A stock solution was made by dissolving 1,1,1 tri-bromoethanol (Aldrich, Poole, UK) 1:1 (w/v) in amyl alcohol (Aldrich). This was stored at 4°C until immediately before use when a working solution was made by diluting the stock 1:40 in LPS-free sterile PBS, pH 7.4 (Invitrogen).

2.5.1 Induction of airway eosinophilia in mice

Mice were sensitised to ovalbumin (OVA) (Fraction V; Sigma), as described in Figure 2.1 by modification of a previously published protocol (Henderson et al., 2000). All OVA solutions were filter sterilised prior to use (Millex-GV 0.22 μm, Millipore, Livingston, UK). An alum suspension (Alhydrogel 2%, Brenntag Biosector, Fredriksund, Denmark) was used as an adjuvant. To adsorb the OVA to the alum, a 1 mg/ml solution of OVA in PBS (Invitrogen) was added to an equal volume of the alum suspension and mixed using a vortex mixer. Mice were sensitised by administering 200 μl of the OVA/alum mixture, that is, 100 μg alum, by intra-peritoneal (i.p) injection day 0 and day 14. On day 14, at the time of i.p. injection, mice were anaesthetised with 300 – 400 μl avertin i.p. and 100 μg of sterile OVA in 50 μl sterile PBS (Invitrogen) was given intranasally (i.n.) using a micropipette. Mice were again anaethetised with avertin before the administration of three i.n. challenges of 50 μg OVA in 50 μl PBS (Invitrogen) on each of days 25, 26 and 27. Control mice were given PBS in place of OVA in both the sensitisation and challenge stages of the protocol. Mice were sacrificed on day 28 by giving a fatal dose of avertin.

Potential anti-inflammatory compounds were administered on days 25, 26 and 27 of the above protocol. Thymosin beta 4 sulfoxide (gifted by Dr. John Young, Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow) at a dose of 20 μg per mouse was given by i.p. injection 30 minutes before each i.n. challenge, as was 3 mg/kg dexamethasone (water soluble, Sigma). Simvastatin (gifted by Dr. Anne Crilly, Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow), which had been acidified to produce the active compound, was given at a dose of 4 mg/kg or 40 mg/kg i.p. 30 minutes before each i.n. challenge. In separate experiments, simvastatin 40 mg/kg was also given by oral gavage 60 minutes before each i.n. challenge.

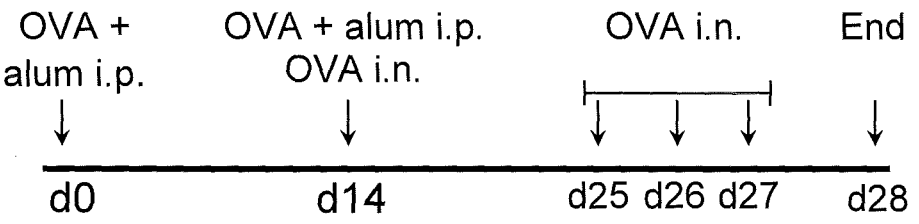


Figure 2.1. Protocol used in the murine model of allergic asthma.

Mice were sensitised and challenged with ovalbumin (OVA) as described in the protocol above. To sensitise the mice, 100 μg of OVA adsorbed to alum was given by intraperitoneal (i.p.) injection on day (d)0 and d14; 100 μg of OVA was also given intranasally (i.n.) on d14. Mice were challenged with 50 μg of OVA on each of d25, d26 and d27. Mice were sacrificed on day 28 and analyses performed.

2.5.2 Bronchoalveolar lavage (BAL)

The thorax was opened carefully and blood was taken by cardiac puncture under direct vision for serum immunoglobulin measurements. The trachea was then exposed and a small transverse incision made in it immediately below the level of the larynx. Polythene tubing (0.58mm ID, 0.78mm OD; VWR International) threaded over a 23 gauge 'blue' needle was inserted into the trachea and held in place using 12 cm blunt forceps (VWR International). The lungs were then lavaged with 2 x 0.5ml of PBS, ensuring that both lungs were seen to inflate during the lavage process and that there was no leakage of lavage fluid from the trachea. The two lavage samples from each mouse were pooled in a 1.5 ml microcentrifuge tube (Thistle Scientific, Uddingston, UK) and kept on ice until processing. The BAL fluid was centrifuged at 400 x *g* for 5 minutes to pellet the cells and the supernatant removed using a 1ml syringe attached to an 18 gauge needle. This allowed the volume of each lavage sample to be estimated to the nearest 0.01 ml. Supernatants were frozen at –20°C until analysis. To remove any contaminating red blood cells, the BAL cell pellet was resuspended in 1 ml of FACS® Lysis Buffer (1:10 dilution in sterile distilled water of commercial stock containing 5% diethylene glycol, 1.5% formaldehyde; BD Biosciences, Cowley, UK) mixed gently by inverting the tube several times, and incubated for 10 minutes at room temperature. The cells were then washed twice in PBS before being resuspended in 1ml of PBS. The cell number was then counted using a haemocytometer. The absolute cell count per lavage was divided by the lavage volume to standardise the cell count per ml of lavage fluid.

Cytospin preparations were then made using a Shandon Cytospin in a similar way to that described for sputum cell cytopreps (Section 2.4.2). Approximately 1 x 10^5 cells were used to make each cytoprep. Cells were centrifuged at 400 rpm for 6 minutes then

fixed in methanol for 10 minutes. Cytopreps were stained with Diff-Quik (Triangle Biomedical Sciences, Skelmersdale, UK), a staining kit for rapid Romanowsky staining. Some slides were also stained specifically for eosinophils using Chromotope 2R (VWR International) following the protocol of the Department of Veterinary Pathology, University of Bristol, UK (www.bris.ac.uk/Depts/PathandMicro/CPL/carbol.html). Differential cell counting was done using standard morphological criteria. Four hundred cells were counted per slide. Differential cell counts were expressed as a percentage of the number of cells then multiplied by the total cell count per ml to give an absolute cell number per ml.

2.5.3 Lung histology

2.5.3.1 Preparation of tissue sections

After BAL sampling had been completed, the lungs were removed from the thoracic cavity by careful dissection. Polythene tubing was again inserted into the trachea, as described above, and the lungs inflated with 1ml of 10% neutral buffered formalin (10% (v/v) of 37% aqueous solution of formaldehyde (Aldrich), 30mM $NaH_2PO_4.H_2O$, 45 mM Na_2HPO_4, pH 7). The trachea was tied with 2-0 silk suture (Ethicon, Edinburgh, UK) and the lungs immediately immersed in 10% neutral buffered formalin for at least 72 hours. After fixation, the left lung was then dissected free, embedded in paraffin and 6 μm sections cut (kindly performed by Mr. Roderick Ferrier, Department of Pathology, Western Infirmary, Glasgow). Sections were stained with haematoxylin and eosin using a standard method.

2.5.3.2 Quantification of lung inflammation

Quantification of lung inflammation on histological sections was determined using a semi-quantitative method (Sur et al., 1999). Total lung inflammation was defined as the sum of peribronchial and perivascular scores. Inflammation was graded on the scale 0, none; 1, mild, 2, moderate; 3, marked and 4, severe. An increment of 0.5 was used when severity of inflammation fell between two integers. Sections were scored by a single observer blinded to the treatment group of the mice.

2.6 Isolation of lymphocytes from murine thoracic lymph nodes

Sensitisation of mice to OVA produced a response in thoracic lymph nodes. To assess this further, lymph nodes from the paratracheal and subcarinal areas were obtained by careful dissection after the lungs had been removed from the thoracic cavity. Nodes to be used for culture or analysis by flow cytometry were stored in complete RPMI on ice until processing. Other lymph nodes were placed in RNA*later*™ (Ambion, Austin, USA), a solution with a high salt concentration which inhibits RNase activity, and used later to obtain RNA for RT-PCR analysis of gene expression.

A single cell suspension was obtained from murine thoracic lymph nodes, obtained as described above, by gently pressing the lymph nodes through sterile Nytex using a 2ml syringe plunger. The cell suspension was washed three times in complete RPMI, passed again through Nytex to remove cellular aggregates and debris and then resuspended in an appropriate volume of complete medium. An accurate cell count was then made using a haemocytometer.

Proliferation assays for thoracic lymph node cells were performed in triplicate in U-bottom 96 well culture plates (Nunc, Rochester, USA) at 2 x 10^5 cells/well in 100 µl complete RPMI with 10% FCS. Additional cell stimulating agents or medium were added in volumes of 100 µl to give a final culture volume of 200 µl per well. Cells were stimulated with a final concentration of 500 – 1000 µg/ml OVA or 5 µg/ml Concanavalin A (Sigma) in culture medium. Cell culture was allowed to proceed for 96 hours with 1 µCi of [^{3}H]-thymidine (Amersham, Little Chalfont, UK) in a volume of 25 µl being added during the final 8 hours of culture. Plates were harvested onto a glass fibre filter (Packard, CT, USA) using a Micromate 196 Harvester (Packard). [^{3}H]-thymidine incorporation was measured using a Matrix 96 Direct Beta Counter (Packard). Cytokine production by thoracic lymph node cells was measured using supernatants derived from parallel triplicate cultures in flat-bottom 96 well culture plates (Nunc) in the presence or absence of stimuli; these culture supernatants were harvested after 72 hours. Supernatants were frozen at –20°C until cytokine assays were performed in single batches (Section 2.8).

2.7 Flow cytometry

2.7.1 Analysis of murine lymphocyte phenotype

The phenotype of murine lymph node lymphocytes was determined by flow cytometric analysis using double immunofluorescence staining. The antibodies used are described in Table 2.1. Cells suspended 1 x 10^6 in 100 µl of serum free complete RPMI, were placed in a 12 x 75 mm polystyrene tube (Falcon 2052, BD Biosciences). The cells were pelleted by centrifugation at 400 x *g* for 5 minutes and then resuspended in 50 µl of a 1:50 (v/v) dilution of Fc Block in FACS buffer (1x PBS, pH 7.5, 5% (w/v) FCS and

Antibody specificity	Conjugate	Host	Isotype	Source
CD3	PE	Rat	IgG2b, κ	BD Biosciences
CD4	PE	Rat	IgG2a, κ	BD Biosciences
CD4	FITC	Rat	IgG2a, κ	Sigma
CD8	FITC	Rat	IgG2a, κ	BD Biosciences
CD11a	FITC	Rat	IgG2a	Caltag Laboratories
CD44	FITC	Rat	IgG2b	Caltag Laboratories
CD45RB	FITC	Rat	IgG2a, κ	BD Biosciences
CD62L	PE	Rat	IgG2a, κ	BD Biosciences
CD16/32/FcγR (Fc Block)	-	Rat	IgG2b, κ	BD Biosciences

Table 2.1. Antibodies used in FACS analysis of murine lymphocyte phenotype.

The antibodies used in the FACS analysis of murine lymphocyte phenotype are listed above.

FITC – fluoroescein isothiocyanate; PE – phycoerythrin

Antibody	Conjugate	Host species	Source
IgG2a	FITC	Rat	Caltag Laboratories
IgG2a	PE	Rat	BD Biosciences
IgG2b	FITC	Rat	Caltag Laboratories
IgG2b	PE	Rat	Caltag Laboratories

Table 2.2. Negative control antibodies used in flow cytometry.

The negative control antibodies used in the FACS analysis of murine lymphocyte phenotype are listed above.

FITC – fluoroescein isothiocyanate; PE – phycoerythrin

0.1% (w/v) sodium azide) and incubated for 10 minutes at room temperature. Antibody diluted to an appropriate concentration in 50 µl FACS buffer was then added to each tube and the samples incubated at 4°C for 30 minutes. Negative control primary antibodies of appropriate isotype and conjugate, as listed in Figure 2.2, were added to parallel tubes and similarly processed. Cells were then washed by adding 3 ml of FACS buffer to each tube followed by centrifugation at 400 x *g* for 5 minutes. Cells were washed with 1 ml FACSFlow (BD Biosciences) followed by centrifugation at 400 x *g* for 5 minutes before being resuspended in 300 µl FACSFlow and analysed by flow cytometry (FACScan, BD Biosciences). Gates were set for lymphocytes using forward and side light scatter parameters. The percentages of fluoroscein isothiocyanate (FITC) or phycoerythrin (PE) positive cells were generated using Cellquest software (BD Biosciences).

2.8 Enzyme linked immunosorbance assay (ELISA) for cytokines

2.8.1 Human IL-18 ELISA

Human IL-18 was measured in sputum cell supernatants using a commercially available kit (Diaclone SAS, Besancon, France). The assay was performed using commercial reagents following the manufacturer's protocol. The assay recognised both pro-IL-18 and mature IL-18. The sensitivity of the ELISA was 20 pg/ml.

2.8.2 Murine cytokine ELISA protocol.

Murine IFN-γ, IL-4, IL-5 and IL-6 were quantified in BAL fluid or culture supernatants using paired capture and biotinylated detection antibodies for each cytokine (BD Biosciences). Details of the concentrations of antibodies used and the lower limit of detection of each assay are given in Table 2.3. Immulon 4 micro-ELISA plates (Dynex Technologies, Ashford, UK) were coated overnight at 4°C with the appropriate concentration of capture antibody in 0.1 M $NaHCO_3$, pH 8.2. The working volume was 50 µl per well. The plates were washed four times with 1xPBS (140 mM NaCl, 4 mM NaH_2PO_4, 5 mM KCl, 1.5 mM KH_2PO_4, pH 7.4) containing 0.05% Tween 20 (wash buffer) and blocked for 2 hours at room temperature with 200 µl of 10% (v/v) FCS in PBS (ELISA buffer). After four washes with wash buffer, 50 µl samples were added in duplicate to the plate. Serial twofold dilutions of recombinant cytokine (BD Biosciences) in duplicate were also added as standards. The plates were then incubated for two hours at room temperature or overnight at 4°C to allow binding between cytokine and antibody. After this, 50 µl of biotinylated detection antibody in ELISA buffer was added after four washes and samples were again incubated for one hour at room temperature. The plates were washed six times before 50 µl of 1:1000 dilution of ExtrAvidin (Sigma) in ELISA buffer was added to each well. After incubation for 30-60 minutes at room temperature the plates were washed 8 times and then developed using 100 µl per well of TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, USA). Plates were incubated at room temperature in the dark until sufficient colour reaction product was visible. The plates were then read at 630 nm on a MRX II microplate reader (Dynex Technologies), always within 1 hour of the TMB being added.

Cytokine	Capture Antibody (μg/ml)	Detection Antibody (μg/ml)	Lower Limit of Detection of Assay (pg/ml)
IL-4	2	2	10
IL-5	2	2	10
IL-6	1	1	10
IL-18*	5	0.3	40
IFN-γ	1	0.5	40
Eotaxin#	0.4	0.08	10

Table 2.3. Antibodies used in murine cytokine ELISAs.

The antibodies used for the quantification of murine cytokines by ELISA in bronchoalveolar lavage fluid and cell culture supernatants are listed above.

All antibodies were purchased from BD Biosciences, Cowley, UK, except:

*, purchased from Perprotech, London, UK.

#, purchased from R and D Systems, Abingdon, UK.

Murine IL-18 was assayed following the above protocol. Paired antibodies for this cytokine were purchased Perprotech, see table 2.3, and recombinant murine IL-18 (R&D Systems, Abingdon, UK) was used as a standard. The range of the standard curve was 80 pg/ml – 80 ng/ml.

Murine eotaxin was assayed using paired capture and biotinylated detection antibodies (R&D systems), see table 2.3, using a recombinant murine eotaxin standard (R&D Systems). Modifications to the above protocol were as follows: capture antibody coating buffer was PBS, pH 7.4; blocking buffer 1% bovine serum albumin (BSA)(Sigma), 5 % sucrose and 0.05% NaN_3 (Sigma); assay diluent 0.01% BSA, 0.05% Tween 20 in Tris-buffered saline (20 mM Trizma base, 150 mM NaCl), pH 7.3. The range of the standard curve was 8 pg/ml – 1000 pg/ml. After incubation with TMB substrate for 30 minutes the reaction was stopped at 30 minutes by the addition of 50 µl 1 M H_3PO_4. Plates were read at 450 nm with 570 nm correction.

2.9 Murine serum immunoglobulin measurements

2.9.1 Serum OVA-specific IgG1, IgG2a and IgE levels

Murine serum OVA-specific IgG1, IgG2a and IgE levels were determined using a modification of the ELISA protocol described above, and of a previously published method (Garside et al., 1995). At least five washes with ELISA wash buffer were done between each step of the protocol. Immunolon 4 micro-ELISA plates were coated at 4°C overnight with 50 µl of a 10 µg/ml solution of OVA in a coating buffer of 0.05 M carbonate/bicarbonate buffer (50mM $NaHCO_3$, 50mM Na_2CO_3, pH 9.5). The plates

were then blocked with 200 µl of ELISA wash buffer containing 10% (v/v) FCS for 1 hour. Serum was diluted appropriately in wash buffer, then 50 µl of the diluted serum added across the top row of the plate, leaving column 12 with buffer alone. The starting dilutions were 1 in 250 for serum IgG1 assays, 1 in 50 for Ig2a assays and 1 in 20 for OVA-specific IgE assays. The samples were then serially diluted with wash buffer through rows two to eight of the plate. IgG1 samples were diluted 1:3 each time and IgG2a and IgE samples diluted 1:2. Samples were then incubated at room temperature for 1.5 hours. Biotinylated anti-mouse IgG1, IgG2a and IgE (BD Biosciences) antibodies were added to the appropriate plates at a concentration of 0.5 µg/ml in 50 µl wash buffer. The plates were incubated for 1 hour at room temperature. Thereafter, 50 µl of a 1:1000 dilution of ExtrAvadin in wash buffer was added to each well and incubated for 30 minutes at room temperature. The plates were developed using 100 µl of TMB substrate and then read at 630 nm, as described in section 2.8.2 above. The mean optical density for a designated serum dilution for each of OVA-specific IgG1, IgG2a and IgE was recorded to give a comparison of the serum levels of these antibodies among different treatment groups.

2.9.2 Measurement of serum total IgE levels

Total murine serum IgE levels were measured using the OptiEIA ELISA Mouse IgE Set (BD Biosciences) following the manufacturer's guidelines. The working volume was 100 µl. At least 4 washes were performed between each step. Maxisorp micro-ELISA plates (Nunc) were coated with a 1:250 dilution of capture antibody in 0.1 M carbonate/bicarbonate buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 9.5) and incubated at 4°C overnight. Plates were blocked by incubating with 200 µl of ELISA buffer for 1

hour at room temperature. Mouse serum samples diluted 1:200 in ELISA buffer or the supplied IgE standard were then incubated for 2 hours at room temperature. Samples were tested in duplicate. The standard curve ranged from 1.6 ng/ml to 100 ng/ml. Detection antibody and detection agent, both at a dilution of 1:250 in ELISA buffer, were then added and samples incubated for 1 hour at room temperature. TMB substrate was used to develop the ELISA. This reaction was stopped at 30 minutes by the addition of 50 µl 1 M H_3PO_4. Plates were then read at 450 nm with 570 nm correction.

2.10 Immunocytochemisty

Sputum cytospin preparations were made using a Shandon cytospin device as described in Section 2.4.2. Polylysine coated microscope slides (VWR International) were used to help maintain cell adherence to the slides. Cells from some sputum samples were cultured for 24 hours in complete DMEM containing 10% FCS in the presence or absence of 100 ng/ml LPS from *Salmonella enteriditis* (Sigma) at a cell concentration of 2 x 10^5 cells in 200 µl. Cells were harvested from the plates by gentle pipetting then washed twice in PBS to remove debris before cytospins were prepared as above.

2.10.1 Human IL-18 immunocytochemistry

Frozen cytospin slide preparations were allowed to come to room temperature before being washed in PBS for 5 minutes. Endogenous peroxidase activity was then blocked using 0.5% hydrogen peroxide (Sigma) (v/v) in methanol (VWR international) for 30 minutes. The slides were washed twice for 10 minutes in PBS at room temperature. A

wax ring was drawn round the tissue section using an ImmEdge™ pen (Vector Laboratories Ltd., Peterborough, U.K.) and 100µl of blocking serum (20% (v/v) human serum (Scottish Antibody Production Unit (SAPU), Lanarkshire, UK) and 20% (v/v) horse serum (SAPU) in PBS) placed over each cell preparation and incubated for 30 minutes at room temperature. The blocking serum was then removed by tapping the slides gently on tissue paper and 100 µl of a 1µg/ml solution of polyclonal mouse anti-human IL-18 serum (D4/D1, gifted from Dr. Andrew Jackson, CRUK, London) in assay diluent (2% (v/v) horse serum and 2% (v/v) human serum in PBS) applied over each cytoprep. The samples were incubated at 4°C overnight. Samples were allowed to come to room temperature and then washed twice in PBS for 5 minutes each time. The secondary antibody solution, 2.5 µg/ml biotinylated horse anti-mouse IgG (DAKO, Ely, U.K.) in assay diluent, was applied for 30 minutes at room temperature. The slides were then washed twice in PBS at room temperature. After this an alkaline phosphatase complex (Vectastain Elite ABC Kit, Vector Laboratories Ltd.) was applied and the samples incubated for 30 minutes at room temperature. The samples were again washed twice for 5 minutes in PBS before the addition of diaminobenzidine substrate (DAB, Vector Laboratories Ltd.) and the slides viewed microscopically until the stain developed. The slides were then washed in PBS for 5 minutes and then in tap water for 5 minutes before counter-staining lightly with Harris haematoxylin (VWR International) for 5 minutes. Samples were dehydrated by successive 5 minute washes in 70% ethanol, 95% ethanol and 100% etahnol and then twice for ten minutes in xylene before being mounted using DPX (VWR International).

Specificity of staining of the primary anti-serum for human IL-18 was demonstrated by pre-incubation with recombinant human IL-18 (produced in the Department of Immunology, University of Glasgow) which neutralised binding (Mrs. S Kitson, Centre

for Rheumatic Diseases, Royal Infirmary, Glasgow) or by the use of 1 μg/ml mouse IgG1 (DAKO, Ely, UK) as a negative control. A mouse anti-human CD68 monoclonal antibody (DAKO) was used as a positive control. The secondary antibody used did not bind to sputum cells in the absence of primary antibodies at the concentrations indicated in the above protocol. The alkaline phosphatase conjugate, either alone or in the presence of secondary antibody, did not bind sputum cells directly.

2.11 Reverse transcription – polymerase chain reaction (RT-PCR)

Induced sputum cells were pelleted by centrifugation at 400 x *g* for 5 minutes and then immediately resupended in 350 μl of cell lysis buffer (from NucleoSpin RNA II purification kit; Macherey-Nagel, Düren, Germany) containing 1% (v/v) 2-mercaptoethanol, lysed by repetitive mixing with a micropipette and then stored at –70°C until use. Murine lymph nodes, which had been stored in RNA*later*™ at –70°C were removed from the RNA*later*™, dried on tissue paper and then placed in 350 μl of lysis buffer 1% 2-mercaptoethanol in a 1.7ml microcentrifuge tube and homogenised using a Kontes Pellet Pestle Cordless Motor (Kontes Glass Company, New Jersey, USA). Lysates were again stored at –70°C until required.

2.11.1 RNA purification

Total cellular RNA was extracted using NucleoSpin RNA II columns (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. Briefly, cells are lysed in a solution containing a large amount of chaotropic ions which inhibit RNase activity and

favour the binding of RNA to the silica membrane of the Nucleospin RNA column. Contaminating DNA is then removed by a DNase I solution which is applied directly onto the silica membrane during the preparation. Washing steps with two different buffers remove salts, metabolites and macromolecular cellular components before the RNA is eluted under low ionic strength conditions with RNase-free water. RNA from sputum cell pellets was eluted in 40 µl of RNase-free water while murine lymph node RNA was eluted in a volume of 60 µl of RNase-free water (Braun, Melsungen, Germany). The RNA concentration of each sample was determined using the 260:280 nm absorbance ratio of each solution by UV spectrometry. RNA samples were stored at –70°C until required.

2.11.2 DNase treatment of RNA samples

Some RNA samples purified using the NucleoSpin RNA II columns were found to have residual DNA contamination. As this would decrease the efficiency and accuracy of subsequent PCR reactions, further treatment with DNase was done using the DNA-*free*™ kit (Ambion, Austin, USA). To 16 µl of RNA solution was added 2 µl (4 units) of DNase I and 2 µl of 10 x DNase buffer (100mM Tris-Cl pH 7.5, 25mM $MgCl_2$, 1 mM $CaCl_2$). Samples were incubated at 37°C for 30 minutes. The reaction was stopped by the addition 5 µl of the supplied DNase Inactivation Reagent to the sample and the sample mixed by repetitive pipetting. The tubes were incubated for 2 minutes at room temperature, the tube being flicked once during the reaction to re-disperse the DNase Inactivation Reagent. Samples were then centrifuged at 10,000 x *g* for 1 minute to pellet the DNase Inactivation Reagent. The RNA-containing supernatant was then removed and either used immediately for reverse transcription reactions or placed in a fresh tube and stored at –70°C until use.

2.11.3 Reverse Transcription

Cloned (c) DNA was generated using the Superscript II RT system (Invitrogen). 1-2 ug of RNA, in a volume of ≤ 10 µl was added to 2-3 µl (100 – 150 ng) of random hexamers (Invitrogen) and the volume made up to 12 µl by the addition of the appropriate volume of RNase-free H_20. This mixture was heated at 70°C for 10 minutes in a heating block and then placed on ice for at least one minute. During this time a mixture of 4 µl 5X First Strand Buffer (250mM Tris-HCl pH 8.3, 375mM KCl, 15mM $MgCl_2$), 2 µl 0.1mM and 1 µl of mixed dNTP stock (10mM each of dATP, dGTP, dCTP and dTTP, pH 7) per sample was prepared. This mixture (7 µl) was added to the RNA/random hexamer mixture and incubated at 42°C for 2 minutes. Superscript II RT (1 µl (200 units)) and reverse transcription undertaken by successive incubations at 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes.

2.11.4 Quantitative polymerase chain reaction

Quantitative 'Taqman™' real-time PCR (Gibson et al. 1996) was performed according to the manufacturer's instructions (PE Applied Biosystems, Foster City, USA) and as described by Overbergh et al (1999). This is a PCR method that allows rapid and accurate quantitation of gene transcription levels from small samples. The principle of this method is outlined in Figure 2.2.

Primers and fluorgenic probes (listed in Table 2.4) were designed by Dr. Carol Campbell, Department of Immunology, University of Glasgow, using the Primer

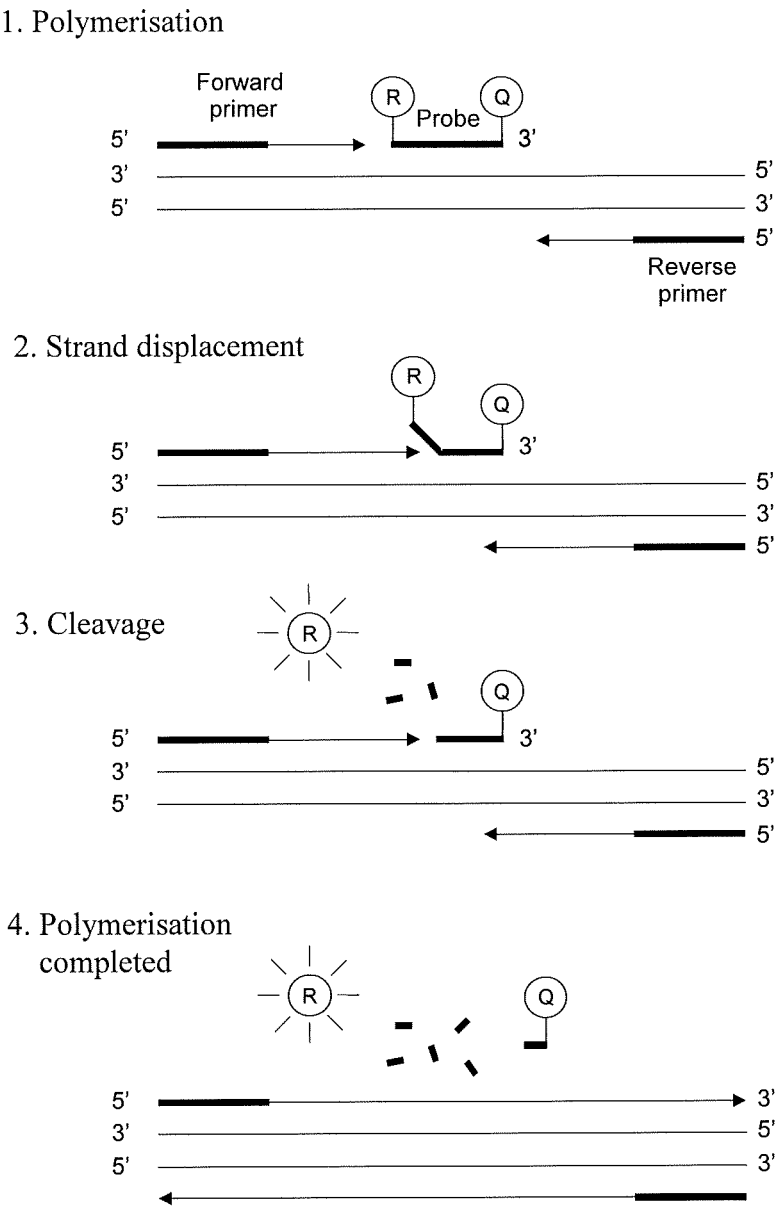


Figure 2.2. Taqman real-time RT-PCR.

The Taqman probe is an oligonucleotide with a 5' reporter dye (R) and a 3' quencher (Q). In an intact probe, the proximity of the quencher to the reporter causes suppression of reporter fluorescence. During PCR, the probe is cleaved by the 5' → 3' exonuclease activity of Taq DNA polymerase separating the reporter from the quencher. Reporter fluorescence increases and can be used to determine the quantity of specific product generated. The 3' end of the probe is blocked to prevent extension of the probe during PCR.

Table 2.4. Taqman Primers and Probes.

Primers: F = forward; R = reverse

Probes: 5' FAM (6- carboxy-fluorescein) labelled

3' TAMRA (6-carboxy-tetramethyl rhodamine; quencher) labelled

Murine IL-4	F primer: 5'- ACA GGA GAA GGG ACG CCA T – 3' R primer: 5'- GAA GCC CTA CAG ACG AGC TCA – 3' Probe: 5' FAM -TCC TCA CAG CAA CGA AGA ACA CCA CA – TAMRA 3'
Murine IL-5	F primer: 5'– AGC ACA GTG GTG AAA GAG ACC TT - 3' R primer: 5'- TCC AAT GCA TAG CTG GTG ATT T - 3' Probe: 5' FAM - CTG TTG ACA AGC AAT GAG ACG ATG AGG -TAMRA 3'
Murine IL-13	F primer: 5'- AGA CCA GAC TCC CCT GTG CA – 3' R primer: 5'- TGG GTC CTG TAG ATG GCA TTG – 3' Probe: 5' FAM - CGG GTT CTG TGT AGC CCT GGA TTC C -TAMRA 3'
Murine IL-18	F primer: 5' - CAA ATC ACT TCC TCT TGG CCC - 3' R primer: 5'- AAC AAA GTC TGG CCT GTA TCC AA - 3' Probe: 5' FAM - CTG CCA TGT CAG AAG ACT CTT GCG TCA ACT.- TAMRA 3'
Murine CIITA*	F primer: 5'- CGC TTC CCG GAA GAG CAT– 3' R primer: 5'- TCC AAA TAG TTC AGT GAG GTC CTA GA – 3' Probe: 5' FAM - CTA TGG ACT CAA AGC ACA GGA AGC TAG TGC C - TAMRA - 3'

* MHC-II transactivator

Murine HPRT	F primer: 5'- GCA GTA CAG CCC CAA AAT GG – 3' R primer: 5'- AAC AAA GTC TGG CCT GTA TCC AA – 3' Probe: 5' FAM – TAA GGT TGC AAG CTT GCT GGT GAA AAG GA – TAMRA – 3'
Human IL-18	F primer: 5'- GGC CTC TAT TTG AAG ATA TGA CTG ATT – 3' R primer: 5' CCT CTA GGC TGG CTA TCT TTA TAC ATA C – 3' Probe: 5' FAM – TGA CTG TAG AGA TAA TGC ACC CCG GAC C – TAMRA 3'
Human HPRT	Purchased from PE Applied Biosystems UK (Warrington, UK)

Express™v1.0 program purchased from PE Biosystems or purchased directly from PE Biosystems. PCR reactions were performed in the ABI-prism 7700 Sequence Detector, which contains a Gene-Amp PCR system 9600 (PE Biosystems). PCR amplification was performed in a total volume of 25 µl, containing 1 µl of sample cDNA, 50 mM KCl, 19 mM Tris-HCl (pH 8.3), 10 µM EDTA, 200 µM of each of dATP, dCTP and dGTP and 400 µM dUTP, 3.5 mM $MgCl_2$, 300 nM each primer, 200 nM fluorgenic probe, 0.625 U AmpliTaqGold™ and 0.25 U Amperase Uracil N-Glycosylase (PE Biosystems). Each reaction was performed in triplicate using the following conditions: 2 minutes at 50°C then 10 minutes at 94°C, followed by a total of 45 two temperature cycles of 15 seconds at 94°C then 1minute at 60°C.

Data analysis was performed using the Sequence Detection v1.6.3 software (PE Biosystems). The threshold cycle, Ct, was calculated for each sample. This represents the PCR cycle at which an increase in fluorescence above a set threshold level can first be detected (Figure 2.3). Expression of the gene of interest in a sample was normalised by comparison to the expression of a reference reporter gene, HPRT. Firstly, the ΔCt was obtained by subtracting the Ct value of HPRT from the Ct value of the gene of interest. The formula $2^{-\Delta Ct}$ was then used to calculate a value for the fold increase in gene expression relative to HPRT. Multiplication of this value by 100 gives expression of the gene of interest as a percentage of HPRT. The positive error is the standard deviation of the difference, $s = \sqrt{(s_1^2 + s_2^2)}$ where s_1 and s_2 are the standard deviations of the Cts of the HPRT and the gene of interest.

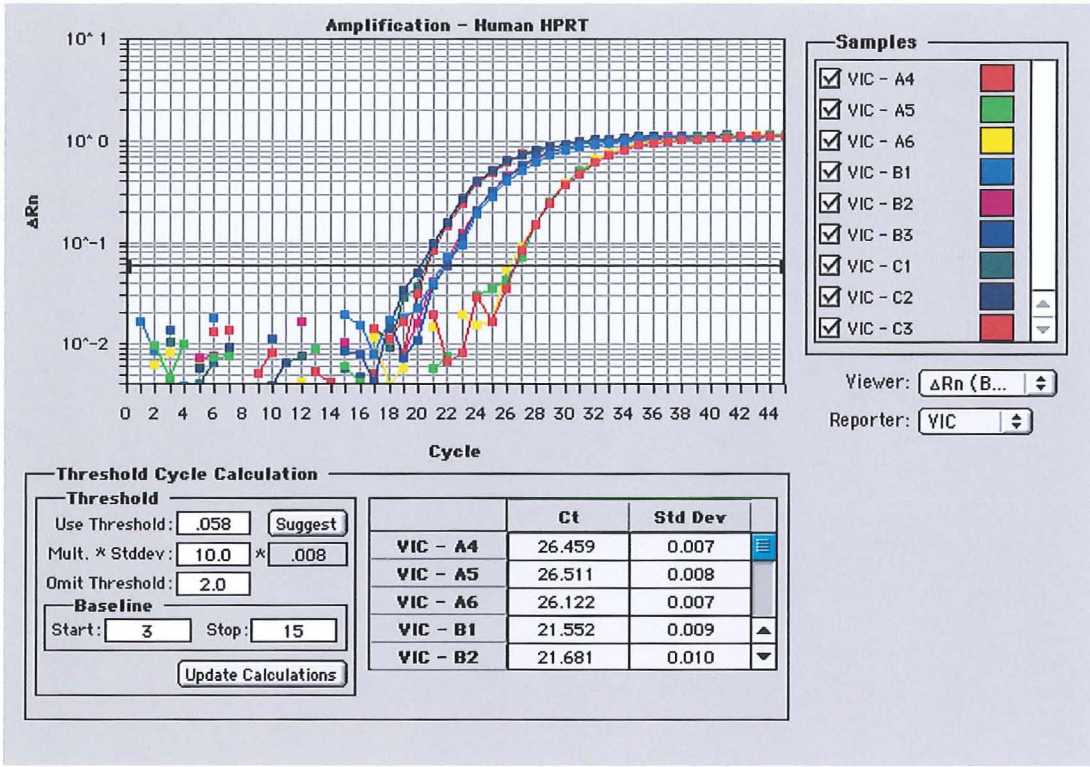


Figure 2.3. Typical Taqman amplification plots.

The amplification plot shows how the fluorescence emission (normalised Reporter, Rn) varies with the PCR cycle number. Initially the fluorescent signal is below the detection limit of the Sequence Detector. As the PCR reaction proceeds the signal can be detected as it continues to increase in direct proportion to the amount of specific amplified product. The Rn reaches a plateau when the ratio of polymerase enzyme to PCR product decreases preventing the amount of PCR product from increasing exponentially. The Ct (threshold cycle) is determined during the exponential phase (threshold indicated by the black line). Three triplicate samples are shown (A4-A6, B1-B3 and C1-C3).

2.12 Photomicrography

Photographs of microscope slides were taken using a Leica DM RB microscope (Leica Microsystems (UK) Ltd., Milton Keynes, UK) connected to a digital camera. Images were stored using ImageAxis Software (assisted by Mr. Peter Kerrigan, Department of Pathology, Western Infirmary, Glasgow).

2.13 Statistical analysis

Data were collated and statistical analyses performed using a statistical software package (Minitab Statistical Software, Minitab Inc., State College, PA, USA). Results are expressed as median and interquartile range for non-parametric data or mean and standard error for normally distributed data. The Student's *t*-test and analysis of variance (ANOVA) were used to compare data sets with normal distribution. Non-parametric statistics were used (Mann-Whitney *U* test) for comparison between data sets with skewed distribution. Spearman rank correlation test was used to correlate cytokine levels in sputum with cell counts and serum IgE levels. Significance was accepted at a level of $p < 0.05$.

Chapter 3

The effect of cigarette smoking on interleukin-18 levels in induced sputum from asthmatic and normal subjects

Data from this chapter has been accepted for publication,

in McKay et al., (2004) *Clin Exp Allergy*.

3.1 Introduction

Atopic asthma is a chronic inflammatory condition of the airways associated with elevated serum IgE levels. It is characterised pathologically by inflammatory infiltrates in the bronchial walls containing eosinophils, T lymphocytes and mast cells (Holt et al., 1999). Th2–type T helper lymphocytes and the cytokines they secrete, such as interleukin (IL)-4, IL-5 and IL-13, are thought to play a key role in the initiation and perpetuation of this airway inflammation (Robinson et al., 1992, Barnes et al., 1998). An alteration in the balance between a Th2 response and a Th1 response, where lymphocytes produce interferon gamma (IFN-γ), may predispose to the development of allergic asthma (Umetsu et al., 2002).

IL-18, originally called interferon-gamma inducing factor, is a pro-inflammatory cytokine secreted by activated macrophages. In association with IL-12 it plays an important role in the development of a Th1 response and in the production of IFN-γ from T cells and NK cells (Okamura et al., 1995). However, IL-18 can also promote Th2 responses, depending on the surrounding cytokine environment (Xu et al., 2000, Nakanishi et al., 2001) and induce IgE production (Hoshino et al., 2000, Yoshimoto et al., 2000). It is able to induce IL-4, IL-13 and histamine production in basophils and mast cells in association with IL-3 (Yoshimoto et al., 1999) and, in combination with IL-2, induces IL-4 and IL-13 secretion from NK and naïve T lymphocytes (Hoshino et al., 199b) and NKT cells (Yoshimoto et al., 2003).

IL-18 is expressed in the human airway epithelium (Cameron et al., 1999) and is also produced by human alveolar macrophages (Shigehara et al., 2001). As yet, there are conflicting reports on the actions of IL-18 in both human asthma and animal models of

this disease. Serum IL-18 levels have been observed to be elevated in asthmatics with stable disease (El-Mezzein et al., 2001) and during acute asthma exacerbations (Tanaka et al., 2001a) while, in contrast, IL-18 levels in BAL fluid from asthmatics (Ho et al., 2002) and IL-18 mRNA expression in the airway epithelium of asthmatics (Cameron et al., 1999) have been found to be reduced when compared to normal subjects. In animal models of asthma, IL-18 promoted allergic airway inflammation when given alone (Wild et al., 2000) but had anti-allergic properties when administered with IL-12 (Hofstra et al., 1998).

In order to assess further the role of IL-18 in airway inflammation in asthma the objectives of the present study were to identify and quantify IL-18 levels in sputum samples from asthmatic and normal subjects and to assess its cellular distribution. Induced sputum is a non-invasive method of sampling airway cells and secretions which allows the identification of pro-inflammatory factors *ex vivo*. Cigarette smoking alters airway inflammation in asthma and changes the profile of cytokines secreted in induced sputum (Chalmers et al., 2001, Takanashi et al., 1999) and so the effect of smoking on sputum IL-18 levels was also investigated.

3.2 Patient details and pulmonary function

Sixty six asthmatic (31 smokers, 35 non-smokers) and forty normal (20 smokers, 20 non-smokers) subjects participated in the study. Table 1 presents the age, gender and spirometry (forced expiratory volume in 1 second, FEV_1, and percentage predicted FEV_1) in the different study groups. Smoking asthmatics had a significantly lower percentage predicted FEV_1 than non-smoking asthmatics ($p < 0.01$). However, smoking asthmatics had a higher percentage reversibility after bronchodilators ($p < 0.01$). The daily dose of inhaled steroid taken by smoking asthmatics was not significantly different from that of non-smoking asthmatics (median (IQR): smokers 800 (300 – 1300) μg *vs* non-smokers 800 (800 – 1600) μg, beclomethasone diproprionate or equivalent). There was no difference in the ages of all the groups.

3.3 Serum total IgE levels

Atopy was assessed by raised total serum IgE level (>120 International Units/ml). Forty nine percent (17/35) of non-smoking asthmatics and sixty five percent (20/31) of smoking asthmatics were atopic. Of the normal subjects, fifteen percent (3/20) of non-smokers and twenty percent (4/20) of smokers were atopic.

Serum total IgE levels (Table 1) were significantly elevated in asthmatics (154 (69 – 434) kU/l, $n = 66$) when compared to controls (35 (13-88) kU/l, $n = 40$, $p < 0.001$). In the normal control group smokers had significantly higher levels of serum total IgE (smokers 59 (26 – 112) kU/l *vs* non-smokers 15 (0-54) kU/l, $p < 0.01$), but in the asthma group the smokers did not have significantly higher total IgE levels than the non-smokers.

Table 3.1. Subject characteristics.

Age, sex, smoking history, spirometry and serum IgE levels for the subject groups. Data are presented as median (interquartile range). One pack year equals 20 cigarettes smoked per day for one year. *, $p < 0.05$; **, $p < 0.01$ compared to corresponding non-smoking group. Statistical significance was assessed by Mann-Whitney U test.

	Controls	Asthma patients		
		All	Non-steroid-treated	Steroid-treated
Non-smokers				
n	20	35	17	18
Age (yr)	33 (26 - 45)	38 (31 - 48)	34 (30 - 44)	44 (31 - 52)
Sex (M/F)	10/10	19/16	10/7	9/9
Baseline FEV_1 (l)	3.8 (2.4 - 4.6)	3.0 (2.3 - 4.0)	3.4 (2.4 - 4.1)	2.9 (2.2 - 3.4)
Baseline FEV_1 (%)	95 (83 - 110)	90 (80 - 101)	89 (8.1 - 101)	95 (44 - 76)
Reversibility (%)	-	7 (1 - 11)	8 (0 - 10)	6 (0 - 18)
Serum total IgE (kU/l)	15 (0 - 54)	125 (51 - 225)	134 (63 - 220)	117 (32 - 387)
Smokers				
n	20	31	12	19
Age (yr)	42 (35 - 45)	38 (34 - 42)	34 (32 - 39)	40 (37 - 45)
Sex (M/F)	8/12	22/9	7/5	15/4
Smoking pack years	28 (15 - 36)	22 (18 - 30)	23 (18 - 30)	20 (18 - 30)
Baseline FEV_1 (l)	2.8 (2.2 - 3.9)	2.7 (2.2 - 3.5)	2.7 (1.8 - 3.5)	2.7 (2.2 - 3.7)
Baseline FEV_1 (%)	88 (76 - 102)	74 (63 - 89)**	73 (63 - 83)**	84 (60 - 92)
Reversibility (%)	-	17 (8 - 25) **	16 (7 - 25) *	19 (8 - 28)
Serum total IgE (kU/l)	59 (26 - 112)**	234 (78 - 499)	230 (116 - 431)	262 (68 - 772)

3.4 Sputum cell characteristics

The total cell number and differential cell count in the induced sputum samples of asthma patients and controls are shown in Table 2. There was no difference in total cell counts between asthmatic and normal subjects in both the smoking and non-smoking groups. Asthmatic smokers had a raised percentage of neutrophils (51.3 (35.8 – 58.4) %) when compared to non-smoking asthmatics (33.2 (22 – 50.5) %, $p < 0.01$) and also a reduction in sputum eosinophilia (smokers 0 (0 – 0.7) % *vs* non-smokers 0.5 (0 – 5.9) %, $p < 0.05$). Smokers had lower lymphocyte proportions in both asthmatic subjects (smokers 0 (0-0.2) % *vs* non-smokers 1 (0 – 3) %, $p < 0.001$) and normal controls (smokers 0 (0 – 0.2) % *vs* non-smokers 0.6 (0 – 3.3) %, $p < 0.01$).

3.5 IL-18 protein levels in induced sputum fluid

IL-18 levels were significantly reduced in cigarette smokers (smokers 20 (0 – 102) pg/ml, $n = 51$, *vs* non-smokers 358 (50 – 876) pg/ml, $n = 55$, $p < 0.001$). This was more pronounced in asthmatics (smokers 47 (40 -64) pg/ml *vs* non-smokers 358 (50 – 876) pg/ml, $p < 0.001$) than in normal subjects (smokers 25 (0 – 78) pg/ml *vs* non-smokers 247 (50 – 656) pg/ml, $p < 0.01$) (Figure 3.1). Within each of the smoking and non-smoking groups there was no significant difference in IL-18 levels between asthmatic and normal subjects, as shown in Figure 3.2. In the asthmatic groups there was no difference between IL-18 levels in those treated with β_2-agonist alone or with β_2-agonist and inhaled corticosteroid (non-smokers: β_2-agonist 156 (23 – 1522) pg/ml *vs* inhaled corticosteroid 671 (118 – 1137) pg/ml, $p = 0.72$; smokers: β_2-agonist 60 (0 – 120) pg/ml *vs* inhaled corticosteroid 0 (0 – 96) pg/ml, $p = 0.61$).

Table 3.2. Sputum differential leukocyte profile.

Total and differential white cell profiles obtained from induced sputum samples from controls and asthmatic patients. Data are presented as median (interquartile range). *, $p < 0.05$, **, $p < 0.01$, when compared to the corresponding non-smoking group. Statistical significance was assessed by Mann-Whitney *U* test.

	Controls	Asthma patients		
		All	Non-steroid-treated	Steroid-treated
Non-smokers	$n = 20$	$n = 35$	$n = 17$	$n = 18$
Total cell yield (x 10^6)	2 (1.1 - 4.2)	2.7 (1 -4.9)	2.6 (0.9 - 4.6)	3.1 (2 - 5)
Macrophages (%)	54.7 (26.3 – 69.6)	60.8 (38.7 - 69)	64 (45.3 - 74)	55 (18.2 - 65)
Neutrophils (%)	37 (26.4 – 60.4)	33.2 (22 - 50.5)	30.2 (17 - 36.3)	36 (26.2 - 60)
Lymphocytes (%)	0.6 (0 - 3.3)	1.0 (0 - 3)	0.8 (0 - 2)	2 (0 - 3.8)
Eosinophils (%)	0 (0 - 0.9)	0.5 (0 - 5.9)	0.5 (0 - 3.1)	1.2 (0 - 6.8)
Smokers	$n = 20$	$n = 31$	$n = 12$	$n = 19$
Total cell yield (x 10^6)	4 (2.5 - 4.7)	3.3 (1.9 - 4.8)	3.9 (1.9 - 7.1)	3.2 (1.9 - 4.8)
Macrophages (%)	53.6 (34.4 - 71.8)	47.2 (40.4 - 64)	48 (37.6 - 60)	47.2 (40.4 - 65.5)
Neutrophils (%)	46.2 (27.7 - 64.9)	51.3 (35.8 - 58.4)**	51.4 (40 - 50.8)**	51.3 (34.4 - 59.6)
Lymphocytes (%)	0 (0 - 0.2)*	0 (0 - 0.2)**	0 (0 - 0.2)**	0 (0 - 0.2)*
Eosinophils (%)	0 (0 - 0.5)	0 (0 - 0.7)*	0.1 (0 - 0.9)	0 (0 - 0.5)

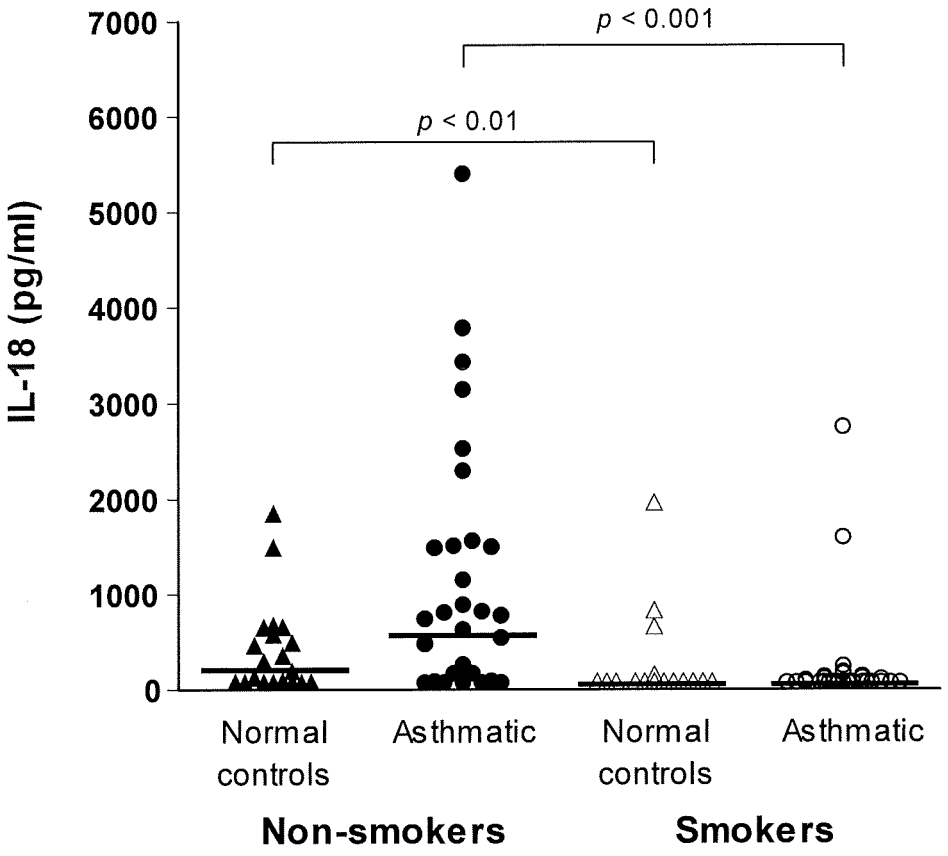


Figure 3.1. IL-18 levels in induced sputum fluid.

IL-18 levels in the fluid phase of induced sputum of normal and asthmatic subjects were assessed by ELISA. Asthmatic and normal smokers had significantly reduced sputum IL-18 levels when compared to the corresponding group of non-smokers. Horizontal bars indicated the median value of each group. Statistical significance was assessed by Mann-Whitney *U* test.

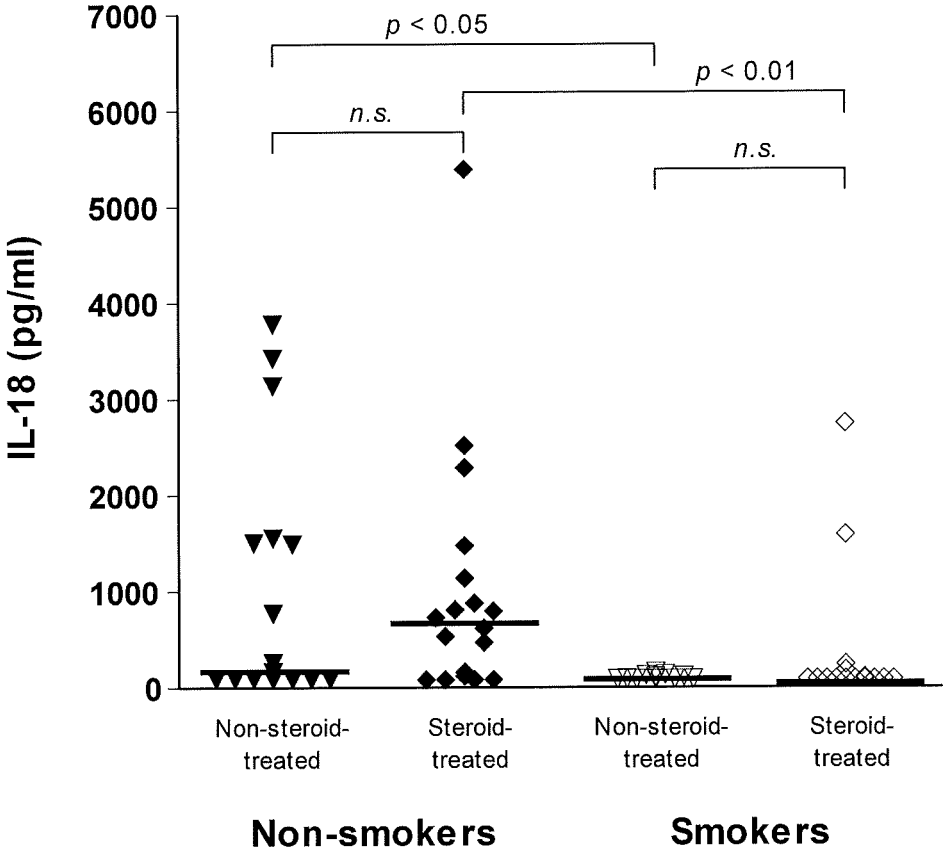


Figure 3.2. Comparison of IL-18 levels in induced sputum fluid between asthmatics treated with or without inhaled corticosteroid.

IL-18 levels in the fluid phase of induced sputum of asthmatic subjects were assessed by ELISA. In both the non-smoking and smoking groups there was no difference in IL-18 levels between asthmatics treated without inhaled corticosteroid (non-steroid-treated; n = 17 non-smokers, n =12 smokers) and those treated with inhaled corticosteroid (steroid-treated; n = 18 non-smokers, n = 19 smokers). For smoking asthmatics treated with or without inhaled corticosteroid, there was a significant reduction in sputum IL-18 levels compared to the corresponding non-smoking group. Horizontal bars indicated the median value of each group. Statistical significance was assessed by Mann-Whitney U test.

There was no correlation between sputum IL-18 levels and any specific cell type in the sputum samples or with serum IgE levels in any of the groups.

3.6 IL-18 mRNA expression in induced sputum cells

Sufficient mRNA was isolated from the sputum cells to allow analysis of IL-18 mRNA expression in 22 normal (10 non-smokers, 12 smokers) and 43 asthmatic (21 non-smokers, 22 smokers) subjects. There was a reduction in the expression of IL-18, expressed as a percentage of HPRT mRNA, in asthmatic smokers when compared to asthmatic non-smokers (smokers 258 (163 – 317) % *vs* non-smokers 385 (221 – 554) %, $p < 0.05$) In addition, there was no significant difference in IL-18 expression between normal subjects and asthmatics in both the smoking and non-smoking groups or between normal smokers and normal non-smokers (smokers 214 (111 - 343) % *vs* non-smokers 356 (110 – 548) %, $p = 0.67$) (Figure 3.3). As illustrated in Figure 3.4, when comparing the asthmatic therapy groups there was no significant difference in IL-18 mRNA levels between those treated with β_2-agonist alone and those treated with β_2-agonist and inhaled corticosteroid (non-smokers: β_2-agonist 364 (157 - 587) % *vs* inhaled corticosteroid 430 (221 – 522) %, $p = 0.57$; smokers: β_2-agonist 286 (159 – 285) % *vs* inhaled corticosteroid 244 (162 - 306) pg/ml, $p = 0.64$).

3.7 IL-18 localisation in sputum cells by immunocytochemistry

The cellular distribution of IL-18 synthesis was visualised in sputum cytospin preparations by immunocytochemistry (Figure 3.5). Positive staining was observed in sputum macrophages. There was no positive staining for IL-18 in sputum neutrophils.

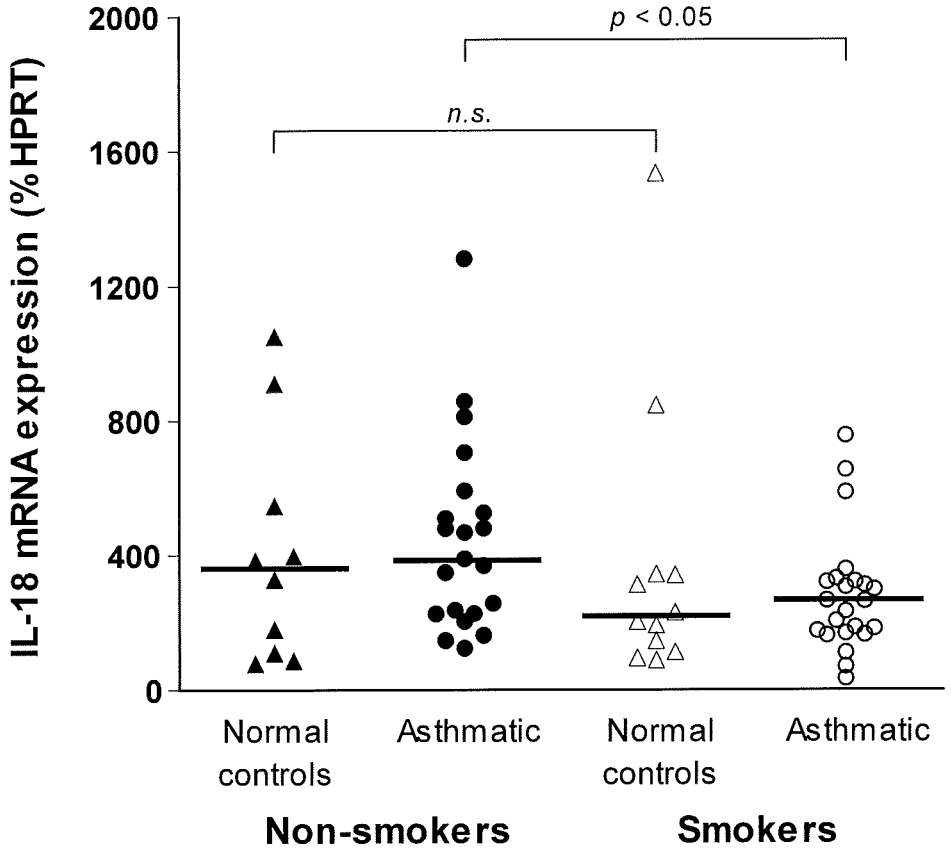


Figure 3.3. IL-18 mRNA expression in induced sputum cells.

IL-18 mRNA expression in the cell pellets of induced sputum samples from normal and asthmatic subjects, comparing non-smokers and smokers. Smoking asthmatics, but not normal smokers, had significantly lower levels of IL-18 expression than the corresponding non-smoking group. Horizontal bars indicated the median value of each group. *n.s.* – non-significant. Statistical significance was assessed by Mann-Whitney *U* test.

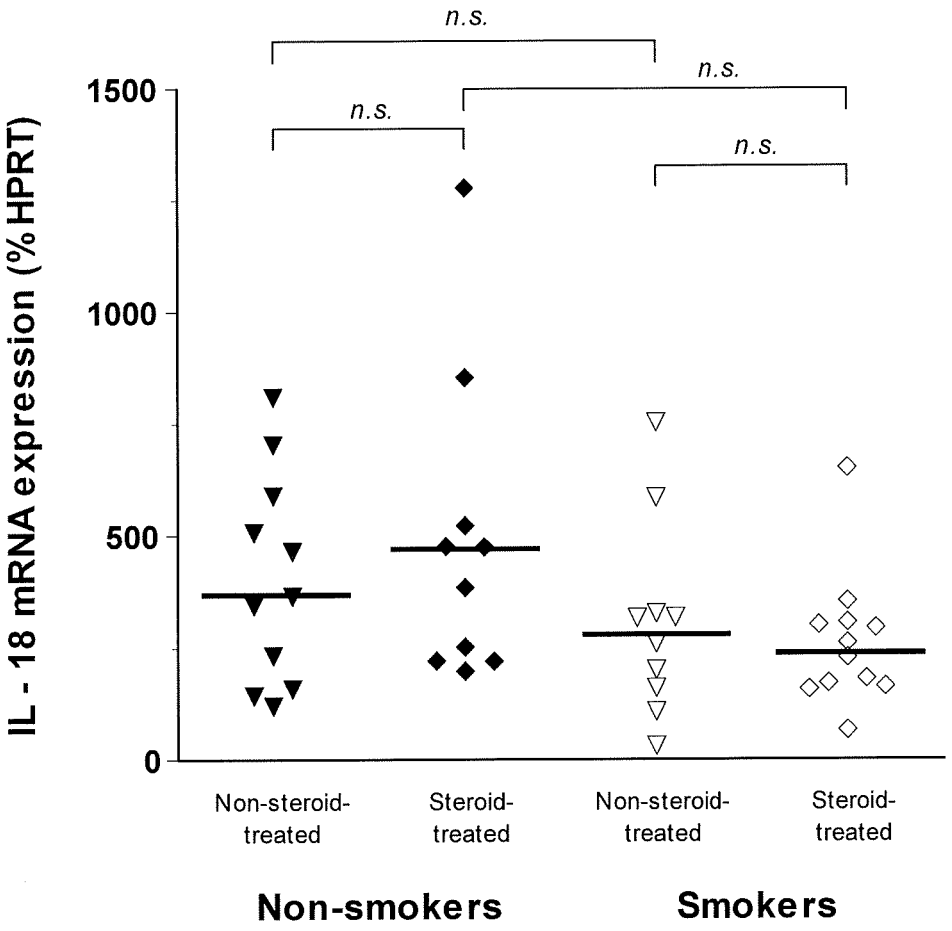


Figure 3.4. Comparison of IL-18 mRNA levels in induced sputum cells between asthmatics treated with or without inhaled corticosteroid.

IL-18 mRNA in induced sputum cells from asthmatic subjects were assessed by quantitative PCR. In both the non-smoking and smoking groups there was no difference in IL-18 mRNA levels between asthmatics treated without inhaled corticosteroid (non-steroid treated; n = 11 non-smokers, n = 10 smokers) and those treated with inhaled corticosteroid (steroid treated; n = 10 non-smokers, n = 12 smokers). In smoking asthmatics treated with or without inhaled corticosteroid, there was no significant reduction in sputum cells IL-18 mRNA levels compared to the corresponding non-smoking group. Horizontal bars indicated the median value of each group. Statistical significance was assessed by Mann-Whitney U test.

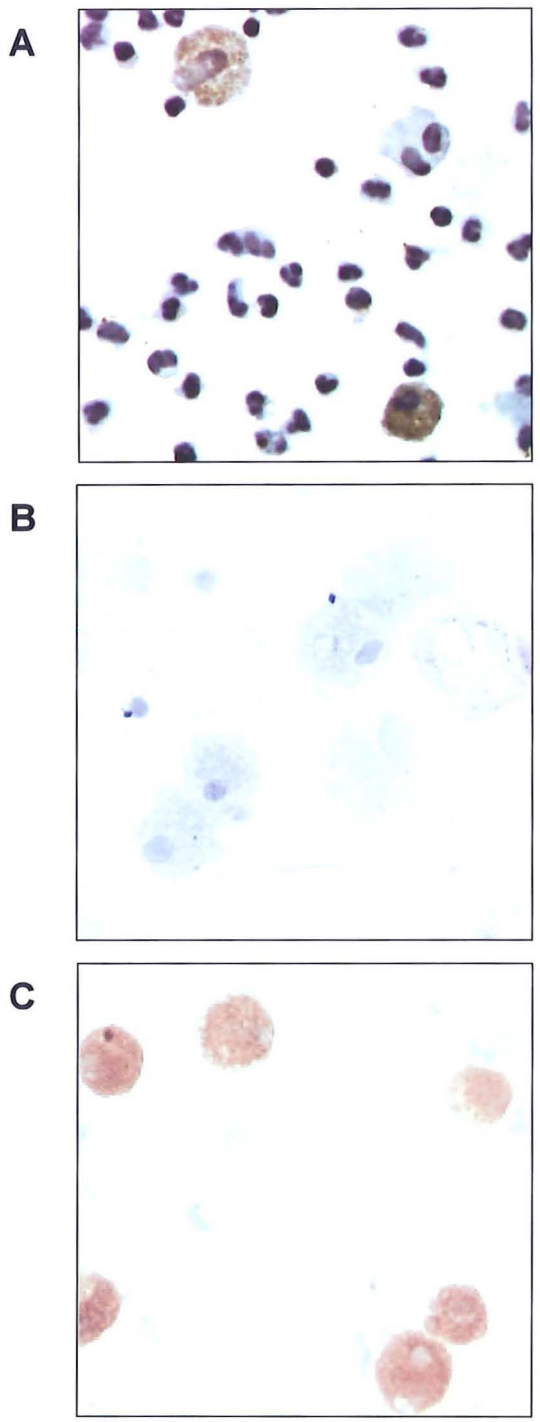


Figure 3.5. Immunocytochemistry of IL-18 in sputum cells.

Cells from an asthmatic subject were stained with **(A)** anti-human IL-18, **(B)** normal murine IgG1 control or **(C)** anti-human CD68. Positive staining was observed to be brown. Staining was observed only in sputum macrophages. Images are shown at magnification x 400.

3.8 Chapter discussion

In the above study it is demonstrated that IL-18 is detectable in the induced sputum fluid of both asthmatic and normal subjects. IL-18 levels do not significantly differ between asthmatic and normal subjects, but IL-18 levels were significantly reduced by cigarette smoking in both these groups. In asthmatics, but not normal subjects, the reduction in IL-18 levels in sputum fluid was associated with reduced IL-18 mRNA expression in induced sputum cells. IL-18 production was localised to sputum macrophages.

IL-18 is a cytokine which has the ability to promote both Th1 and Th2 responses depending on the surrounding cytokine environment (Xu et al., 2000, Nakanishi et al., 2001). There is increasing evidence that IL-18 may have a pro-inflammatory action in allergic disease. In murine models of asthma, IL-18 administered alone can promote allergen-induced eosinophil influx into the airways (Kumano et al., 1999, Campbell et al., 2000, Wild et al., 2000) and increase serum IgE levels (Hoshino et al., 2000). In human studies, serum IL-18 levels were elevated in asthmatic patients during acute exacerbations (Tanaka et al., 2001a) in stable asthmatics (El-Mezzein et al., 2001) and in those with a related allergic condition, atopic dermatitis (El-Mezzein et al., 2001, Yoshizawa et al., 2002). Also, IL-18 levels were found to be raised in nasal secretions from patients with allergic seasonal rhinitis (Verhaeghe et al., 2002). Polymorphisms in the IL-18 gene have been associated with asthma (Higa et al., 2003a). In contrast, IL-18 had anti-allergic properties when administered with IL-12 in a murine asthma model (Hofstra et al., 1998) and IL-18 deficient mice had increased airway eosinophilia (Kodama et al., 2000).

In the current study, the first evidence that IL-18 can be detected in induced sputum fluid is presented. There was no significant difference in sputum IL-18 levels between asthmatic and normal subjects. However, in non-smokers, IL-18 levels tended to be higher in asthmatics than in normal subjects. Previous clinical studies have shown that IL-18 levels are reduced in bronchoalveolar lavage (BAL) fluid from asthmatics (Ho et al., 2002) and that IL-18 mRNA expression in the airway epithelium was decreased (Cameron et al., 1999) compared to normal controls and patients with sarcoidosis. Since sputum induction provides a sample from the larger airways than BAL (Keatings et al., 1997) and samples a different inflammatory compartment to bronchial biopsies (Rutgers et al., 2000), the results presented above may reflect such differences.

Since cigarette smoking alters airway inflammation in asthma (Chalmers et al., 2001) it was investigated whether smoking altered sputum IL-18 levels. The smoking asthmatics who participated in this study satisfied the criteria for the diagnosis of asthma (American Thoracic Society, 1987), had a median age of 38 years and been symptomatic since their twenties or earlier, and so were felt to be distinct from patients with COPD. Cigarette smoking reduced IL-18 levels in both asthmatic and normal subjects. This was more pronounced in asthmatic smokers and was associated with a decrease in IL-18 mRNA expression. The reason for this is unclear. Smoking is known to suppress the ability of alveolar macrophages to secrete pro-inflammatory cytokines, such as tumour necrosis factor alpha, in response to inflammatory stimuli (McCrea et al., 1994) and has also been shown to reduce the number of cells spontaneously secreting Th1 cytokines in the airways (Hagiwara et al., 2001). A similar suppressive mechanism may be acting on sputum cell IL-18 production. Also, IL-18 is a cytokine highly dependent on proteolytic processing for its activity and extracellular secretion. It is produced initially as an inactive precursor, pro-IL-18, which is cleaved by IL-1β converting enzyme (ICE or caspase-1) into the active protein (Ghayur et al., 1997).

Other proteases, such as neutrophil proteinase-3 (Sugawara et al., 2001) can also be involved in the production of active IL-18 whereas caspase-3 (Akita et al., 1997) can cleave active IL-18 into inactive fragments. Cigarette smoking promotes neutrophil influx into the airways (Chalmers et al., 2001) and increases protease activity in the airways (Vignola et al., 1998). This may contribute to the degradation of sputum IL-18 in smokers, especially in asthmatics.

IL-18 mRNA is highly expressed in cells from induced sputum samples. IL-18 mRNA levels are significantly lower in asthmatic smokers than in asthmatic non-smokers, and a reduction in IL-18 gene expression may therefore contribute to suppression of IL-18 production in smokers. The control of IL-18 gene expression is less well understood. IL-18 mRNA is highly stable and this may explain the high levels detected in sputum cells. The gene is constitutively expressed in human peripheral blood mononuclear cells (Puren et al., 1999) but its mRNA expression can be up-regulated by cytokines such as IFN-γ (Tone et al., 1997). Cigarette smoke may contain compounds with the ability to alter IL-18 gene expression. As there was no significant change in IL-18 mRNA expression in normal smokers it is likely that there is some alteration of both IL-18 mRNA expression and protein processing causing the decrease in sputum IL-18 levels observed.

There was no significant difference in sputum IL-18 levels or sputum IL-18 mRNA expression between asthmatics treated with inhaled β_2-agonist alone and those treated with inhaled β_2-agonist and inhaled corticosteroid in either the smoking or non-smoking groups. In vitro, treatment of PBMC and monocytic cell lines with the steroid prednisolone has been shown to increase the levels of IL-18 mRNA expression and protein release (Moller et al., 2002b). Steroid treatment is known to affect the levels of

several airway cytokines, including other macrophage-derived cytokines, such as IL-12 (Naseer et al., 1997) and IL-15 (Komai-Koma et al., 2001). Since the present study was a cross-sectional study, further longitudinal studies would be necessary to assess fully if steroid therapy could alter IL-18 production in the airways.

Immunocytochemistry localised IL-18 production to sputum macrophages. In association with the IL-18 levels found in induced sputum fluid and the high levels of IL-18 mRNA expression in sputum cells, this suggests that IL-18 is produced directly by the cells in the sputum samples. However, other cells such as the bronchial epithelium, may also contribute to the IL-18 detected by ELISA in induced sputum fluid.

In conclusion, IL-18 can be detected in induced sputum fluid and IL-18 gene expression is also detectable in cells from induced sputum samples. There is no significant difference between sputum IL-18 levels in asthmatic and normal subjects but cigarette smoking significantly reduces levels of IL-18 in both these groups. This reduction may be mediated by alterations in the post-translational processing of IL-18 but direct suppression of IL-18 gene expression could also be involved. IL-18 is a pro-inflammatory cytokine which has the ability to induce Th1 and Th2 cytokine secretion, and as such, may have a pivotal role in the development of airway inflammation in asthma. The reduction in sputum IL-18 levels associated with cigarette smoking may alter of the balance of Th1/Th2 cytokines secretion and contribute to increased airway inflammation. Further studies are necessary to clarify the mechanisms by which sputum IL-18 levels are reduced by cigarette smoking and to determine the precise effect of this reduction on Th1/Th2 cytokine secretion.

Chapter 4

The effect of IL-18 deficiency in a murine model of allergic asthma

4.1 Introduction

In the previous chapter it was reported that IL-18 levels were reduced in the induced sputum fluid of asthmatic and normal smokers. In non-smokers, there was a trend for the levels of IL-18 in induced sputum fluid to be higher in asthmatic subjects. To investigate further the possible role of IL-18 in allergic airway inflammation, a murine model of allergic asthma was used.

Consistent with the ability of IL-18 to induce either Th1 or Th2 lymphocyte responses (Xu et al., 2000), there have been conflicting reports on the actions of IL-18 in experimental asthma models. In association with IL-12, IL-18 reduced antigen-induced airway hyper-responsiveness and eosinophilia in a murine model of allergic asthma (Hofstra et al., 1998). A similar reduction in the airway inflammatory response was observed when IL-18 gene was expressed in the airways using an adenoviral vector (Walter et al., 2001) or when exogeneous IL-18 was administered with sensitisation in an RSV-induced murine asthma model (Zhang et al., 2003). An anti-inflammatory role for IL-18 in these animal models of asthma was supported by the observation that IL-18 deficient C57BL/6 mice had more severe airway inflammation after antigen sensitisation and challenge than wild-type mice (Kodama et al., 2000). In addition, the use of a neutralising antibody to IL-18, resulted in an increase in airway inflammation in a murine model of fungal-induced asthma (Blease et al., 2001). However, IL-18 can increase IL-4 and IL-5 production and airway eosinophilia if given at the time of allergic sensitisation (Wild et al., 2000), and can enhance eosinophil influx to the airways (Kumano et al., 1999, Campbell et al., 2000). When co-administered with antigen challenge, IL-18 has also been demonstrated to induce airway inflammation and AHR in naïve mice which have previously received antigen-specific memory Th1 cells (Sugimoto et al., 2004).

C57BL/6 mice deficient in IL-18 have been shown to have increased airway inflammation in response to antigen (Kodama et al., 2000). Such C57BL/6 mice have a genetic bias to produce Th1 responses. The absence of IL-18 produced a decrease in IFN-γ production, and subsequent failure to suppress the Th2 response. *In vitro*, IL-18 can stimulate the development of Th2 cells and production of IgE (Yoshimoto et al., 2000), and IL-4 and IL-13 secretion from basophils (Yoshimoto et al., 1999), NK cells and naïve T lymphocytes (Hoshino et al., 1999) and NKT cells (Yoshimoto et al., 2003). IL-18 has also been shown enhance antigen-induced production of IL-9, IL-13 and GM-CSF from Th1 cells *in vitro* (Sugimoto et al., 2004).

It was postulated that the ability of IL-18 to promote Th2 responses would be more apparent in a strain of mice in which had a tendency to produce Th2 cytokines. In murine models of *Leishmania major* infection, disease-susceptible BALB/c mice have been shown to have an intrinsic predisposition to produce higher levels of IL-4 and predominant Th2 responses when compared to resistant C57BL/6 mice (Fowell et al., 1998). Therefore, IL-18 gene knockout (ko) mice on a BALB/c background were used in a murine model of asthma in the following studies to assess further the role of IL-18 in allergic airway inflammation.

4.2 Bronchoalveolar lavage cellularity in IL-18 knockout mice

To assess the role of IL-18 in the pathogenesis and development of the inflammatory response in a murine asthma model, BALB/c wild type (WT) mice and BALB/c IL-18 gene knockout (ko) mice were sensitised and then challenged with ovalbumin (OVA). The protocol for OVA sensitisation and challenge is shown in Figure 2.1. This asthma model induced a significant increase in cell numbers and altered the cell profile in bronchoalveolar lavage fluid after antigen challenge. Eosinophils were the predominant cell type observed, but there was also an increase in the influx of other inflammatory cells, such as macrophages, lymphocytes and neutrophils. Histologically, perivascular and peribronchial inflammatory infiltrates, containing eosinophils, with associated mucosal hyperplasia in the bronchial walls were observed in lung tissue sections from mice previously sensitised and challenged with OVA.

4.2.1 Total bronchoalveolar lavage cellularity.

Mice sensitised and challenged with OVA had increased total BAL cellularity when compared to PBS sensitised and treated controls (Figure 4.1). However, total BAL cellularity was significantly reduced in OVA-treated IL-18 ko mice when compared to OVA-treated WT mice ($p < 0.05$).

4.2.2 Bronchoalveolar lavage differential cell count.

Differential cell counts were performed on the BAL fluid samples to determine which cell types in lavage fluid were reduced in OVA-treated IL-18 ko mice. In OVA-treated IL-18 ko mice there was a significant reduction in the numbers of eosinophils present

when compared to OVA-treated WT mice ($p < 0.05$). There was no significant difference in the numbers of macrophages, lymphocytes and neutrophils between these two groups, as shown in Figure 4.2.

4.3 Histological analysis of lung inflammatory infiltrates

Histological analysis of lung tissue was done to assess if the reduction in BAL cellularity and eosinophilia was accompanied by a decrease in total lung inflammation. Representative tissue sections from each of the groups are shown in Figure 4.3. Sections from PBS treated control WT mice (Figure 4.3*A*) and IL-18 ko mice (Figure 4.3*B*) show no evidence of airway inflammation. Both OVA-sensitised and challenged WT mice (Figure 4.3*C*) and IL-18 ko mice (Figure 4.3*D*) have peribronchial and perivascular inflammatory infiltrates containing eosinophils with associated mucosal hyperplasia in the bronchial walls. However, on semi-quantitative scoring of the histological slides (Figure 4.4) there was no significant difference in the severity of the inflammatory infiltrates between WT and IL-18 ko mice.

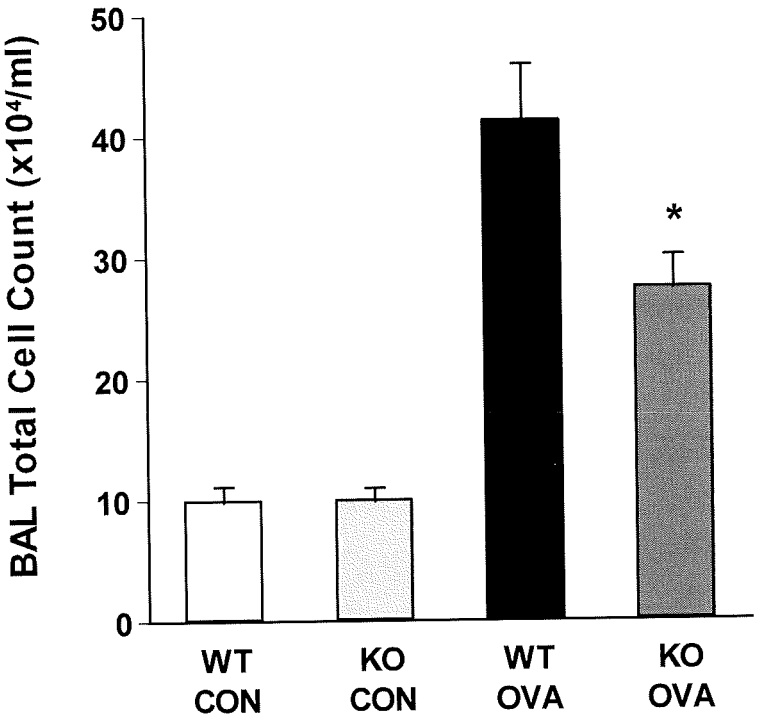


Figure 4.1. Total bronchoalveolar lavage cellularity.

Wild type (WT) and IL-18 knockout (KO) BALB/c mice were sensitised with OVA and then challenged with OVA i.n. on three consecutive days from day 25-27. Control (CON) mice were sensitised and challenged with PBS. BAL cell counts were performed on day 28. In OVA treated mice, total BAL cellularity was significantly reduced in the IL-18 KO group when compared to WT mice. Data are expressed as mean ± SEM. (n = 11 – 16 mice per group). * $p < 0.05$. Statistical significance was assessed by ANOVA.

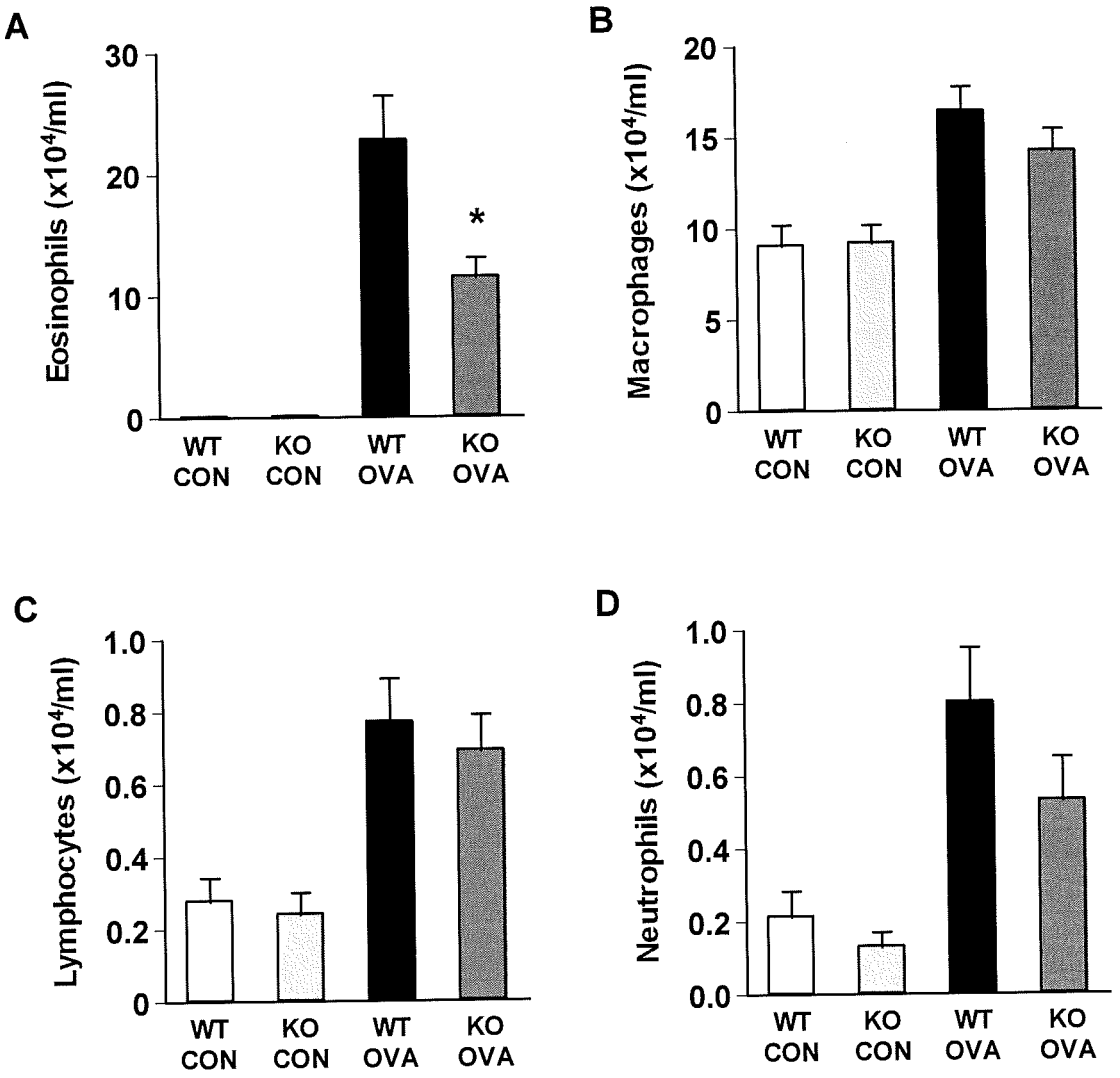
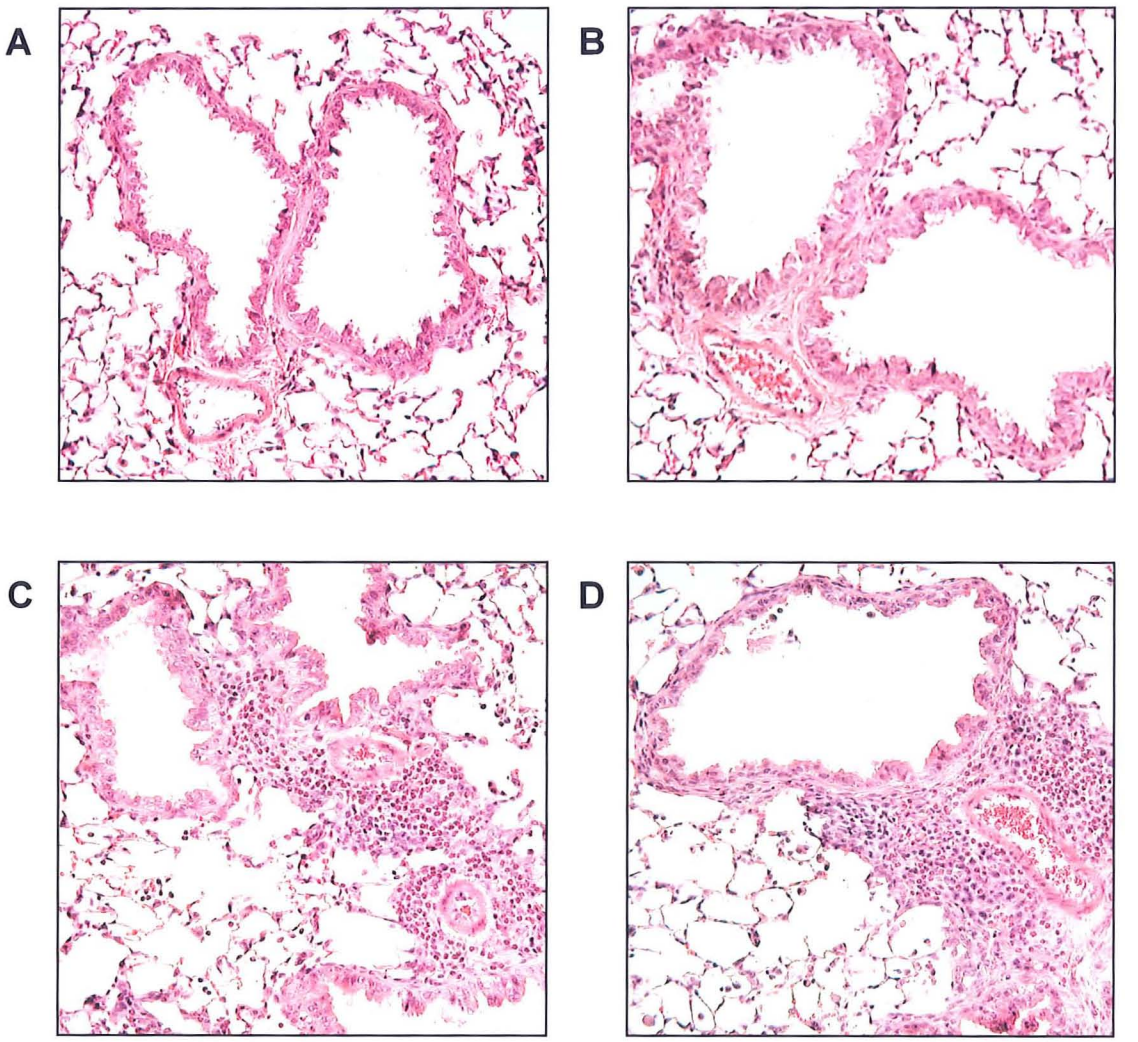


Figure 4.2. Bronchoalveolar lavage differential cell count.

Differential cell counts were determined on the BAL cell counts performed on day 28. In OVA-treated mice, BAL (**A**) eosinophils were significantly reduced in IL-18 knockout (KO) mice when compared to wild-type (WT) mice. **(B)** Macrophage, **(C)** lymphocyte and **(D)** neutrophil numbers were not significantly different between OVA-treated IL-18 knockout and WT mice. Data are expressed as mean ± SEM. (n = 11 – 16 mice per group). * $p < 0.05$. Statistical significance was assessed by ANOVA.



4.3. Histological evidence of lung inflammation in OVA-treated mice.

A representative section is shown from each group of mice. **(A)** Control wild type (WT) and **(B)** control IL-18 knockout mouse given PBS challenges. **(C)** OVA-challenged WT mouse and **(D)** OVA-challenged IL-18 knockout mouse; peribronchial and perivascular infiltrates are seen, with eosinophils present, and mucosal hyperplasia. There was no difference in inflammatory cell infiltrates between OVA challenged WT and IL-18 ko mice. H&E staining, magnification x200.

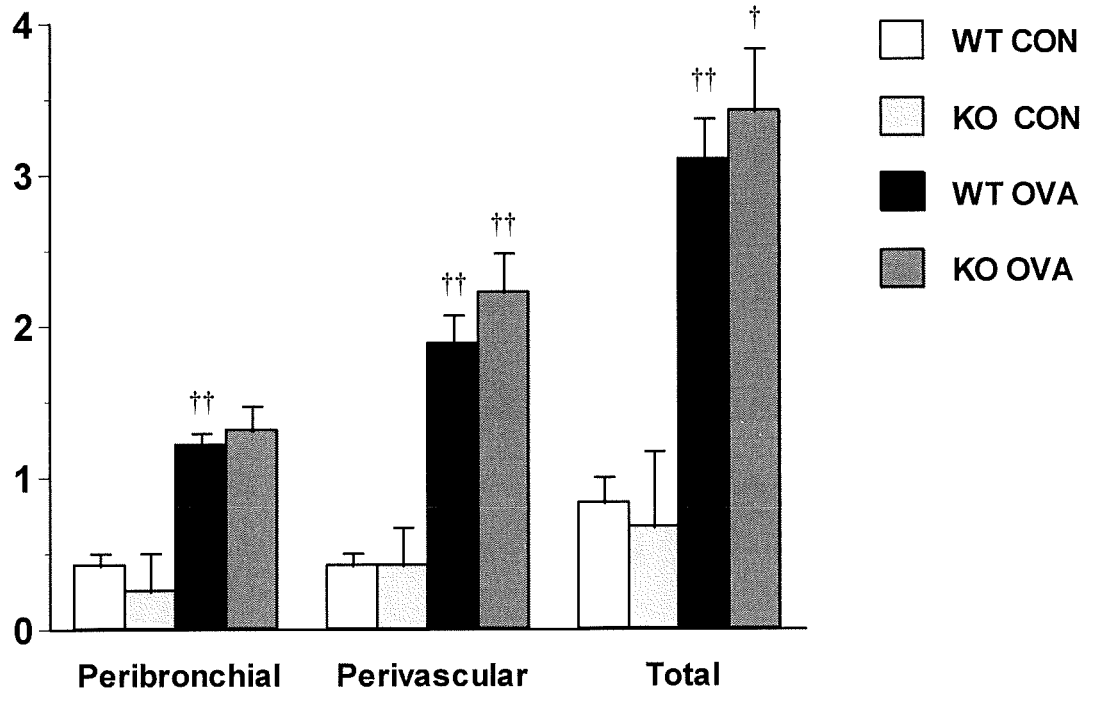


Figure 4.4. Histological score of lung inflammation.

Total lung inflammation on histological sections was determined as the sum of peribronchial and perivascular scores. Inflammation was graded on the scale 0, none; 1, mild, 2, moderate; 3, marked and 4, severe. An increment of 0.5 was used when severity of inflammation fell between two integers. OVA-treated wild-type (WT) and IL-18 knockout (ko) mice had significantly increased perivascular and total lung inflammation compared to the corresponding PBS-treated controls. OVA-treated WT mice also had significantly increased peribronchial inflammation, compared to PBS-treated mice. There was no significant difference in inflammatory scores between OVA-treated WT and IL-18 ko mice. (n = 3-10 mice per group). †, $p < 0.05$, ††, $p < 0.01$ (when compared to corresponding PBS-treated controls). Significance was assessed by Student's *t* test.

4.4 Bronchoalveolar lavage cytokines

Since prior OVA sensitisation and challenge produced an eosinophilia in the BAL fluid, we wished to assess if this was associated with an increase in the levels of cytokines in the lavage fluid, and whether the levels of these cytokines differed between IL-18 ko mice and WT mice. In OVA-treated WT mice the levels of IL-4 in BAL fluid were significantly elevated when compared to PBS-treated WT controls ($p < 0.05$) (Figure 4.5). This was not seen in IL-18 ko mice. When comparing OVA-treated mice, although there was a trend for IL-4 levels in BAL fluid to be lower in IL-18 ko mice, but this did not reach statistical significance ($p = 0.08$). There was no difference in IL-5 levels in BAL fluid between the OVA-treated groups or between the OVA-treated groups and the PBS control groups, Figure 4.5*B*.

Since IL-18 is known to induce eotaxin production in the airways (Campbell et al, 2000), levels of this chemokine were measured in BAL fluid. Only low levels of eotaxin were detected in BAL fluid but WT mice sensitised and challenged with OVA had a small but significant rise in eotaxin levels in BAL fluid when compared to the PBS-treated WT mice (Figure 4.5*C*). There was no significant difference between eotaxin levels in OVA-treated WT and ko mice.

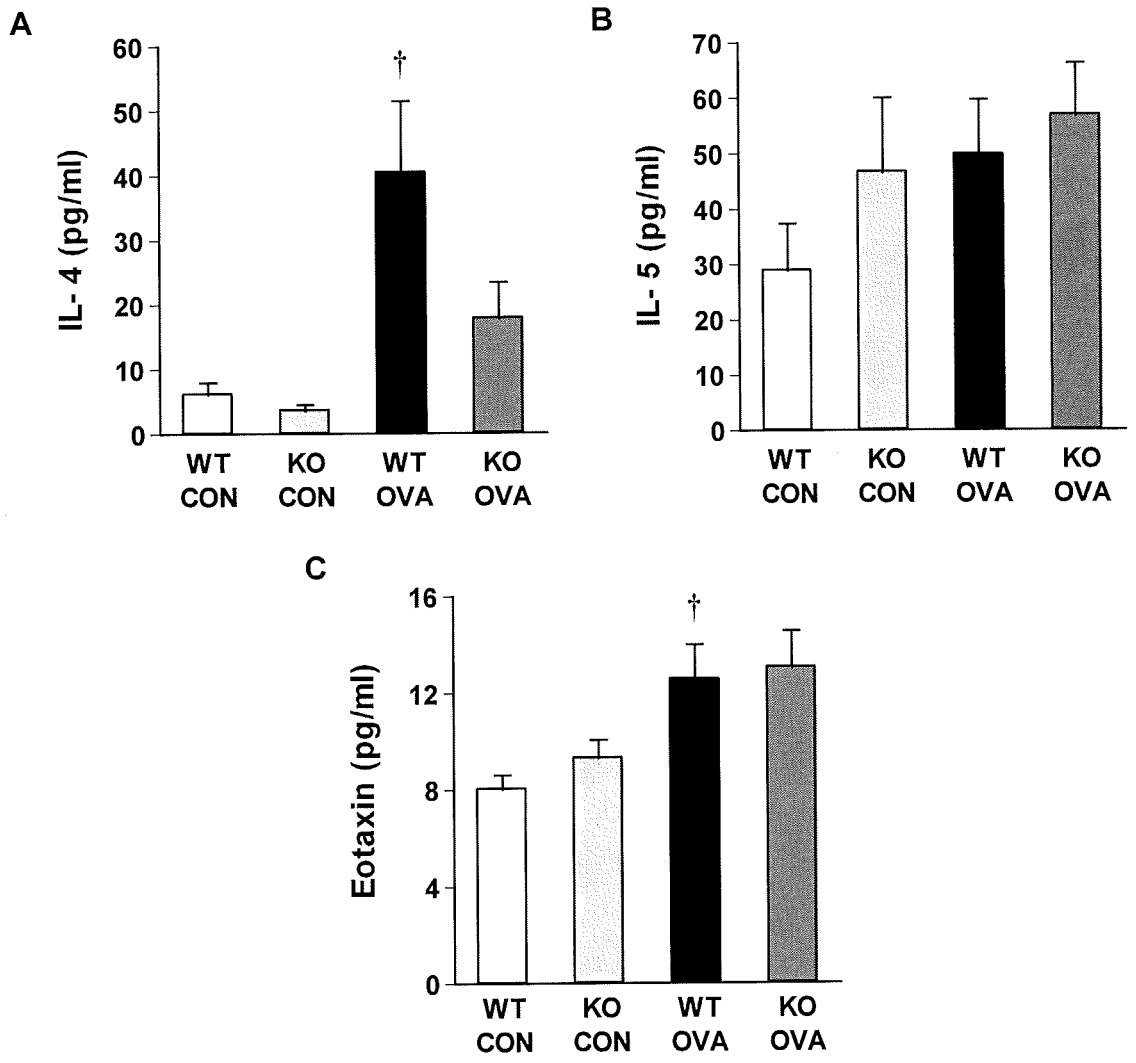


Figure 4.5. Bronchoavelolar lavage cytokine levels.

Bronchoalveolar lavage IL-4, IL-5 and eotaxin levels were determined by ELISA. **(A)** IL-4 levels and **(C)** eotaxin levels were significantly higher in OVA-treated wild type (WT) mice when compared to PBS-treated WT controls but there was no difference in **(B)** IL-5 levels. There was no significant difference in the levels of all cytokines between OVA-treated WT mice and OVA-treated IL-18 knockout (KO) mice. Data is expressed as mean ± SEM. (n = 11 - 16 mice per group). †, $p < 0.05$ (when compared to corresponding PBS treated control). Statistical significance was assessed by ANOVA.

4.5 Serum immunoglobulins

Figure 4.6 shows the serum immunoglobulin levels from control and OVA-treated WT and IL-18 ko mice. OVA sensitisation and challenge increased total serum IgE and OVA-specific IgE levels in both WT and IL-18 ko mice, with no significant difference in the amount of these immunoglobulins found between WT and IL-18 ko mice. Similarly, levels of OVA-specific IgG1 and IgG2a did not differ between these two groups.

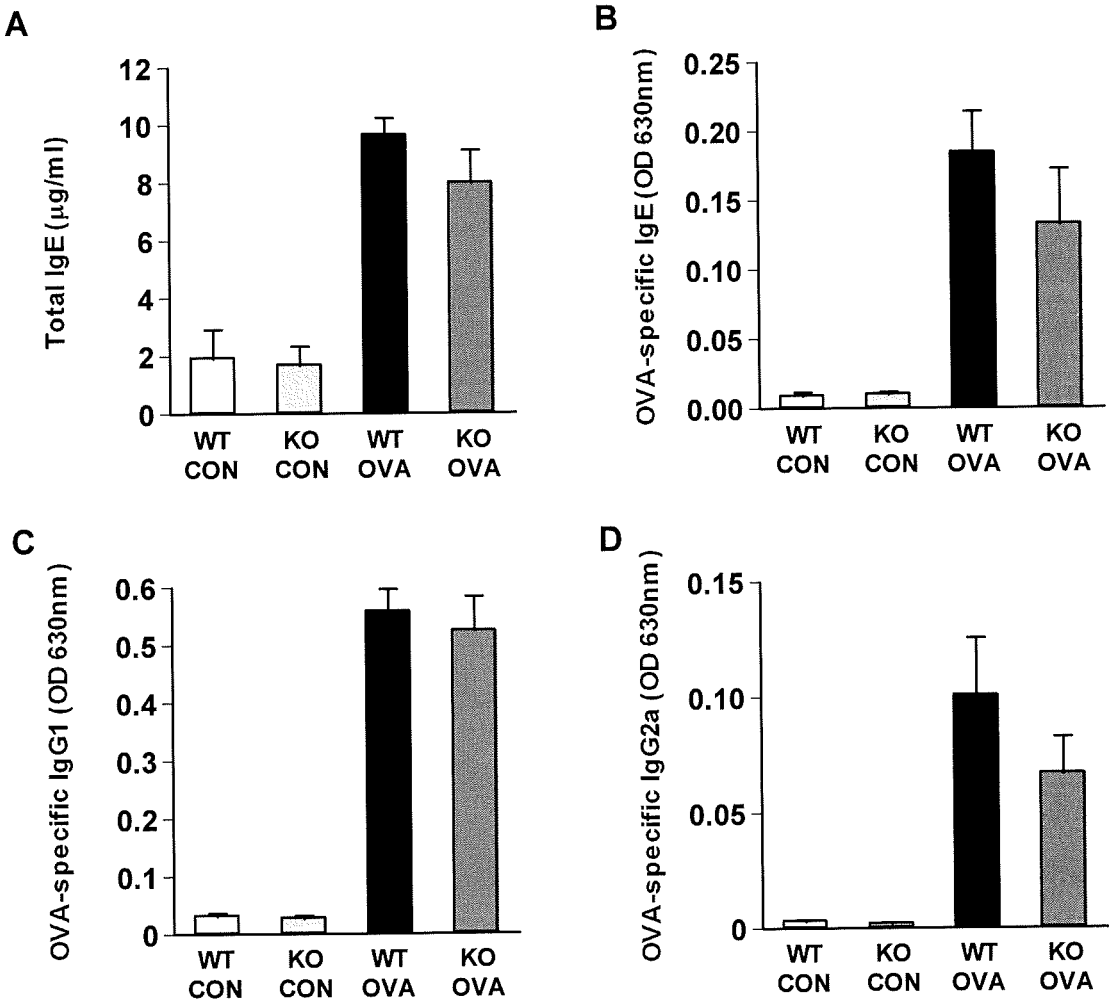


Figure 4.6. Serum immunoglobulin levels.

Serum immunoglobulin levels were assessed by ELISA. **(A)** Serum total IgE levels, **(B)** serum OVA-specific IgE (serum dilution 1/160), **(C)** serum OVA-specific IgG1 (serum dilution 1/60,000) and **(D)** serum OVA-specific IgG2a (serum dilution 1/800) levels were not significantly different between OVA-treated wild type (WT) mice and OVA-treated IL-18 knockout (KO) mice. (n = 7 - 16 mice per group). Data are expressed as mean ± SEM. Statistical significance was assessed by Student's *t* test.

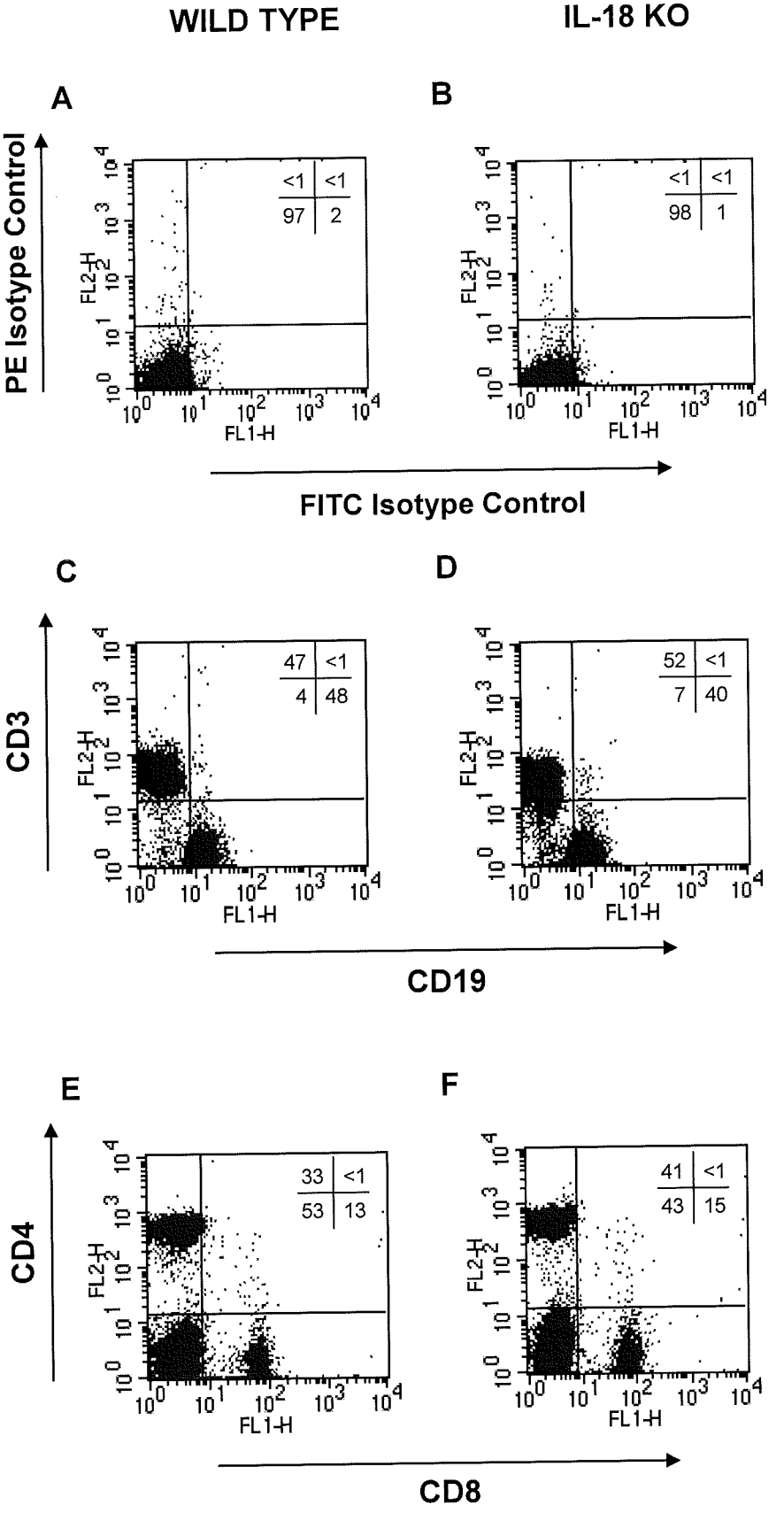
4.6 Flow cytometric analysis of thoracic lymph node cells

To determine if there was any difference in lymphocyte populations during the inflammatory response in IL-18 ko mice, cells from thoracic lymph nodes were isolated from OVA treated WT and IL-18 ko mice on day 28 of the experimental protocol for analysis by flow cytometry (Figure 4.7).

The lymph node cells from IL-18 ko mice had similar proportions of $CD19^+$ B cells and $CD3^+$ T lymphocytes to lymph node cells from WT mice (Figure 4.7*C* and *D*). Of the total lymph node cells, although the IL-18 ko group had a higher proportion of $CD4^+$ lymphocytes than WT mice (Figure 4.7*E* and *F*), the ratio of $CD4^+$:$CD8^+$ cells was similar between the IL-18 ko and WT mice ($CD4^+$:$CD8^+$ ratio – WT 2.5; IL-18 ko 2.7). In addition, the $CD4^+$ from both WT and IL-18 ko mice did not appear to differ in their activation status since levels of CD44, CD11a, CD45RB and CD62L were comparable between the two groups, as illustrated in Figure 4.8.

Figure 4.7. Analysis by FACS of purified thoracic lymph node cells.

Cells were isolated from pooled thoracic lymph nodes (n = 5 mice per group) obtained from WT and IL-18 knockout (ko) mice sensitised and challenged with OVA on day 28 of the experimental protocol. Flow cytometric analysis for lymphocyte cell surface markers was performed. The percentage of cells in each quadrant is displayed at the top right of each plot. **(A)** and **(B)** show the isotype controls WT and IL-18 ko groups, respectively. **(C)** Cells from WT mice have a marginally higher proportion of $CD19^+$ cells, but similar proportions of $CD3^+$ cells compared to **(D)** IL-18 ko mice. **(E)** A marginally lower proportion of $CD4^+$ lymphocytes are present in the lymph nodes of WT mice than **(F)** IL-18 ko mice, but $CD8^+$ levels are similar. Results are shown from single experiment.


WILD TYPE
IL-18 KO
A
B
PE Isotype Control
FITC Isotype Control
<1 | <1
97 | 2
<1 | <1
98 | 1
C
D
CD3
CD19
47 | <1
4 | 48
52 | <1
7 | 40
E
F
CD4
CD8
33 | <1
53 | 13
41 | <1
43 | 15
FL1-H
FL2-H

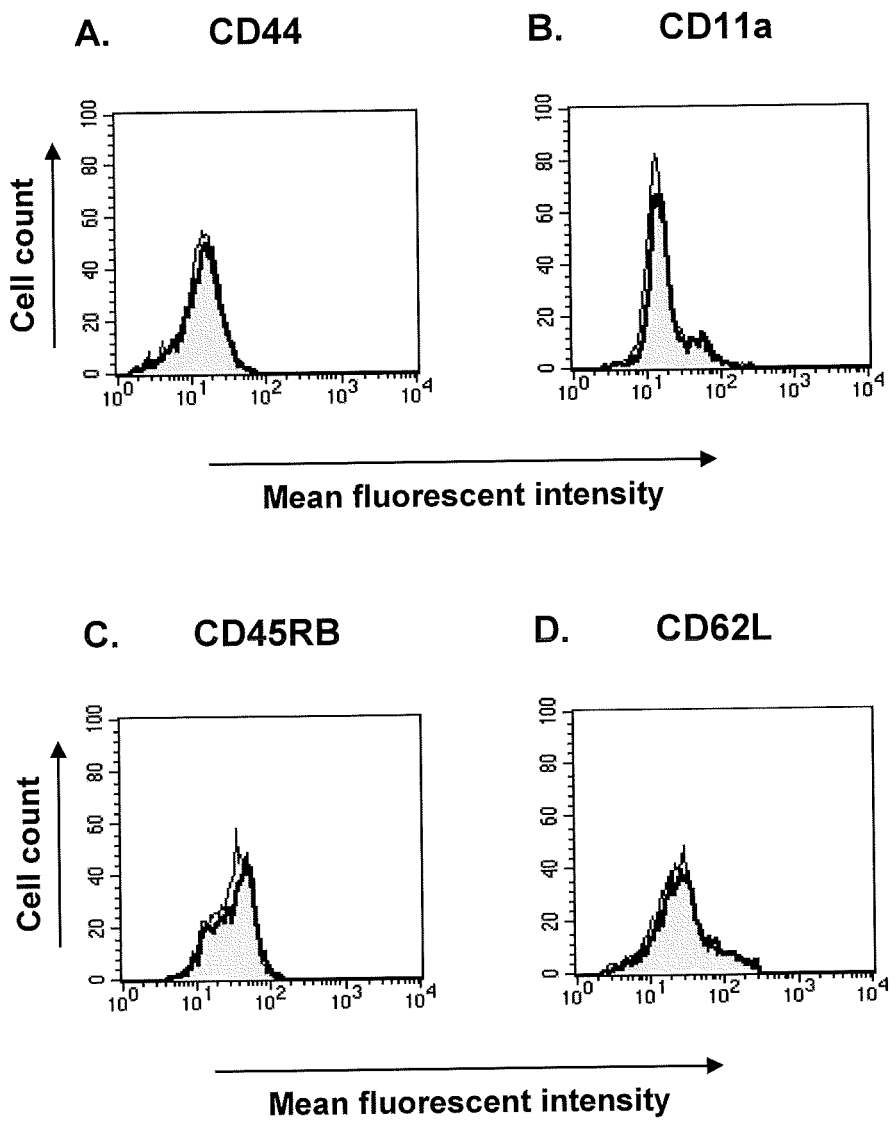


Figure 4.8. Cytometric analysis of cell surface activation markers on $CD4^+$ cells.

Cells were isolated from pooled thoracic lymph nodes (n = 5 mice per group) obtained from WT and IL-18 knockout (ko) mice sensitised and challenged with OVA on day 28 of the experimental protocol. Flow cytometric analysis for lymphocyte cell surface activation markers was performed. The analyses of cells from WT mice are shown by the grey shaded areas, and those from IL-18 ko mice by the thick black line. No difference in the cell surface levels of **(A)** CD44, **(B)** CD11a, **(C)** CD45RB and **(D)** CD62L was seen between the two groups of mice. Results are shown from a single experiment.

4.7 *In vitro* thoracic lymph node cell responses in wild-type and IL-18 knockout mice

The reduction observed in IL-4 levels in BAL fluid suggested that there might be suppression of Th2 responses in IL-18 knockout mice. To investigate this further, cells isolated from thoracic lymph nodes of both WT mice and IL-18 ko mice were cultured and *in vitro* OVA-induced cell proliferation and cytokine production assessed.

4.7.1 *In vitro* OVA-induced cell proliferation in thoracic lymph node cells

The OVA-induced cell proliferation responses in thoracic lymph node cultures are illustrated in Figure 4.9*A*. There was no significant difference in OVA-induced cell proliferation between WT mice and IL-18 ko mice which had previously been sensitised and challenged with OVA. In the experiment shown, spontaneous cell proliferation was significantly higher in IL-18 ko mice ($p < 0.05$). The proliferative response to Concanavalin (Con) A was not significantly different between these groups of mice (Figure 4.10*A*).

4.7.2 *In vitro* OVA-induced cytokine production in thoracic lymph node cells

The OVA-induced cytokine responses, from parallel cultures to those described in Section 4.4.1, are shown in Figures 4.9*B* to *D*. The levels of IL-4 (Figure 4.9*B*), and IFN-γ (Figure 4.9*D*) detected in culture supernatants of lymph node cells from OVA-sensitised and challenged IL-18 ko mice were reduced when compared cells from similarly treated WT mice (IL-4 $p < 0.01$; IFN-γ ($p < 0.05$). The levels of IL-5 (Figure 4.9*D*) were not significantly different between these two groups. The reduction in IL-4

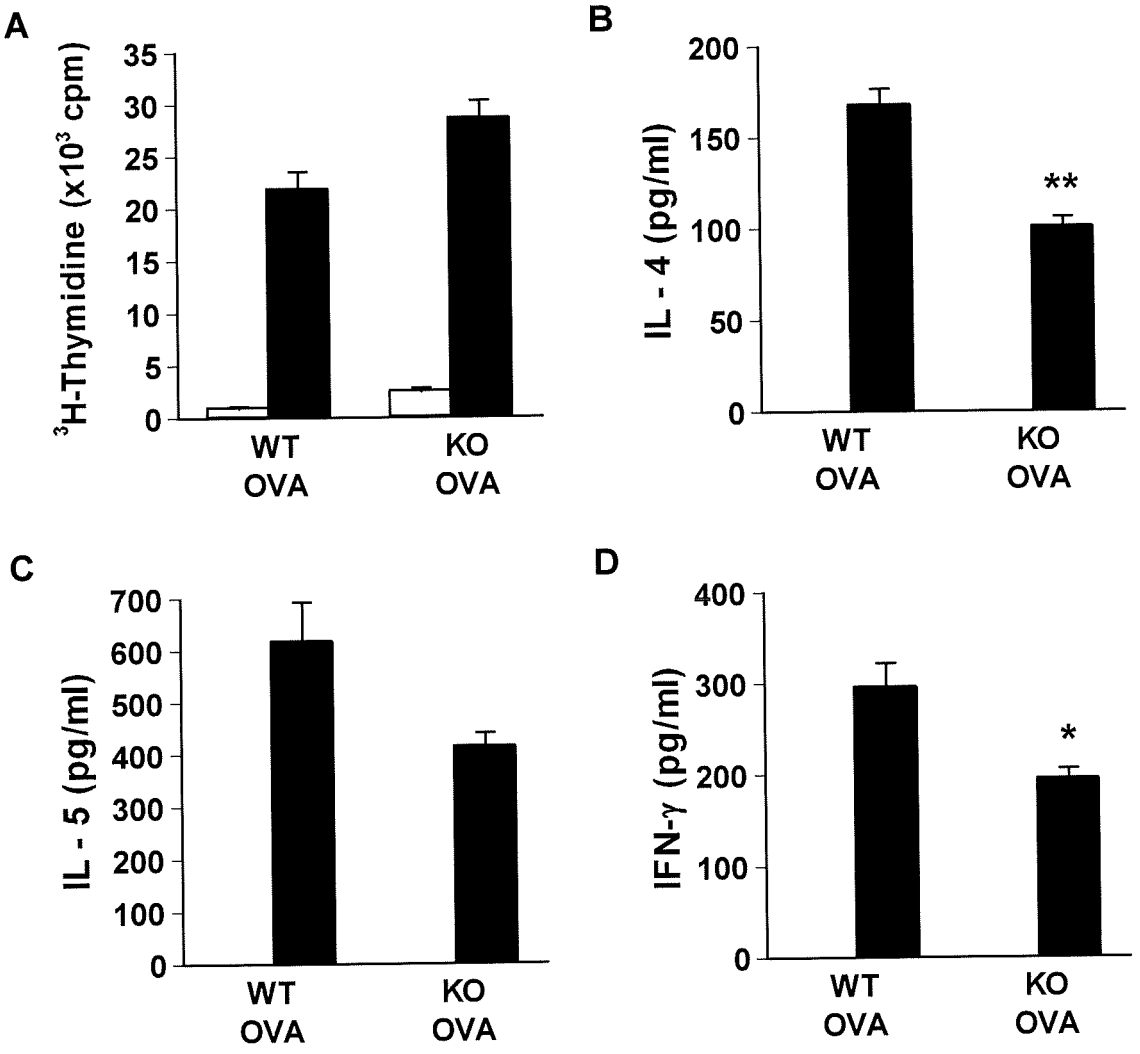


Figure 4.9. Proliferative and cytokine responses in thoracic lymph node cells *in vitro*.

Thoracic lymph node cells (n = 3 mice per group) were harvested from mice on day 28 and cultured with medium alone (□) or OVA (■; 1 mg/ml). **(A)** T cell proliferation was assessed by uptake of [^{3}H]-thymidine after 96 hours of culture; there was no significant difference in cell proliferation between IL-18 knockout (KO) and wild-type (WT) mice. Cytokine levels in cell culture supernatants were assessed by ELISA after 72 hours of culture. **(B)** IL-4 and **(D)** IFN-γ levels were significantly reduced in lymph node cells from IL-18 KO mice compared to WT mice. **(C)** IL-5 levels were not significantly different. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$. Statistical significance was assessed by Student's *t* test.

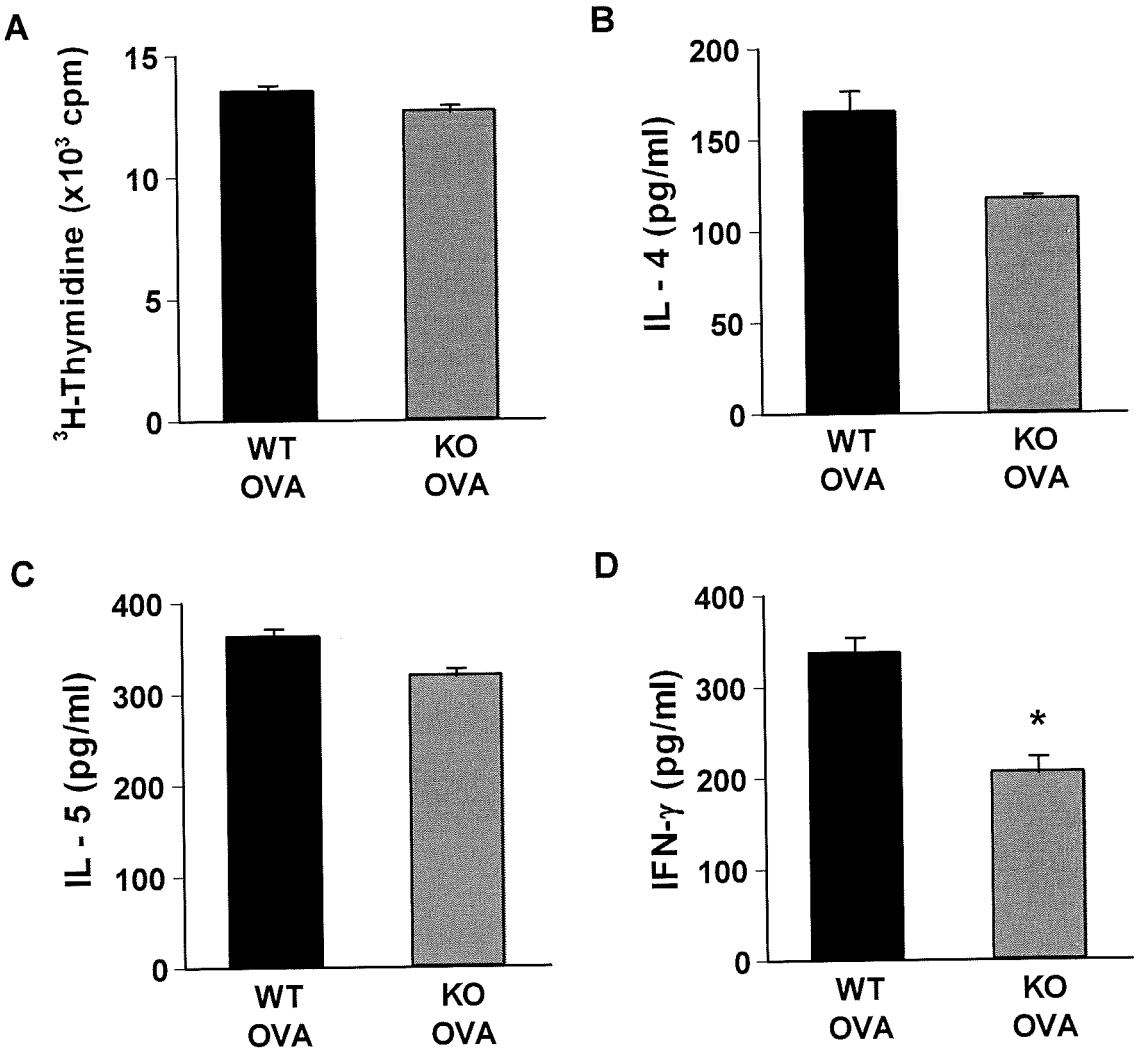


Figure 4.10. Proliferative and cytokine responses in thoracic lymph node cells *in vitro*.

In parallel cultures to those in 4.6 (a), thoracic lymph node cells from (n = 3 mice per group) cultured with 5 μg/ml Concanavalin (Con) A. **(A)** T cell proliferation was assessed by uptake of [^{3}H]-thymidine after 96 hours of culture; there was no significant difference in cell proliferation between IL-18 knockout (KO) and wild-type (WT) mice. Cytokine levels in cell culture supernatants were assessed by ELISA after 72 hours of culture. **(D)** IFN-γ production in response to Con A was significantly reduced in lymph node cells from IL-18 KO mice. There was no significant difference in **(B)** IL-4 and **(C)** IL-5 production. Data are expressed as mean ± SEM. * $p < 0.05$. Statistical significance was assessed by Student's *t* test.

production was antigen-specific, since the amount of IL-4 induced by Con A (Figure 4.10B) was not lower in lymph node cells from IL-18 ko mice. This was not the case for IFN-γ, since the levels of this cytokine in cultures with Con A were also decreased ($p < 0.05$). There was no difference in IL-5 production induced by Con A between these two groups of mice.

4.8 Cytokine mRNA expression in thoracic lymph nodes.

IL-4 mRNA expression in thoracic lymph nodes was determined using quantitative RT-PCR to assess if alterations in its gene expression accompanied the decreased levels of this cytokine detected by ELISA in cell culture supernatants in IL-18 deficient mice. IL-5 mRNA levels were also measured. These results are shown in Figure 4.11.

The level of IL-4 mRNA were increased in OVA-treated WT and IL-18 ko mice when compared to the PBS-treated controls. IL-4 expression was reduced in IL-18 ko mice when compared to the WT group. There was also a reduction in IL-5 mRNA levels in OVA-treated IL-18 ko mice.

The above experiments on thoracic lymph node cells are from pooled lymph nodes from single groups of mice and so statistical analysis could not be performed. Insufficient mice were available to repeat the LN studies to confirm these results and therefore further work would focus on these studies.

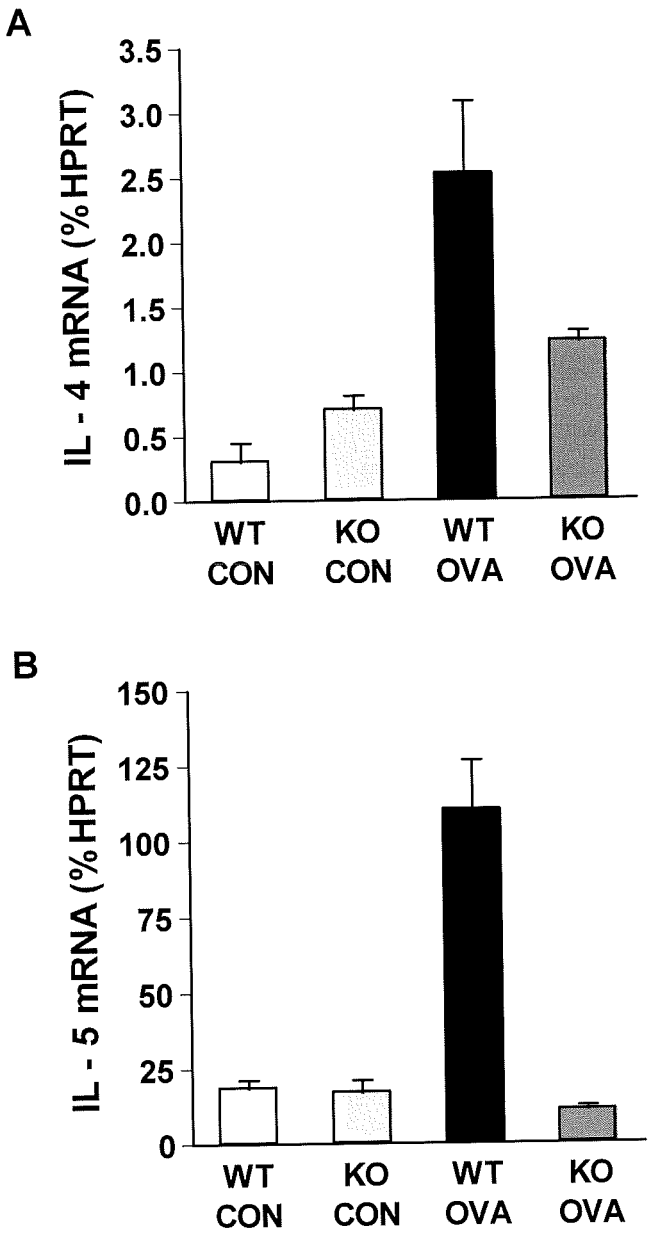


Figure 4.11. Cytokine expression in thoracic lymph node cells.

The levels of IL-4 and IL-5 mRNA expression were determined by the use of RT-quantitative PCR from lymph nodes harvested on day 28. Levels of **(A)** IL-4 and **(B)** IL-5 mRNA were reduced in OVA-treated IL-18 KO mice when compared to WT controls. (Pooled lymph nodes, n = 3-5 mice per group). Data are expressed as mean ± adjusted error.

4.9 Chapter discussion

The above investigations have demonstrated that, in this murine model of allergic asthma, BALB/c IL-18 gene deficient mice have decreased BAL total cell numbers and eosinophilia after antigen sensitisation and challenge when compared to WT mice. The reduction in BAL eosinophilia was not associated with a reduction in inflammatory infiltrates in lung histology. There was a non-significant decrease in IL-4 levels in the BAL fluid of antigen-sensitised and challenged IL-18 ko mice when compared to WT mice. Serum OVA-specific or total IgE levels, or OVA-specific IgG1 and IgG2a levels were not altered in IL-18 gene deficient mice. However, *in vitro* thoracic lymph node cells from IL-18 ko mice produced less IL-4 in response to OVA than cells from WT mice. Also, lower levels of IL-4 mRNA were expressed in thoracic lymph nodes from IL-18 ko mice. These observations suggest that IL-18 has a pro-inflammatory action in this murine model of asthma which is, at least in part, mediated by its ability to stimulate IL-4 secretion.

In murine models of inflammatory arthritis (Wei et al., 2001) and infectious disease (Wei et al., 1999), mice genetically deficient in IL-18 have a decreased ability to mount a Th1 lymphocyte responses with an associated decrease in IFN-γ production. Consistent with these properties, in a murine model of asthma, C57BL/6 IL-18 gene knockout mice had increased airway inflammation after antigen sensitisation and challenge which was proposed to be related to decreased IFN-γ production (Kodama et al., 2000). However, it is now thought that IL-18 can stimulate the development of both Th1 and Th2 responses depending on the surrounding cytokine environment (Xu et al., 2000). This theory has been supported in studies in transgenic mice over-expressing IL-18, where higher serum levels of both IFN-γ and IL-4, and also increased IgE, were observed (Hoshino et al., 2001).

In the present study, BALB/c IL-18 ko mice sensitised and challenged with OVA had reduced total BAL cellularity mainly due to reduced numbers of eosinophils when compared to identically treated WT controls, although there was no associated reduction in inflammatory infiltrates seen on lung histology. This is in contrast to the previous study where C57BL/6 mice lacking IL-18 were used (Kodama et al., 2000); in this study an increase in eosinophil numbers was observed but again, there was no alteration in the influx of other cell types into the airways during this inflammatory response.

The reduction in BAL eosinophilia, but not histological inflammation, seen after antigen challenge in IL-18 ko mice in the current study may reflect alterations in the production of cytokines, chemokines and adhesion molecules which are involved in eosinophil trafficking to the lung and into the bronchial lumen during allergic responses. The numbers of eosinophils within the inflammatory infiltrates was not quantified, and it is possible that these differ between IL-18 deficient and WT mice while the amounts of other inflammatory cells remain unchanged, or are relatively higher. IL-18 given intra-tracheally at the time of antigen challenge promotes eosinophil chemotaxis into the airways by stimulating the production of eotaxin but does not directly stimulate eosinophil migration (Campbell et al., 2000). IL-18 can inhibit airway eosinophilia when given intraperitoneally in relatively low doses at the time of antigen challenge, an effect dependent on the production of systemic IFN-γ (Hofstra et al., 1998, Walter et al., 2001). However, when high doses of IL-18 are given systemically, eosinophil influx into the airways is induced through the production of TNF-α (Kumano et al., 1999), which negates the suppressive effect of IFN-γ on airway inflammation. *In vitro*, IL-18 has been shown to induce the production of the cytokine IL-9, IL-13, GM-CSF, TNF-α and the chemokines RANTES and macrophage inflammatory protein (MIP) 1 alpha, which may all contribute to the recruitment of eosinophils into the lung. Eosinophils express the IL-18Rα chain and are able to produce IL-8 in response to IL-18 (Wang et

al., 2001), and so may respond directly to IL-18 stimulation in other ways, for example by alterations in the expression of adhesion molecules involved in eosinophil migration such as very late activation antigen (VLA)-4 (Sagara et al., 1997). The absence of IL-18 in BALB/c IL-18 ko mice is therefore likely to cause complex alterations in the balance of cytokine and chemokine secretion which may inhibit eosinophil migration into the airways.

Although the absence of IL-18 in the lung in IL-18 ko mice may alter pulmonary cytokine and chemokine production leading to altered BAL and tissue eosinophilia, it is likely that there are also reduced T helper lymphocyte responses in IL-18 gene deficient mice. In the above study, there was a non-significant decrease in IL-4 levels in BAL fluid. However, the reduction in OVA-stimulated IL-4 production from thoracic lymph node cells *in vitro* was significant and was associated with decreased IL-4 mRNA expression in thoracic lymph node cells. The reduction in IL-4 production was antigen-specific, since production of this cytokine by the T-lymphocyte mitogen Concanavalin A was not reduced. These results are consistent with IL-18 having a role in the production of Th2 responses by enhancing the production of IL-4. The ability of IL-18 to stimulate IL-4 production may be important in the development of allergic sensitisation to antigen. Naïve T lymphocytes (Th0) express low levels of IL-18 receptor alpha (IL-18Rα) and, in the presence of IL-2, IL-18 stimulates IL-4 production and Th2 development in these cells (Hoshino et al., 1999). NKT cells express higher levels of IL-18Rα, and also produce IL-4 and IL-13 in response to IL-18 (Yoshimoto et al., 2003), and these NKT cells, and also basophils and mast cells (Yoshimoto et al., 1999) may provide the initial amounts of IL-4 needed to enhance Th2 development. It has previously been shown that the exogenous administration of IL-18 at the time of allergic sensitisation increased the inflammatory airways response (Wild et al., 2000); it may be that the exogenous IL-18 potentiates the production of IL-4 from antigen-

stimulated T lymphocytes to further augment Th2 cell development. Since BALB/c mice have greater propensity to produce IL-4 in response to stimulation with αCD3 than C57BL/6 mice (Xu et al., 2000, Habu et al., 2001), if IL-18 enhances the induction of IL-4 secretion through T cell receptor stimulation *in vivo*, its absence in BALB/c IL-18 ko mice could have a more profound effect on Th2 development than in the C57BL/6 IL-18 gene deficient mice which were used in the study of Kodama et al. (2000).

Th1 cells may contribute the inflammatory response in murine asthma models (Hansen et al., 1999, Randolph et al., 1999a, Randolph et al., 1999b). IL-18 has been shown to induce eosinophilic airway inflammation and AHR in mice which have previously received Th1 memory cells, and to enhance the secretion of cytokines and chemokines, including IL-13 and RANTES, from IFN-γ-producing Th1 cells *in vitro* (Sugimoto et al., 2004). In the lymph node cultures described above, the production of IFN-γ in response to OVA was also reduced. This may indicate that there is also some suppression of Th1 responses in these IL-18 ko mice and that this may contribute to the decrease in airway inflammation observed.

Atopic asthmatic responses are associated with increased levels of serum IgE. *In vitro*, IL-18 can induce IgE production, which is dependent on the presence of $CD4^+$ T cells and IL-4 (Yoshimoto et al., 2000). In mice, NKT cells are also thought to be essential for this response *in vivo* (Yoshimoto et al., 2003). Increased levels of IgE are also seen in mice treated with exogenous IL-18 (Wild et al., 2000) and in transgenic mice overexpressing IL-18 (Hoshino et al., 2001). In contrast, when IL-12 is present, IL-18 inhibits the production of IgE by inducing IFN-γ production (Yoshimoto et al., 1997). In the above experiments there was no significant difference in the levels of total serum IgE or OVA-specific IgE, IgG1 and IgG2a in the IL-18 ko mice which had been treated

with OVA compared to the WT mice. This is comparable to previous studies where no difference in serum IgE levels was observed in C57BL/6 IL-18 ko mice (Kodama et al., 2000) nor serum IgE or IgG1 levels in wild-type BALB/c mice treated with IL-18 and OVA adsorbed to alum (Pollock et al., 2003).

Wei et al. (1999) have previously noted that antigen naïve IL-18 deficient mice, on a CD1 background, had similar levels of $CD4^+$ and $CD8^+$ T lymphocytes, B cells and NK cells as WT mice on flow cytometric analysis. In this current study, flow cytometric analysis of thoracic lymph nodes cells revealed marginally decreased proportion of B lymphocytes in OVA-treated IL-18 ko mice when compared to OVA-treated WT mice. Since immunoglobulin levels are unaffected, the relevance of this is unclear. Also, as there was a relatively larger proportion of $CD4^+$ cells in the thoracic lymph nodes of IL-18 ko mice, it would imply that a decrease in the amount of T helper lymphocytes present does not contribute to the decrease in IL-4 and IFN-γ production observed in lymph node cell cultures. IL-18 is known to up-regulate the cell surface expression of the intracellular adhesion molecules ICAM-1 in monocytes (Stuyt et al., 2003) and VCAM-1 on endothelial cells (Morel et al., 2002), so promoting the influx of leukocytes to sites of inflammation. Although IL-18 may influence the expression of the molecules, the results above suggest that it does not induce alterations in the levels of the lymphocyte adhesion molecules CD44, CD11a and CD62L on murine T helper lymphocytes; this is consistent with the observation that IL-18 does not alter the levels of these molecules on human peripheral blood T cells (Yoshida et al., 2001).

In conclusion, in this murine model of asthma, IL-18 knockout mice had decreased airway eosinophilia, but not tissue inflammation, when compared to wild-type mice after OVA-sensitisation and challenge. There was a tendency for IL-4 levels in BAL fluid to be lower in IL-18 ko mice. OVA-induced IL-4 production was significantly

decreased in cultures of thoracic lymph node cells from OVA-sensitised and challenged IL-18 ko mice when compared to cells from similarly treated WT mice. The above results suggest that IL-18 may have a pro-inflammatory action in allergic airway inflammation which may in part be mediated by decreased IL-4 production. Further work is required to confirm that IL-18 is important in augmenting the secretion of IL-4 during the development of allergic airway inflammation, and to identify the cell types on which IL-18 acts to enhance the secretion of this cytokine. In addition, investigations are needed to elucidate the actions of IL-18 on eosinophil activity and migration, including a comparison of quantification of tissue eosinophilia between OVA-sensitised and challenged IL-18 gene knockout mice and wild-type mice. IL-18 is likely to affect the secretion of several cytokines and chemokines which promote allergic responses, and these should also be studied to clarify the pro-inflammatory role of IL-18 in allergic airway inflammation in order to assess more fully its potential as a therapeutic target in the treatment of human asthma.

Chapter 5

The anti-inflammatory actions of simvastatin in a murine model of allergic asthma

Data from this chapter has been published in

McKay et al., (2004) *J Immunol.* **172**, 2903-8.

5.1 Introduction

Statins are inhibitors of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, in cholesterol biosynthesis. As such, they have been widely used in clinical practice as cholesterol lowering agents to reduce morbidity and mortality from coronary artery disease (Scandinavian Simvastatin Survival Study, 1994, Shepherd et al., 1995). There is evidence from clinical studies (Albert et al., 2001, Joukhadar et al., 2001) and *in vitro* experiments (Crisby et al., 2001, Sparrow et al., 2001) that statins have additional anti-inflammatory properties in atherosclerotic disease which are unrelated to their lipid lowering activity. There are likely to be several molecular mechanisms through which statins exert their immunomodulatory effects (Takemoto and Liao, 2001) but these have not yet been fully elucidated. Statin treatment has the potential to modify T lymphocyte-driven disease through the ability to allosterically inhibit the interaction between the cellular adhesion molecules lymphocyte function-associated antigen (LFA)-1 and intercellular adhesion molecule (ICAM)-1 (Weitz-Schmidt et al., 2001) and decrease IFN-γ-induced expression of major histocompatibility complex (MHC)-II on antigen presenting cells (Kwak et al., 2000). Also, by inhibiting the production of L-mevalonic acid and its metabolites, statins prevent the isoprenylation of signalling molecules such as Ras and Rho which are involved in lymphocyte activation (Czyzyk et al., 2003, Greenwood et al., 2003). Statins may therefore have beneficial effects in a broad range of inflammatory conditions.

Atherosclerotic plaques contain large numbers of lymphocytes which are mainly of T helper (Th)-1 type (Frostegard et al., 1999) which characteristically secrete IFN-γ. Since statins inhibit inflammation in plaques, recent studies have focussed on the potential ability of statins to modulate Th1-predominant disease, such as rheumatoid arthritis

(Dolhain et al., 1996) and multiple sclerosis (Lassmann et al., 2001). In animal models of both these conditions statins have had an immunosuppressive action (Leung et al., 2003, Youssef et al., 2002, Aktas et al., 2003). In contrast, evidence is lacking as to whether statins might modulate inflammation in which Th2 lymphocytes, secreting interleukin (IL)-4, IL-5 and IL-13, are important.

Treatments targeted at inhibiting the function of LFA-1 have been effective in reducing airway eosinophilia in a murine model of allergic asthma (Winn et al., 2001) and sputum eosinophilia after allergen challenge in asthmatic patients (Gauvreau et al., 2003). Since simvastatin can inhibit LFA-1/ICAM-1 interaction, we sought to establish whether this drug could modulate inflammatory responses in a murine model of allergic asthma, a Th2-driven condition. In this study it is shown for the first time that simvastatin can effectively suppress acute eosinophilic airway inflammation and Th2 cytokine secretion.

5.2 The effect of simvastatin on bronchoalveolar lavage cellularity

In order to determine if simvastatin treatment could influence the development of allergic airway inflammation, the murine model of allergic asthma used in Chapter 4 was used. BALB/c mice were sensitised and challenged with OVA, following the protocol outlined in Figure 2.1. Simvastatin was given by either intraperitoneal injection 30 minutes before each antigen challenge, or oral gavage 60 minutes before each allergen challenge, on days 25 – 27 of the experimental protocol. Control mice were treated with PBS. Experiments were terminated on day 28.

5.2.1 The effect of intraperitoneal simvastatin treatment on total bronchoalveolar lavage cellularity

In initial experiments, simvastatin was given by intrperitoneal injection before each intranasal challenge with OVA. Simvastatin at 40 mg/kg i.p., but not at 4 mg/kg, produced a significant reduction in BAL total cell count (Figure 5.1).

5.2.2 The effect of intraperitoneal simvastatin on bronchoalveolar lavage differential cell count

Simvastatin at 40 mg/kg i.p. but not at 4 mg/kg produced a reduction in BAL eosinophilia and macrophage count, as shown in Figure 5.2 *A* and *B*. At either dose, simvastatin did not alter BAL lymphocyte or neutrophil numbers (Figure 5.2 *C* and *D*).

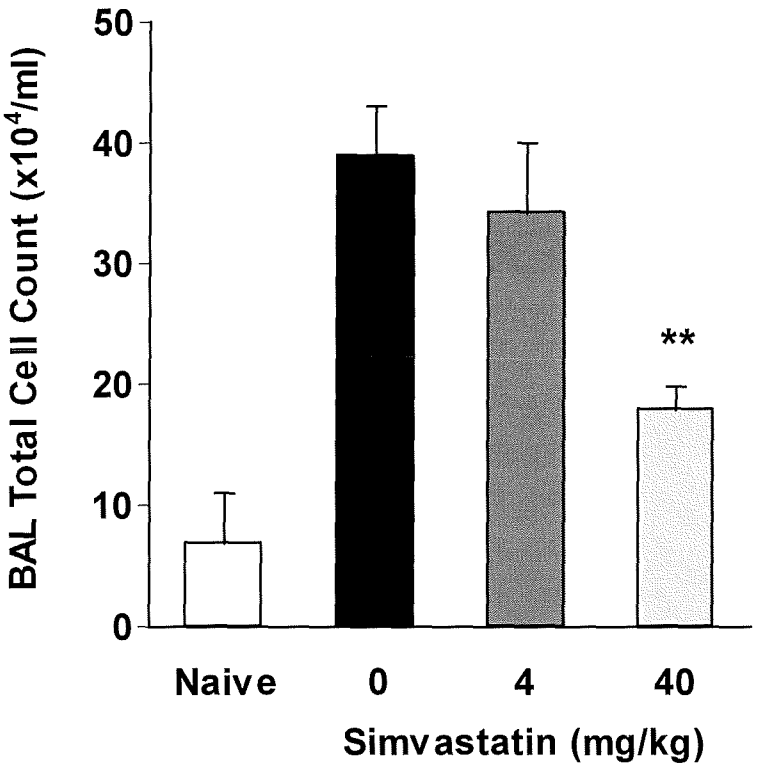


Figure 5.1. Total bronchoalveolar lavage cellularity after intraperitoneal simvastatin treatment.

BALB/c mice were sensitised with OVA and then challenged with OVA i.n. on three consecutive days from day 25-27. Simvastatin 40 mg/kg or 4 mg/kg i.p. was given 30 minutes before each antigen challenge. Control mice were given PBS. Bronchoalveolar lavage (BAL) cell counts were performed on day 28. Treatment with simvastatin 40 mg/kg resulted in a significant reduction in BAL total cell count. Data are expressed as mean ± SEM. (n = 7-14 mice per group). **$p < 0.01$. Statistical significance was determined by ANOVA.

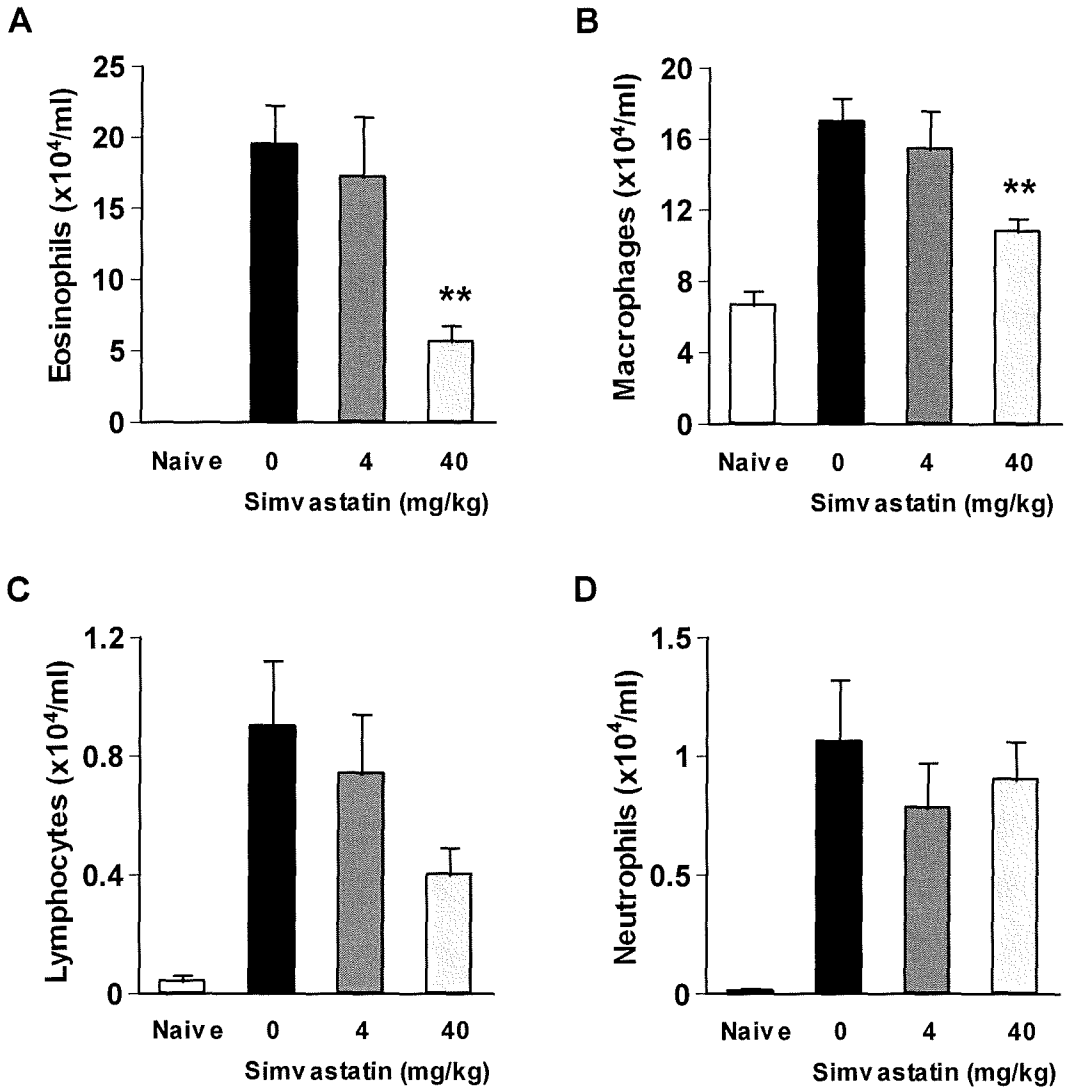


Fig. 5.2. Bronchoalveolar lavage differential cell count after treatment with simvastatin intraperitoneally.

BALB/c mice were sensitised with OVA and then challenged with OVA i.n. on three consecutive days from day 25-27. Simvastatin 40 mg/kg or 4 mg/kg i.p. was given 30 minutes before each antigen challenge. Control mice were given PBS. Bronchoalveolar lavage (BAL) cell counts were performed on day 28. Treatment with simvastatin 40 mg/kg resulted in a significant reduction in BAL **(A)** eosinophil and **(B)** macrophage numbers. There was no significant difference in BAL **(C)** lymphocyte or **(D)** neutrophil levels. Data are expressed as mean ± SEM. (n = 7-14 mice per group). $^{**}p < 0.01$. Statistical significance was determined by ANOVA.

5.2.3 The effect of oral simvastatin on bronchoalveolar lavage total cellularity

As simvastatin is orally administered in clinical use, it was important to determine if a similar anti-inflammatory effect could be obtained by giving the drug enterally. When simvastatin 40 mg/kg was given by oral gavage (o.g) before the allergen challenges on days 25 to 27, a significant decrease in BAL total cellularity was again observed (Figure 5.3).

5.2.4 The effect of oral simvastatin on bronchoalveolar lavage differential cell count

These results are shown in Figure 5.4. Treatment with simvastatin 40 mg/kg orally produced a reduction in BAL eosinophilia (Figure 5*A*). but no reduction was seen in the amounts of macrophages, lymphocytes or neutrophils in the lavage fluid (Figure 5.4*B-D*).

5.3 Histological analysis of lung inflammatory infiltrates

Histological analysis demonstrated that there was a reduction in inflammatory infiltrates in the lungs of mice treated with i.p. simvastatin 40 mg/kg. Representative photomicrographs of this are shown in Figure 5.5. This reduction in inflammatory infiltration in these mice, but not mice treated with simvastatin 4mg/kg intraperitoneally, was confirmed by histological scoring (Figure 5.6*A*). Similarly in mice treated with simvastatin 40 mg/kg orally, although there was a reduction in perivascular inflammation on histological scoring, there was no significant decrease in total lung inflammatory scores (Figure 5.6*B*).

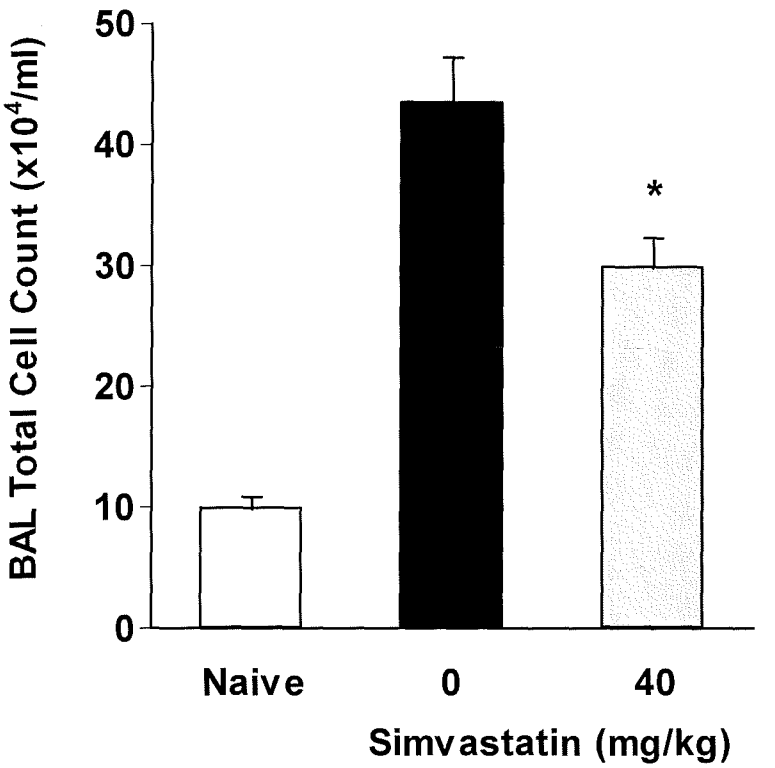


Figure 5.3. Bronchoalveolar lavage total cell count after treatment with simvastatin orally.

BALB/c mice were sensitised with OVA and then challenged with OVA i.n. on three consecutive days from day 25-27. Simvastatin 40 mg/kg. was given by oral gavage 60 minutes before each antigen challenge. Control mice were given PBS. Broncholaveolar lavage (BAL) cell counts were performed on day 28. Treatment with simvastatin 40 mg/kg by oral gavage resulted in a significant reduction in BAL total cell count. Data are expressed as mean ± SEM. (n = 8 – 16 mice per group). $^{**}p < 0.01$. Statistical significance was determined by ANOVA.

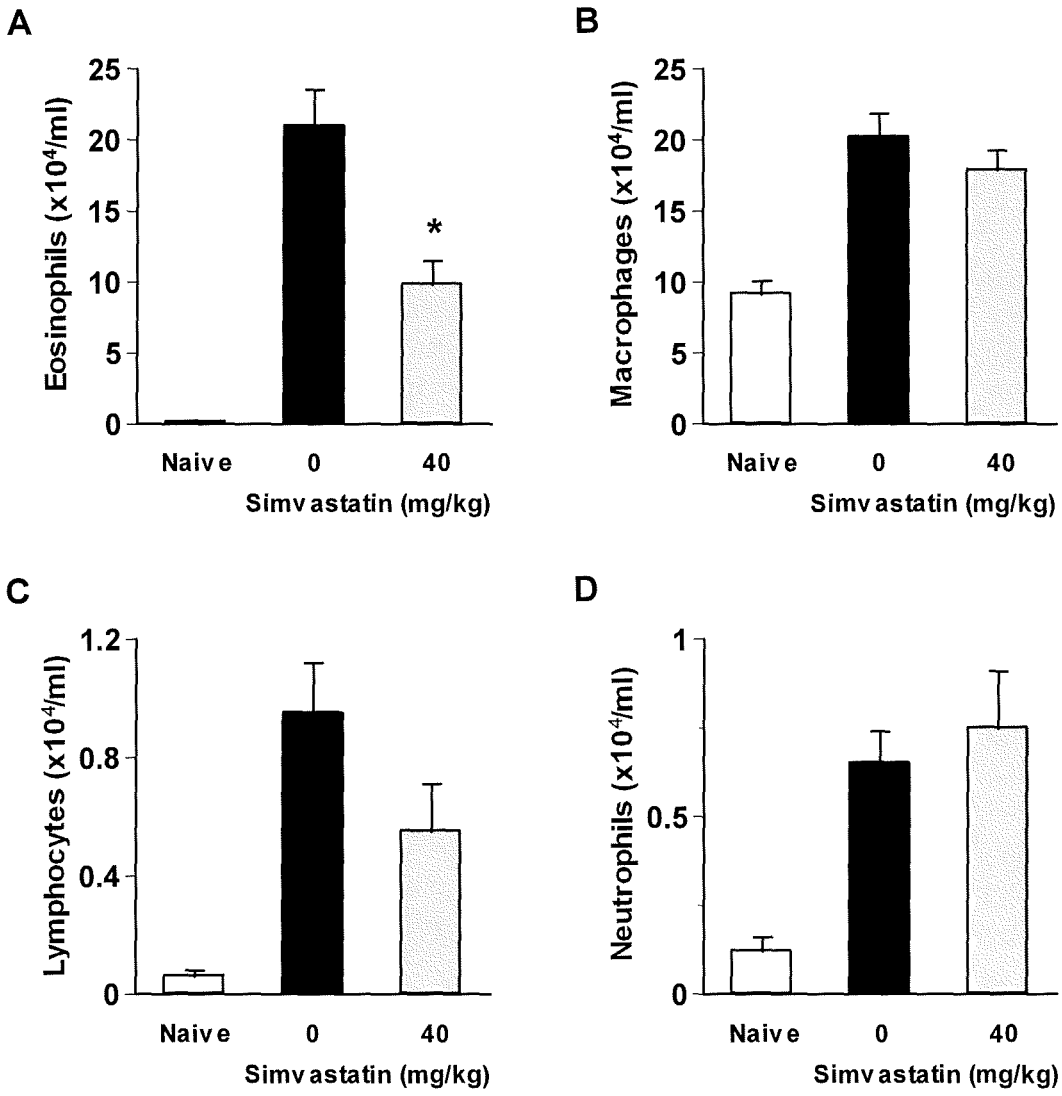


Fig. 5.4. Bronchoalveolar lavage differential cell count after treatment with simvastatin orally.

BALB/c mice were sensitised with OVA and then challenged with OVA i.n. on three consecutive days from day 25-27. Simvastatin 40 mg/kg was given by oral gavage 60 minutes before each antigen challenge. Control mice were given PBS. Broncholaveolar (BAL) cell counts were performed on day 28. Treatment with simvastatin resulted in a significant reduction in BAL **(A)** eosinophil numbers. There was no significant difference in BAL **(B)** macrophage, **(C)** lymphocyte or **(D)** neutrophil levels. Data are expressed as mean ± SEM. (n = 8 - 16 mice per group). $^{**}p < 0.01$. Statistical significance was determined by ANOVA.

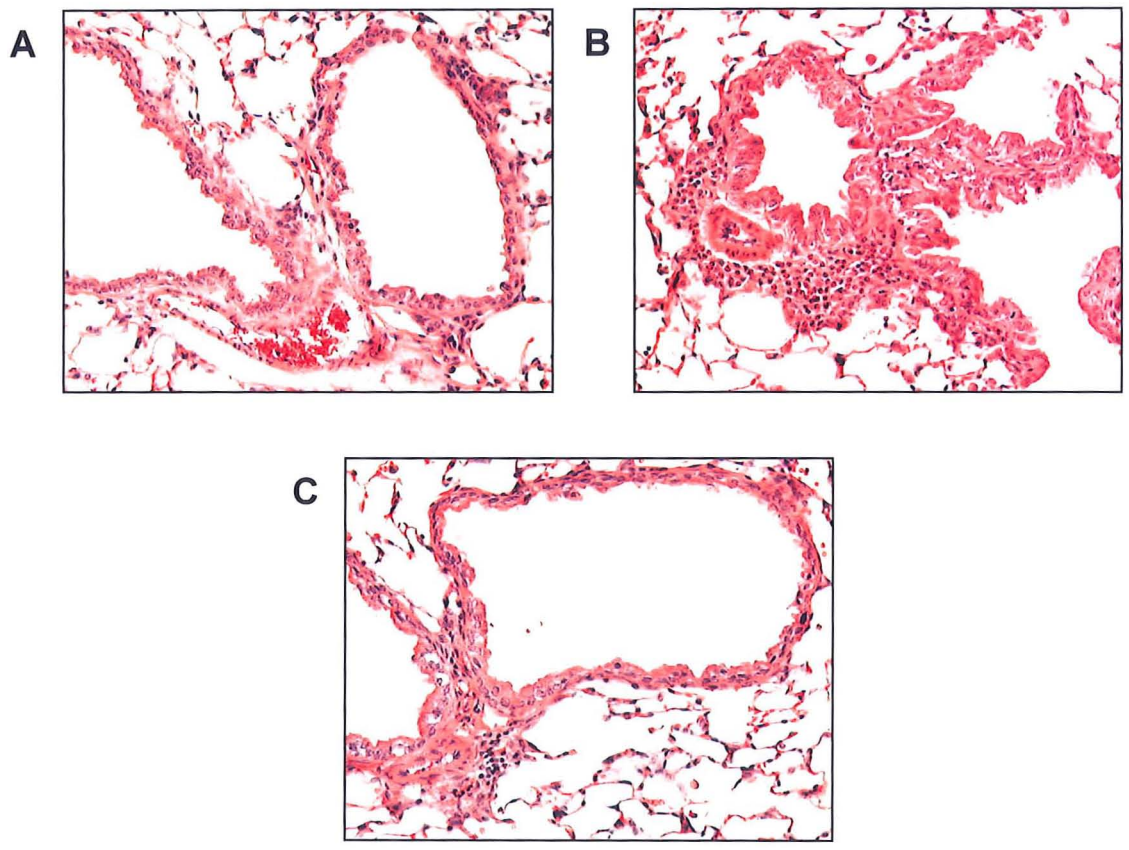


Fig. 5.5. Histological evidence of decreased lung inflammation in mice treated with simvastatin 40 mg/kg intraperitoneally.

A representative section is shown from each group of mice (n = 3-5 per group). (**A**) Naïve mouse, given PBS challenge. (**B**) OVA challenged mouse; peri-bronchial and peri-vascular inflammatory infiltrates are seen, with eosinophils present, and mucosal hyperplasia. (**C**) OVA challenged plus treatment with simvastatin 40 mg/kg i.p; a reduction in inflammatory infiltrates is seen compared with **(B)**. H&E staining, magnification x200.

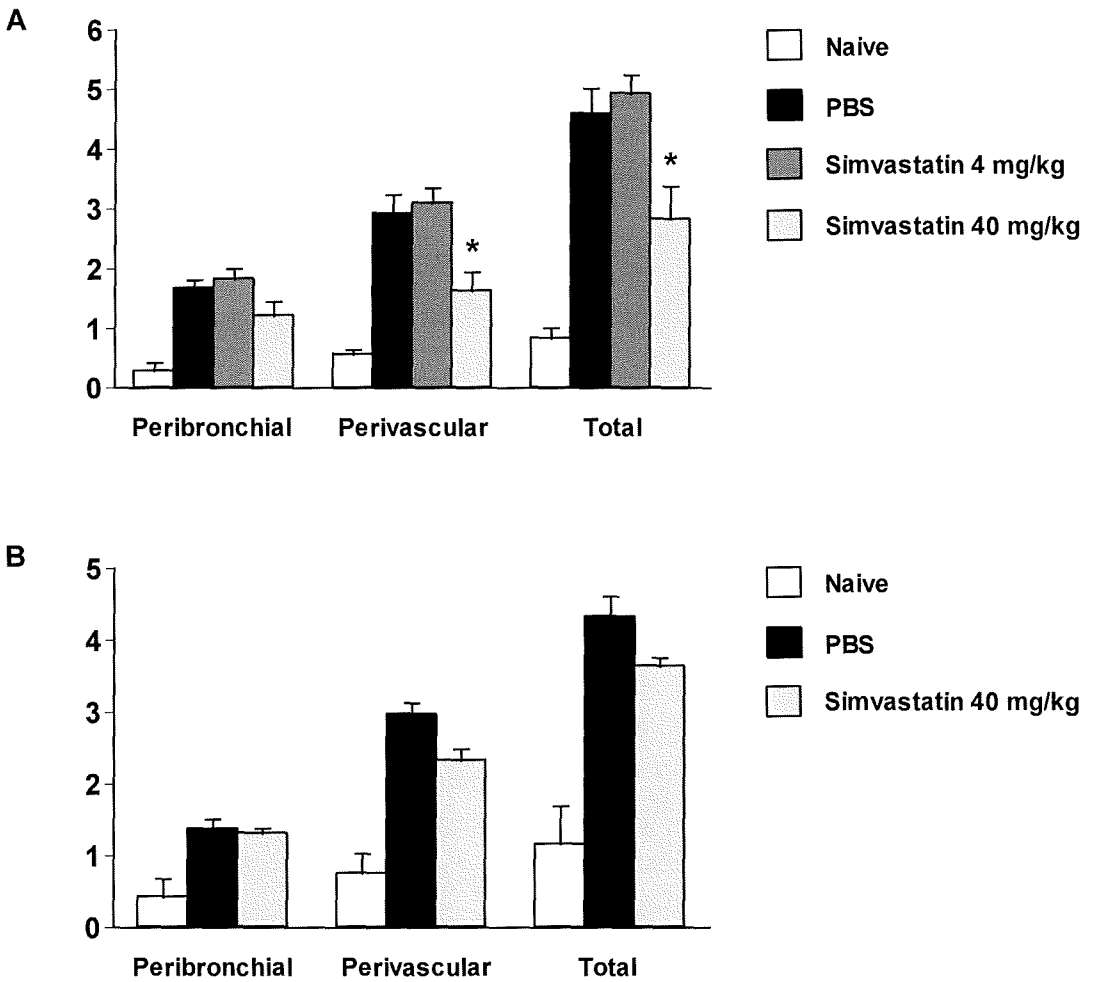


Figure 5.6. Histological score of lung inflammation in mice treated with simvastatin. Total lung inflammation on histological sections was determined as the sum of peribronchial and perivascular scores. Inflammation was graded on the scale 0, none; 1, mild, 2, moderate; 3, marked and 4, severe. An increment of 0.5 was used when severity of inflammation fell between two integers. **(A)** Intraperitoneal treatment with simvastatin 40 mg/kg, but not 4 mg/kg, produced a significant reduction in perivascular and total lung inflammation when compared to PBS-treated mice. **(B)** Mice treated with simvastatin 40 mg/kg by oral gavage had a significant reduction in perivascular inflammation, but total lung inflammatory scores were not significantly different from control mice given PBS. Data are expressed as mean ± SEM. * $p < 0.05$. (n = 3 -7 mice per group). Statistical significance was assessed by Student's *t* test.

5.4 Bronchoalveolar lavage cytokines

In order to establish if the reduction in BAL total cellularity and eosinophilia produced by simvastatin treatment was accompanied by decreased Th2 cytokine production, the levels of IL-4 and IL-5 in BAL fluid were measured. Since statin treatment has previously been shown to increase IL-18 secretion from human peripheral blood monocytes (Montero et al., 2000), IL-18 levels in BAL fluid were also determined, as were the levels of the chemokine eotaxin, a chemoattractant for eosinophils.

Intraperitoneal administration of simvastatin produced a dose-related reduction in the levels of IL-4 and IL-5 in BAL fluid (Fig. 5.7*A* and *B*) when compared to PBS-treated control mice (IL-4 – simvastatin 4 mg/kg $p < 0.05$, simvastatin 40 mg/kg $p < 0.01$; IL-5 – simvastatin 4 mg/kg $p < 0.01$, simvastatin 40 mg/kg $p < 0.01$). However, the levels of IL-18 (Figure 5.7*C*) were not significantly different between PBS-treated mice and simvastatin treated mice, nor between naïve mice and OVA-sensitised mice. Levels of the chemokine eotaxin (Figure 5.7*D*) tended to be higher in OVA-sensitised and challenged mice but again, intraperitoneal simvastatin treatment did not significantly alter its levels in BAL fluid. IFN-γ was not detectable (data not shown).

Oral simvastatin treatment did not significantly alter the levels of IL-4, IL-5, IL-18 or eotaxin in bronchoalveolar lavage fluid, as shown in Figure 5.8.

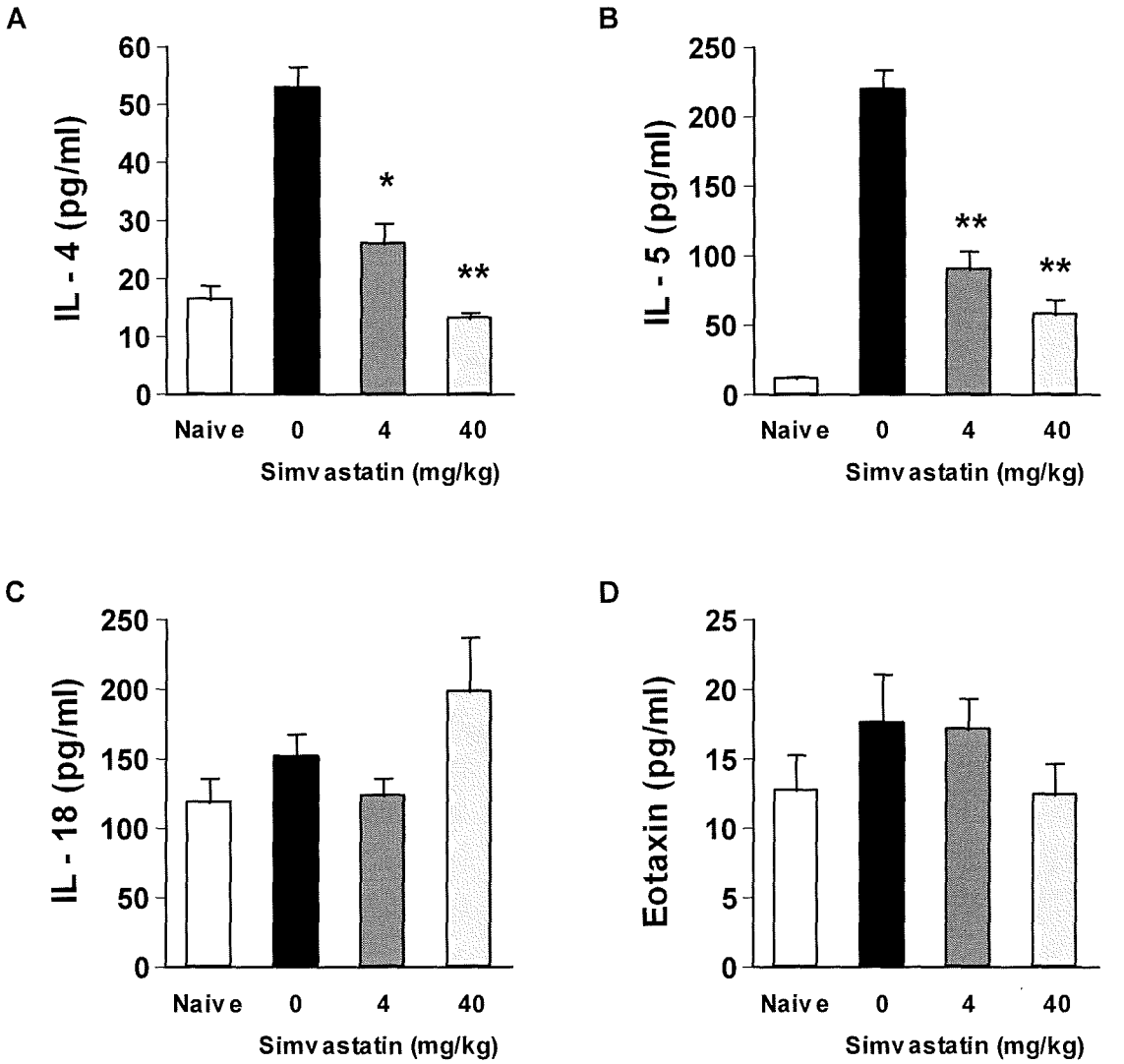


Figure 5.7. Bronchoalveolar lavage cytokines in mice treated with simvastatin intraperitoneally.

Levels of IL-4, IL-5 and IL-18 and the chemokine eotaxin in bronchoalveolar lavage (BAL) fluid were determined by ELISA. **(A)** IL-4 levels and **(B)** IL-5 levels were significantly reduced by treatment with simvastatin 4 mg/g and 40 mg/kg i.p. compared to PBS-treated controls. Levels of **(C)** IL-18 and **(D)** the chemokine eotaxin were not significantly different in simvastatin-treated mice compared to control mice treated with PBS. Data are expressed as mean ± SEM. (n = 6 -11 mice per group). * $p < 0.05$, ** $p < 0.01$. Statistical significance was assessed by ANOVA.

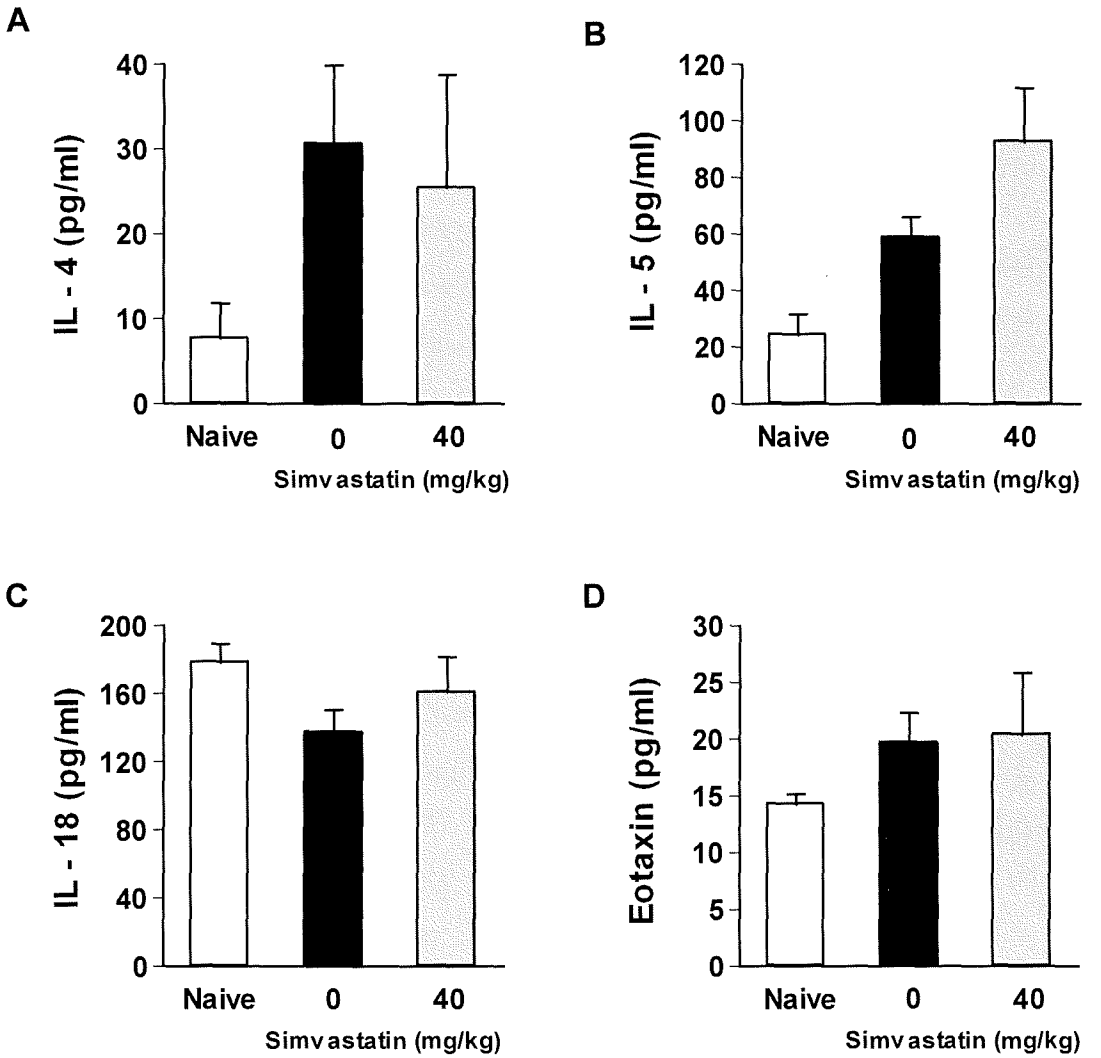


Figure 5.8. Bronchoalveolar lavage cytokines in mice treated with simvastatin orally.

Levels of the cytokines IL-4, IL-5 and IL-18 and the chemokine eotaxin in bronchoalveolar (BAL) fluid were determined by ELISA. **(A)** IL-4 levels, **(B)** IL-5, **(C)** IL-18 and **(D)** eotaxin levels were not significantly altered by treatment with simvastatin 40 mg/kg by oral gavage. Data are expressed as mean ± SEM. (n = 8 -14 mice per group). Statistical significance was assessed by ANOVA.

5.5 Serum immunoglobulin levels

Figure 5.9 shows the serum total IgE and IgG subclass profiles. Treatment with simvastatin intraperitoneally at the time of antigen challenge did not significantly affect serum levels of total IgE or OVA-specific IgG1 or IgG2a. Levels of these immunoglobulins were elevated in all OVA-treated groups compared to naïve mice.

Similarly, oral simvastatin treatment did not significantly alter the amount of these immunoglobulins compared to PBS-treated mice (data not shown).

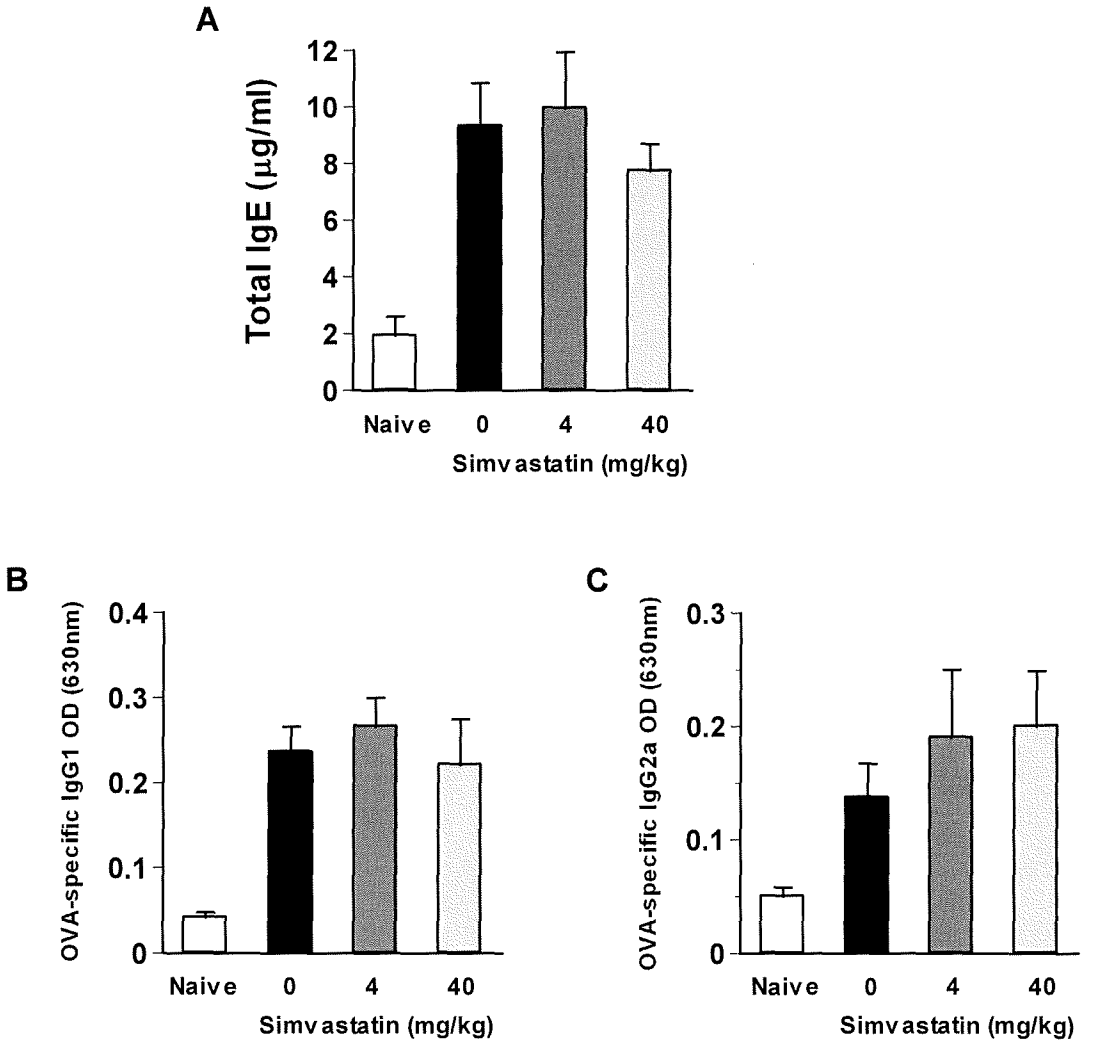


Figure 5.9. Serum immunoglobulin levels in mice treated with simvastatin intraperitoneally.

Serum immunoglobulin levels were assessed by ELISA. Treatment with simvastatin 4 mg/kg or 40 mg/kg intraperitoneally did not alter **(A)** serum total IgE levels (n = 6 mice per group) when compared to PBS-treated controls. In addition, **(B)** Serum OVA-specific IgG1 (serum dilution 1/180,000) and **(C)** serum OVA-specific IgG2a levels (serum dilution 1/800) were also unaffected by treatment with simvastatin compared to mice treated with PBS alone (OVA) (n=6 mice per group). Data are expressed as mean ± SEM. Statistical significance was assessed by Student's *t* test.

5.6 *In vitro* thoracic lymph node cell responses following the *in vivo* administration of simvastatin

Since there was a reduction in IL-4 and IL-5 levels in BAL fluid, this suggested that there was suppression of Th2 lymphocyte responses. To investigate this further, cells isolated from the lymph nodes of mice previously treated with simvastatin, both intraperitoneally and orally were cultured *in vitro* and OVA-induced cell proliferation and cytokine production assessed.

5.6.1 *In vitro* OVA-induced cell proliferation in thoracic lymph node cells

Figure 5.10 demonstrates the proliferative responses of thoracic lymph node cells to OVA and Concanavalin (Con) A. Neither intraperitoneal simvastatin, at both the higher and lower doses, nor oral simvastatin treatment altered cell proliferation in response to OVA or Con A.

5.5.1 *In vitro* OVA-induced cytokine responses in thoracic lymph node cells

Treatment with both simvastatin 4 mg/kg and 40 mg/kg intraperitoneally resulted in a significant decrease in OVA-induced IL-4 and IL-5 production from thoracic lymph node cells compared to cells from PBS-treated controls (IL-4 – s imvastatin 4 mg/kg $p < 0.05$, simvastatin 40 mg/kg $p < 0.01$; IL-5 - simvastatin 4 mg/kg $p < 0.01$, simvastatin 40 mg/kg $p < 0.01$) (Figure 5.11*A* and *B*, respectively). Treatment with oral simvstatin did not affect the levels of these cytokines (Figure 5.11*C* and *D*).

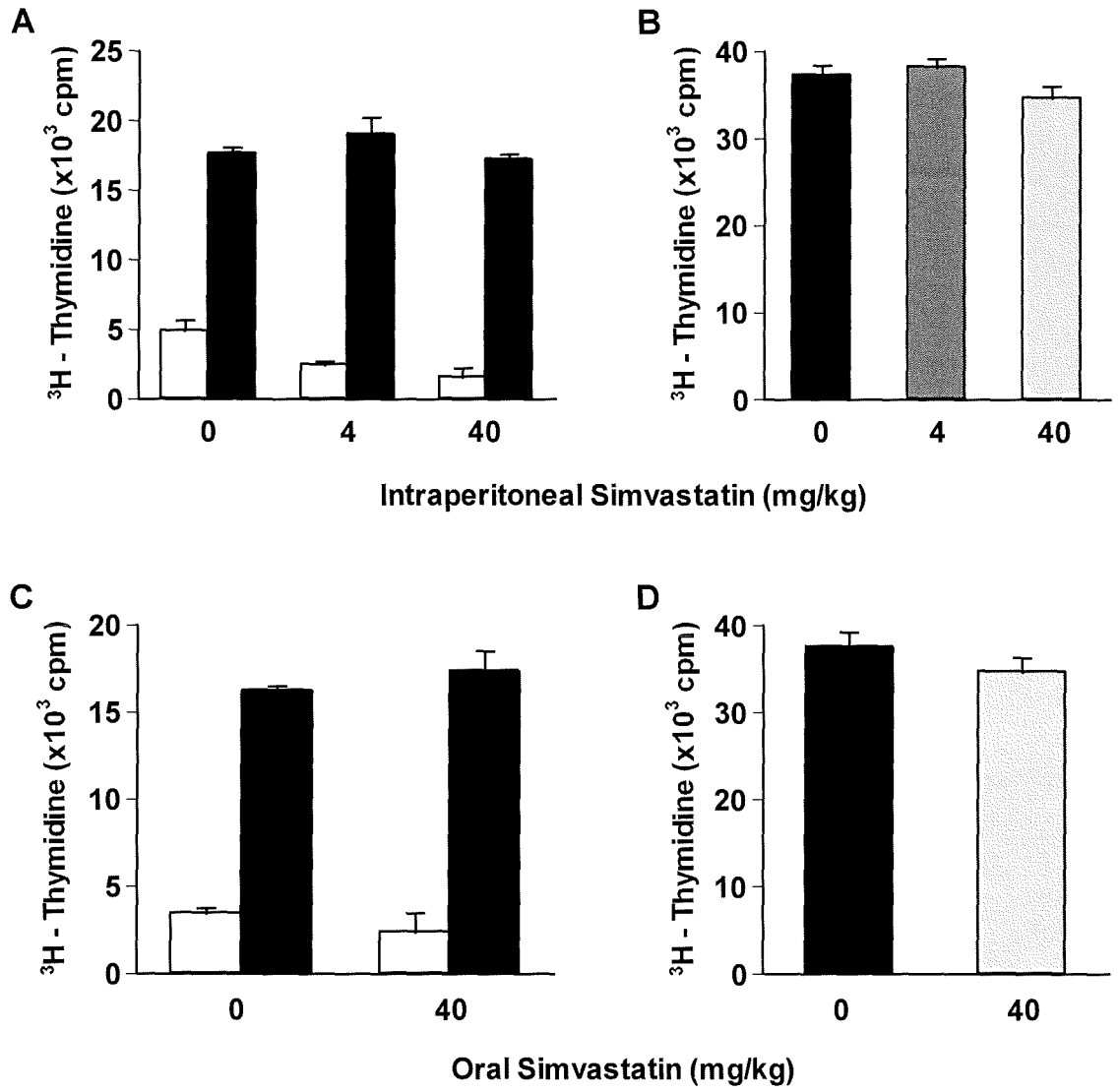


Figure 5.10. Proliferative responses in thoracic lymph node cells *in vitro*.

Thoracic lymph node cells (n = 3 mice per group) were harvested from mice on day 28 and cultured for 96 hours with **(A)** and **(C)** medium alone (□) or OVA (■; 500 μg/ml) or **(B)** and **(D)** 5 μg/ml Concanavalin A. T cell proliferation was assessed by uptake of [^{3}H]-thymidine. **(A)** Mice treated with simvastatin 4 mg/kg or 40 mg/kg intraperitoneally or **(C)** simvastatin 40 mg/kg orally had no significant reduction in OVA-induced cell proliferation and spontaneous cell proliferation when compared to mice treated with PBS alone. Similarly neither **(B)** intraperitoneal nor **(D)** oral simvastatin treatment reduced the proliferative response to Concanavalin A. Data are expressed as mean ± SEM. Statistical significance was assessed by ANOVA.

Con A-induced production of IL-4 and IL-5 from thoracic lymph node cells is illustrated in Figure 5.12. For the experiment shown, IL-4 production significantly decreased from thoracic lymph node cells from mice treated with simvastatin 4 mg/kg intraperitoneally. Treatment with simvasatatin 40 mg/kg i.p. or orally did not alter ConA-induced IL-4 production. IL-5 production was not affected by simvastatin treatment intraperitoneally or orally (Figure 5.12*B* and *D*).

As shown in Figure 5.13*A* and *C*, OVA-induced IFN-γ production was significantly reduced by all conditions of simvastatin treatment (simvastatin i.p. 4 mg/kg and 40 mg/kg, $p < 0.01$; simvastatin orally 40 mg/kg $p < 0.05$). Similarly, thoracic lymph node cells from mice treated with simvastatin 40 mg/kg i.p or orally produced less IL-6 in response to OVA (both $p < 0.05$), than cells from PBS treated mice, Figure 5.13*B* and *D.* IFN-γ and IL-6 production may therefore be differentially regulated by the pharmacological actions of simvastatin, compared to that of IL-4 and IL-5.

Previous *in vivo* simvastatin treatment, either intraperitoneally or orally, did not significantly affect the production of IFN-γ or IL-6 in response to ConA in thoracic lymph node cells *in vitro* (Figure 5.14).

For the above data, in the majority of cases, prior simvastatin treatment appears to specifically suppress antigen-induced cytokine production from thoracic lymph node cells.

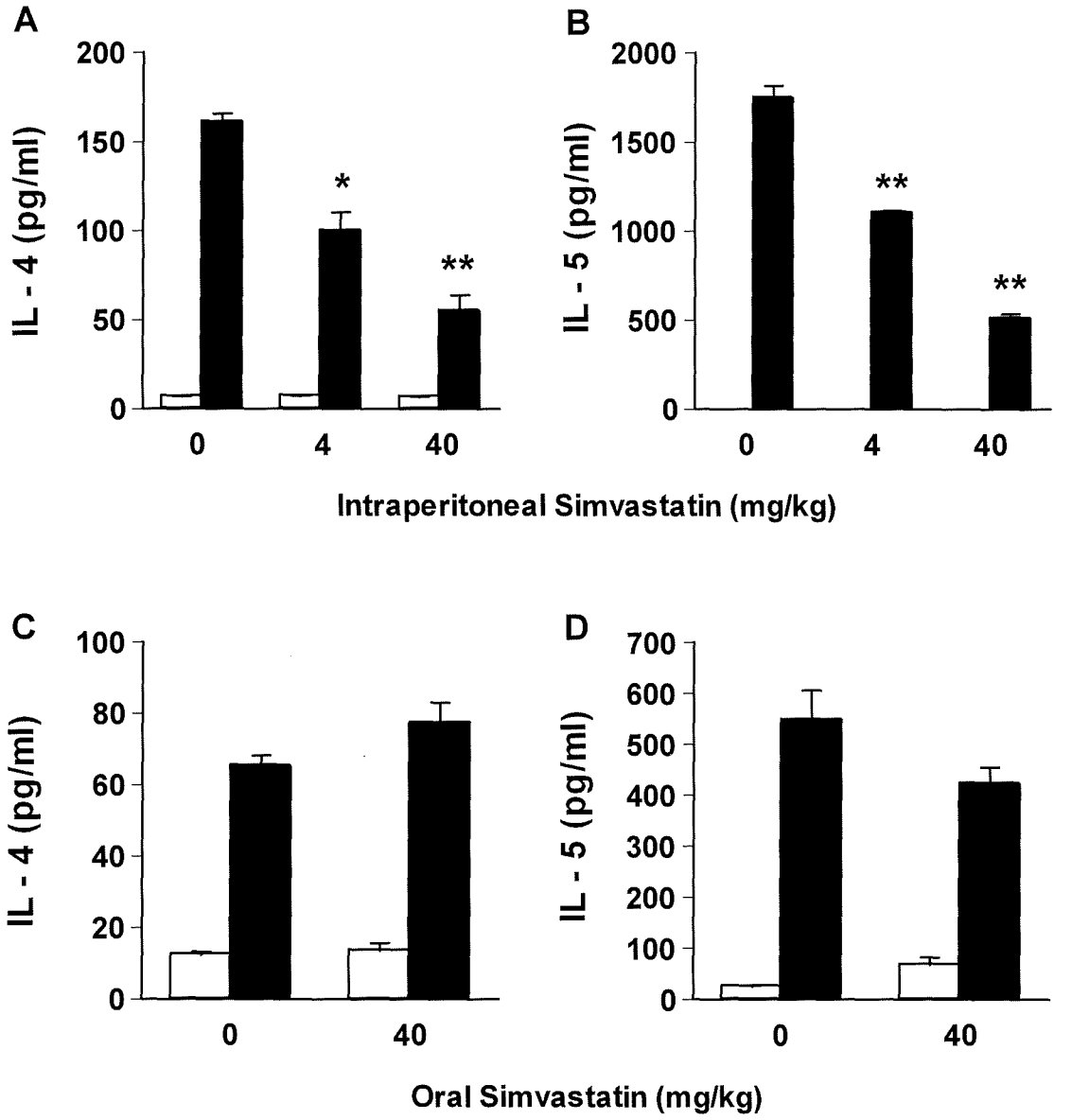


Figure 5.11. OVA-induced IL-4 and IL-5 responses in thoracic lymph node cells *in vitro*.

In parallel cultures to those in Figure 5.10, thoracic lymph node cells (n = 3 mice per group) were harvested from mice on day 28 and cultured for 72 hours with medium alone (□) or OVA (■; 500 μg/ml). Cytokine levels in cell culture supernatants were assessed by ELISA. **(A)** IL-4 and **(B)** IL-5 production in response to OVA were significantly reduced in lymph node cells from mice treated with simvastatin 4 mg/kg and 40 mg/kg intraperitoneally when compared to cells from mice treated with PBS alone. **(C)** IL-4 and **(D)** IL-5 production were not reduced in cells from mice treated with simvastatin 40 mg/kg orally. Data are expressed as mean ± SEM. ** $p < 0.01$. Statistical significance was assessed by ANOVA.

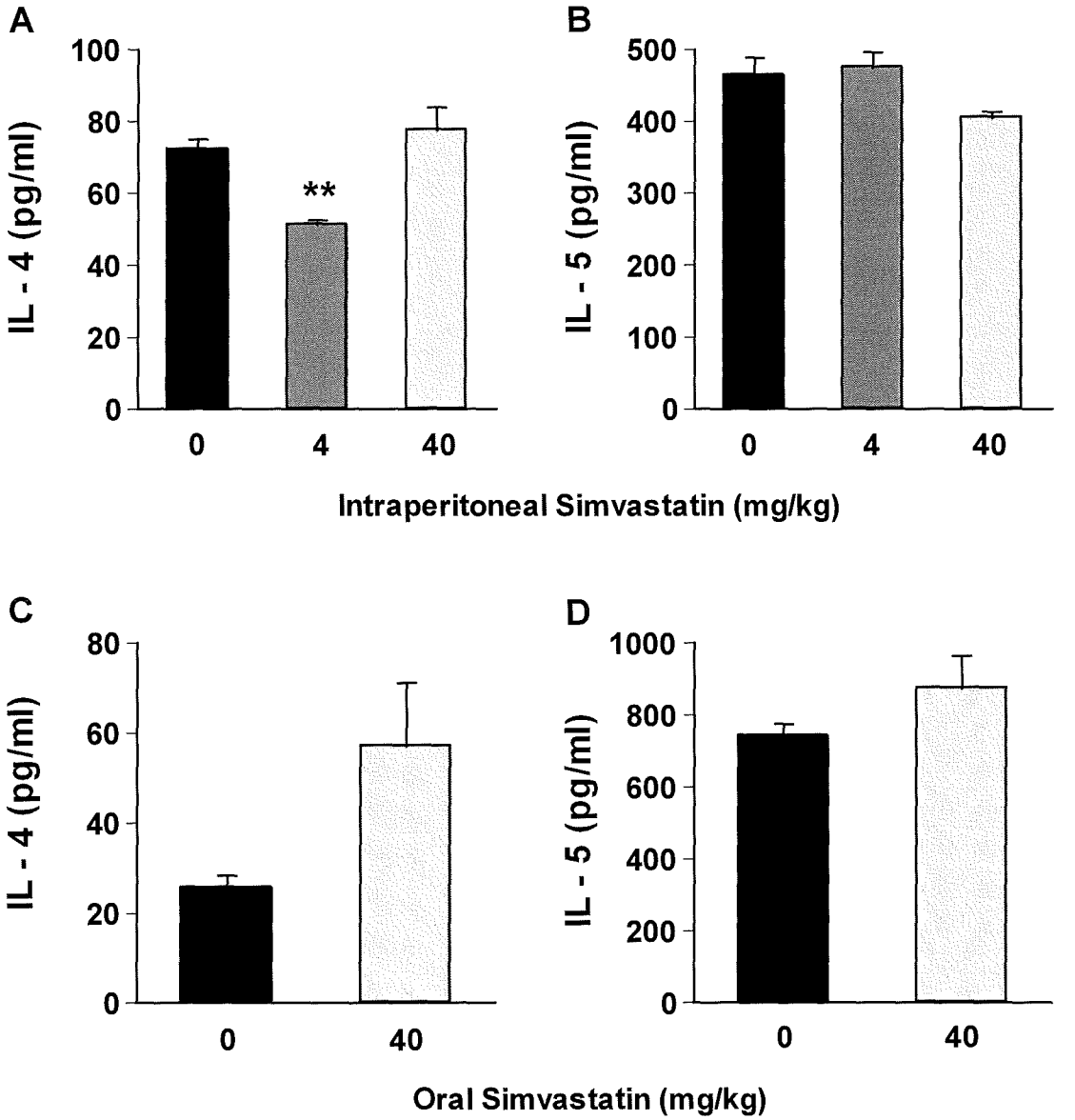


Figure 5.12. Concanavalin A-induced IL-4 and IL-5 responses in thoracic lymph node cells *in vitro*.

In parallel cultures to those in Figure 5.10, thoracic lymph node cells (n = 3 mice per group) were harvested from mice on day 28 and cultured for 72 hours with Concanavalin (Con) A 5 μg/ml. Cytokine levels in cell culture supernatants were assessed by ELISA. **(A)** IL-4 production in response to Con A was significantly reduced in mice treated intraperitoneally with simvastatin 4 mg/kg, but not 40 mg/kg, compared to cells from PBS-treated controls. **(B)** IL-5 production was not affected by intraperitioneal simvastatin treatment. **(C)** IL-4 and **(D)** IL-5 production were not significantly reduced in cells from mice treated with simvastatin 40 mg/kg orally. Data are expressed as mean ± SEM.

** $p < 0.01$. Statistical significance was assessed by ANOVA.

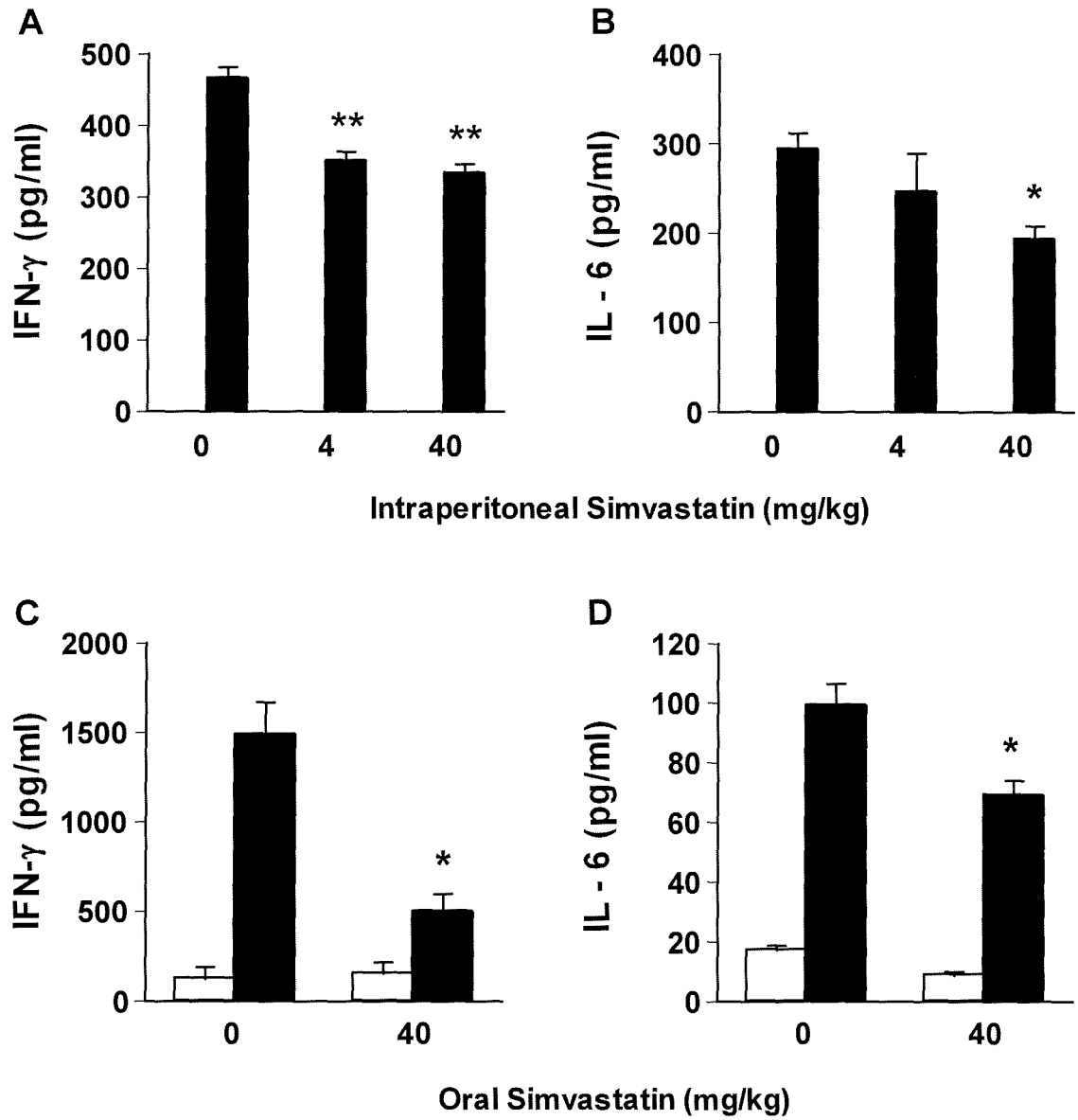


Figure 5.13. OVA-induced IFN-γ and IL-6 responses in thoracic lymph node cells *in vitro*.

In parallel cultures to those in Figure 5.10, thoracic lymph node cells (n = 3 mice per group) were harvested from mice on day 28 and cultured for 72 hours with medium alone (□) or OVA (■; 500 μg/ml). Cytokine levels in cell culture supernatants were assessed by ELISA. IFN-γ production in response to OVA was reduced in thoracic lymph node cells from mice treated with **(A)** simvastatin 4 mg/kg and 40 mg/kg intraperitoneally and **(C)** simvastatin 40 mg/kg orally, when compared to cells from PBS treated mice. OVA-induced IL-6 production was reduced in cells from mice treated with **(B)** simvastatin 40 mg/kg intraperitoneally and **(D)** 40 mg/kg orally. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$. Statistical significance was assessed by ANOVA.

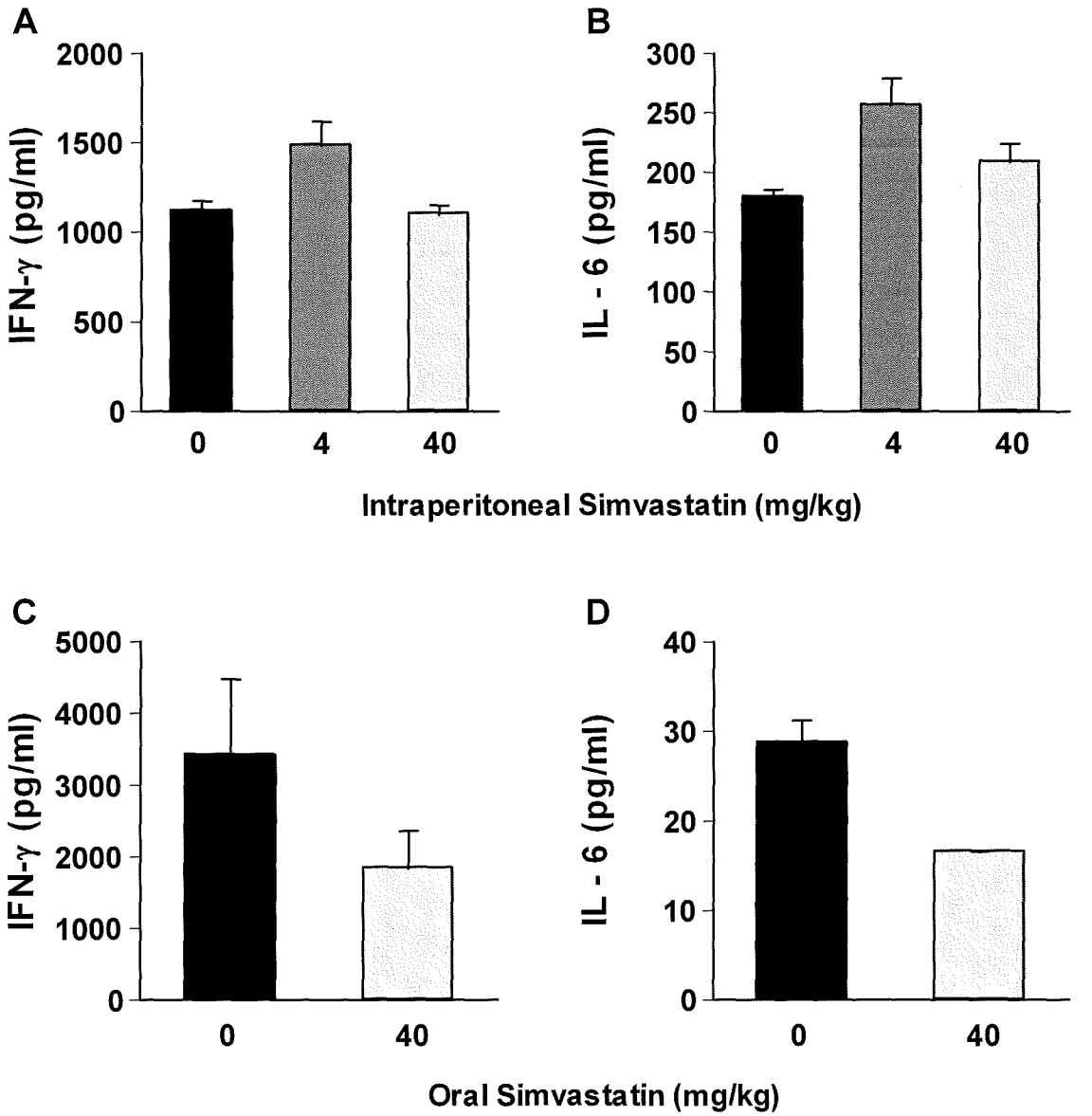


Figure 5.14. Concanavalin A –induced IFN-γ and IL-6 responses in thoracic lymph node cells *in vitro*.

In parallel cultures to those in Figure 5.13, thoracic lymph node cells (n = 3 mice per group) were harvested from mice on day 28 and cultured for 72 hours with Concanavalin (Con) A 5 μg/ml. Cytokine levels in cell culture supernatants were assessed by ELISA. **(A)** IFN-γ and **(B)** IL-6 production in response to Con A was not significantly different in mice treated intraperitoneally with simvastatin 4 mg/kg,or 40 mg/kg compared to cells from PBS-treated controls. Similarly, **(C)** IFN-γ and **(D)** IL-6 production were not significantly different in cells from mice treated with simvastatin 40 mg/kg orally compared to cells from control mice. Statistical significance was assessed by ANOVA.

5.7. Gene expression in mice treated with simvastatin intraperitoneally

5.7.1 Gene expression in thoracic lymph nodes

Since previous treatment with simvastatin intraperitoneally influenced OVA-induced production of IL-4 and IL-5 in cultures of thoracic lymph node cells, the gene expression of these cytokines was assessed in thoracic lymph nodes taken from mice on day 28 of the experimental protocol. As shown in Figure 5.15*A,* the expression of IL-4 mRNA tended to be higher in OVA-sensitised and challenged mice when compared to naïve mice but simvastatin treatment did not significantly alter its levels. There was no significant difference in IL-5 amongst any of the groups (Figure 5.15*B*). Similarly, the expression of IL-18 did not differ significantly between naïve nor OVA-sensitised and challenged mice, or with simvastatin treatment (Figure 5.15*C*).

Statins have previously been shown to suppress IFN-γ-induced MHC-II expression on antigen presenting cells through suppression of the transcription factor MHC-II transactivator (CIITA) (Kwak et al., 2000), and so levels of CIITA mRNA expression in thoracic lymph nodes were also determined. There was no significant difference in the levels of CIITA expression between simvastatin-treated and PBS treated mice (Figure 5.15*D*).

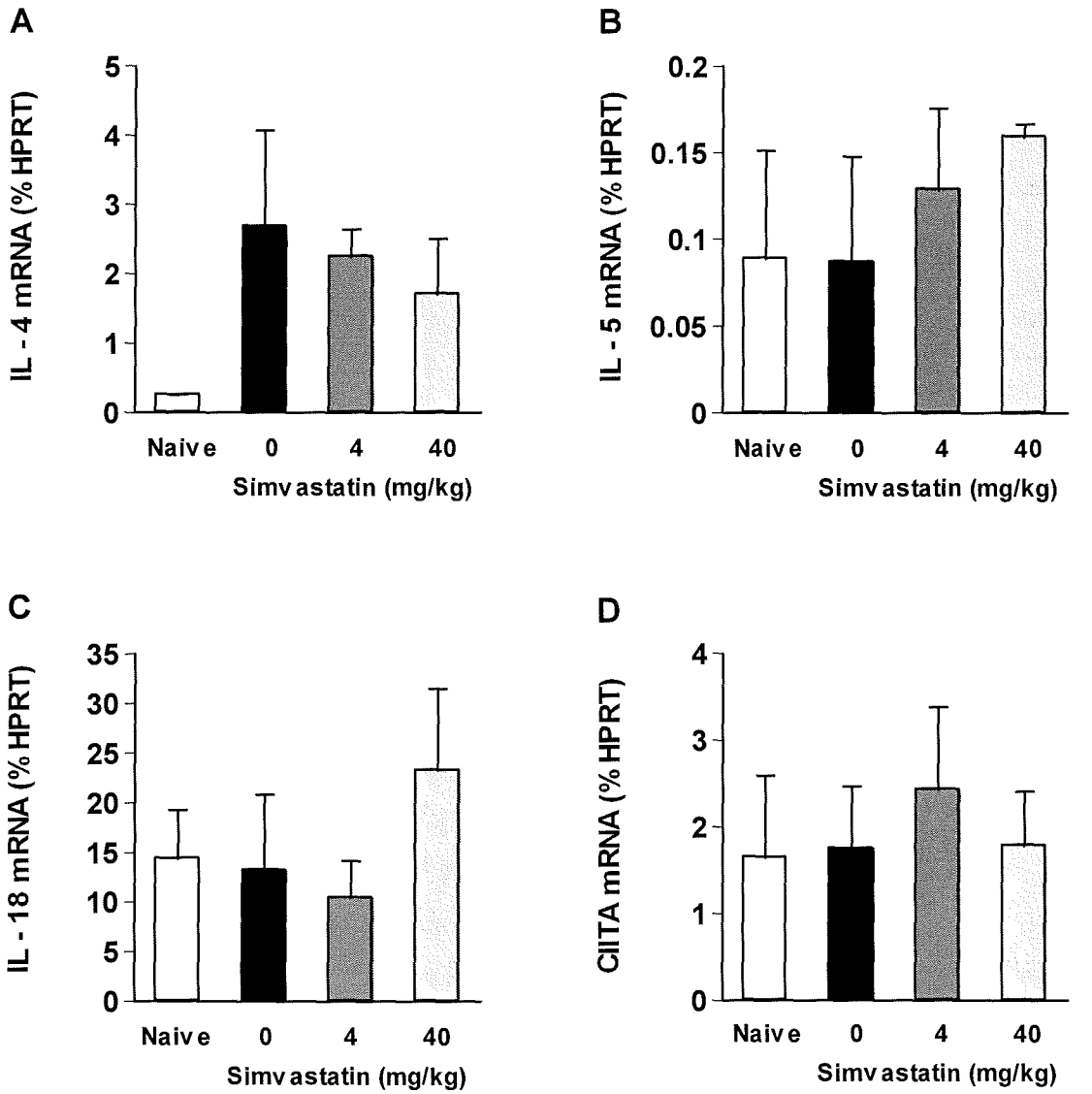


Figure 5.15. Gene expression in thoracic lymph nodes from mice treated with simvastatin intraperitoneally.

The mRNA expression of the cytokines IL-4, IL-5 and IL-18 and the MHC class II transactivator (CIITA) in pooled thoracic lymph nodes (n = 3–5 mice per group) was determined by the use of RT-quantitative PCR on day 28. There was no significant difference in the levels of **(A)** IL-4, **(B)** IL-5, **(C)** IL-18 or **(D)** CIITA mRNA expression in thoracic lymph nodes from naïve mice, OVA-sensitised mice treated with PBS and OVA-senstised mice treated with simvastatin 4 mg/kg or 40 mg/kg intraperitoneally. Data are expressed as mean ± adjusted error.

5.7.2 IL-18 expression in the lungs of mice treated with simvastatin intraperitoneally

As shown above in Section 5.4, IL-18 protein levels in BAL fluid did not significantly differ between naïve mice and OVA-sensitised and challenged mice, or with simvastatin treatment. To assess if this correlated with constant levels of pulmonary IL-18 gene expression, quantitative PCR was used to determine IL-18 mRNA levels in lung tissue. There was a trend for IL-18 mRNA expression to be far higher in antigen-naïve mice, which had been treated with alum and PBS, than in OVA-sensitised mice. Although simvastatin treatment appeared to increase the levels of IL-18 expression, this was not significant when compared to PBS-treated, OVA-challenged mice (shown in Figure 5.16, below).

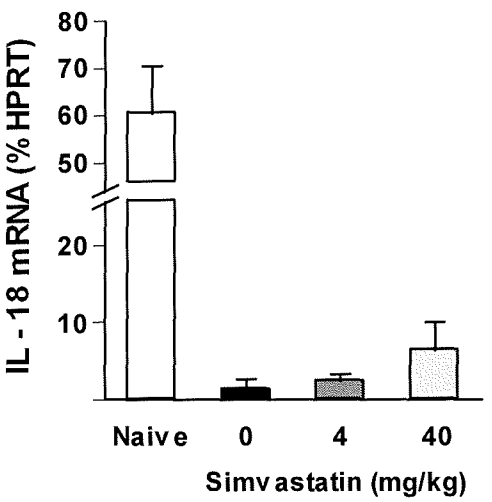


Figure 5.16. IL-18 expression in the lungs of mice treated with simvastatin intraperitoneally.

IL-18 mRNA expression was determined by the use of RT-quantitative PCR on day 28. There was a trend for the levels of IL-18 mRNA expression was significantly reduced in OVA-sensitised and challenged mice compared to naïve controls. Data are expressed as mean ± SEM. (n = 3-6 mice per group). Statistical significance was assessed by ANOVA.

5.8 Chapter discussion

The prevalence of asthma is rising (Umetsu et al., 2002). Consequently, there is an increased need for the development of new agents for its treatment, especially for patients who respond poorly to conventional therapy. In this study we have shown that simvastatin has an effective anti-inflammatory action in a murine model of allergic airway inflammation. In mice previously sensitised to OVA, simvastatin treatment, either orally or intraperitoneally, reduced the total inflammatory cell infiltrate and eosinophilia in bronchoalveolar lavage (BAL) fluid in response to inhaled OVA challenge. Simvastatin therapy i.p. was also associated with a reduction in IL-4 and IL-5 levels in BAL fluid and, at higher doses, a histological reduction in inflammatory infiltrates in the lungs. OVA-induced IL-4 and IL-5 secretion was reduced in thoracic lymph node cultures from simvastatin-treated mice. Simvastatin treatment did not alter serum total IgE or OVA-specific IgG1 and IgG2a levels. Therefore, statins, or similar agents may have potential as therapeutic agents in human asthma.

Simvastatin has previously been shown to have an acute anti-inflammatory action in carrageenan-induced footpad swelling in mice (Sparrow et al., 2001) and in thioglycolate-induced peritoneal inflammation (Weitz-Schmidt et al., 2001). In both of these models the inflammatory infiltrate is predominantly neutrophils. Statins have not previously been shown to have an inhibitory action on eosinophilic infiltration. In this current study, the anti-inflammatory effect of simvastatin is, at least in part, mediated through a suppressive action on T lymphocytes since OVA-specific IL-4, IL-5, IL-6 and IFN-γ secretion were reduced in thoracic lymph node cultures from mice treated with simvastatin intraperitoneally. A reduction in BAL fluid IL-4 and IL-5 levels was also observed in these mice. Similarly, IL-4 and IL-5 secretion in thoracic lymph node cell

cultures in response to OVA was reduced in simvastatin-treated mice; this was not associated with a reduction in IL-4 and IL-5 mRNA expression in thoracic lymph nodes. The reduction in Th2 cytokine production in thoracic lymph node cultures was not accompanied by an increase in the secretion of IFN-γ, a Th1 cytokine. Indeed, IFN-γ production was also reduced in lymph node cultures. There is now evidence that Th1 cells (Hansen et al., 1999, Randolph et al., 1999a, Randolph et al., 1999b) and IFN-γ secretion (Ford et al., 2001) may exacerbate airway inflammation in asthma. Therefore, there may also be suppression of Th1 cells, and hence IFN-γ levels, contributing to the decrease in inflammation seen. This result corresponds with the previous observation in murine collagen-induced arthritis (CIA), where a decrease in Th1 cytokines was not associated with an increase in Th2 cytokine secretion (Leung et al., 2003). This is in contrast to that seen in murine experimental allergic encephalitis (EAE), where statin treatment increased the Th2 bias in antigen-stimulated lymph node cultures (Youssef et al., 2002, Aktas et al., 2003) while reducing a Th1 response. In these studies, atorvastatin was used, and the immunomodulatory effects of this drug may differ from those of simvastatin.

In contrast to the CIA and EAE inflammatory models, a reduction in antigen-induced cell proliferation in lymph node cells was not observed in the present study. These other models were of chronic inflammatory conditions and statin treatment was continued for at least 15 days after the last dose of antigen. In this current study, simvastatin was only given for three days and was not given after the last challenge with OVA. This shorter exposure to statin therapy may explain the failure to suppress cell proliferation. This result suggests there may be divergent mechanisms by which statins inhibit cytokine secretion and cell proliferation.

Simvastatin treatment at a dose of 40 mg/kg i.p. reduced BAL eosinophil and macrophage numbers. This might reflect the reduction in Th2 lymphocytes responses, but a direct suppressive effect of the simvastatin on eosinophils and macrophages cannot be excluded. Eosinophils and macrophages both express LFA-1 and so simvstatin may have a direct effect on trafficking of these cells into the airways (Watkins et al., 1996, Watanabe and Fan, 1998). IL-5-mediated Ras activation is important in eosinophil survival (Hall et al., 2001) and simvastatin may inhibit the activity of this signalling molecule in this model. Statins have also been shown to modify the secretion pro-inflammatory cytokines, such as macrophage chemotactic protein (MCP)-1 and IL-8, in macrophages (Kothe et al., 2000).

Simvastatin has previously been shown to suppress IFN-γ-induced MHC II expression on antigen presenting cells through suppression of the transcription factor CIITA (Kwak et al., 2000). However, CIITA expression in thoracic lymph nodes was not significantly different between simvastatin-treated and PBS-treated mice, suggesting that general suppression of inducible class II MHC expression was not an important contributing factor in the production of these immunosuppressive actions of simvastatin in this inflammatory model.

It was important to establish that simvastatin could have an anti-inflammatory effect when administered orally, since this is the route of administration of the drug in clinical practice. Although oral therapy produced an anti-inflammatory effect, this was less pronounced than with i.p. administration of the same dose. This is likely to be due to first-pass hepatic metabolism of the drug after absorption from the gastrointestinal tract, where several metabolites may be produced (Prueksaritanont et al., 1997) so reducing the effective dose of simvastatin available.

The doses of simvastatin used in this study are higher than those used in man. Statin doses comparable to those used here are commonly used in rat/murine studies (Sparrow et al., 2001, Dimmeler et al., 2001, Ni et al., 2001, Youssef et al., 2002, Aktas et al., 2003) since there is rapid up-regulation of of HMG-CoA reductase with statin treatment in rodents (Kita et al., 1980). In a previous study in murine CIA, a dose of simvastatin 40 mg/kg i.p. did not produce a reduction in cholesterol levels, and liver function tests were similar between placebo and simvastatin-treated mice (Leung et al., 2003).

In conclusion, the current investigations demonstrate that simvastatin treatment is effective in reducing BAL total cellularity and eosinophilia in a murine model of allergic asthma. Treatment with simvastatin also decreases IL-4 andIL-5 levels in BAL fluid when given intraperitoneally and, at higher doses, is associated with a histological reduction in pulmonary inflammatory infiltrates. OVA-induced IL-4 and IL-5 secretion was reduced in thoracic lymph node cultures from simvastatin-treated mice. The immunomodulatory effects of simvastatin are likely to occur through several different anti-inflammatory pathways and these mechanisms require further elucidation. In particular, the effects of simvastatin on sensitisation to antigen and on airway inflammation after prolonged administration will be of interest. Clinical studies are also necessary to assess if simvastatin has therapeutic potential in allergic asthma.

Chapter 6

The anti-inflammatory actions of thymosin beta 4 sulfoxide in a murine model of allergic asthma

6.1 Introduction

The beta thymosins are a group of 5 kDa peptides which have the principal intracellular activity of binding monomeric G-actin to inhibit its polymerisation into F-actin (Nachmias, 1993). Thymosin β4, the most abundant of this group, is released from cells at sites of inflammation and cell damage. Extracellularly, thymosin β4 has anti-inflammatory properties supplementary to its actin-binding ability. Thymosin beta 4 has been shown to promote keratinocyte migration *in vitro* and accelerate skin wound healing in animal models (Malinda et al., 1999, Philp et al., 2003). Similarly, thymosin β4 stimulated conjunctival cell migration and corneal wound healing (Sosne et al., 2001, Sosne et al., 2002a, Sosne et al., 2002b). Oxidation of the methionine residue at position 6 of thymosin β4 forms thymosin β4 sulfoxide (Tβ4SO), which has been identified as the factor reducing fMLP-induced neutrophil chemotaxis in supernatants derived from steroid-treated monocytes *in vitro* (Chettibi et al., 1994, Young et al., 1999). The oxidation of thymosin β4 to Tβ4SO greatly enhances its wound-healing ability in an *in vitro* assay, and also its anti-inflammatory actions in carrageenen-induced footpad inflammation in mice, a neutrophil-predominant inflammatory model (Young et al., 1999). LTβ4, a splice variant of thymosin β4 derived from lymphocytes, which contains two oxidised methionine residues, had anti-inflammatory activity in murine models of the T lymphocyte mediated-conditions, irritant contact dermatitis and allergic contact dermatitis (Girardi et al., 2003).

Glucocorticoids are widely used as anti-inflammatory agents in the treatment of human asthma. They suppress the production of pro-inflammatory cytokines such as IL-4, IL-5 (Robinson et al., 1993) and IL-13 (Naseer et al., 1997) by down-regulating their gene

expression and decrease the numbers of inflammatory cells present in the airways (Barnes and Adcock, 2003).

Neutrophils are the predominant inflammatory cell observed in carrageenin-induced footpad inflammation and in models of wound healing. Since eosinophils are also of granulocytic lineage, it was postulated that Tβ4SO may have an anti-inflammatory action in a murine model of allergic asthma, an eosinophil-predominant inflammatory model. The ability of glucocorticoids to induce the release of Tβ4SO from monocytes may contribute to their anti-inflammatory properties, and so the effect of Tβ4SO on airway inflammation was compared to that of the glucocorticoid dexamethasone.

6.2 The effect of thymosin beta 4 sulfoxide on bronchoalveolar lavage cellularity

The anti-inflammatory effects of Tβ4SO were assessed using the same murine asthma model as in the previous chapters. The protocol for OVA-sensitisation and challenge is detailed in Figure 2.1. Mice were treated with either Tβ4SO 20 μg (an approximate dose of 1 μg/g) or dexamethasone 3 mg/kg by i.p. injection thirty minutes before the intra-nasal challenge with OVA on days 25 to 27. Control mice were treated with PBS. Experiments were terminated on day 28.

6.2.1. Total bronchoalveolar lavage cellularity

Treatment with Tβ4SO resulted in a significant reduction in the total number of cells present in BAL fluid when compared to control mice which had been treated with PBS alone (Figure 6.1, $p < 0.01$). Dexamethasone treatment also significantly reduced BAL total cellularity ($p < 0.05$).

6.2.2 Bronchoalveolar lavage differential cell count

Differential cell counts were performed on the BAL fluid samples to determine which cell types in lavage fluid were being inhibited by Tβ4SO and dexamethasone. Both these compounds significantly suppressed eosinophil numbers (Figure 6.2*A*, $p < 0.01$ for Tβ4SO and dexamethasone). Tβ4SO also significantly reduced macrophage (Figure 2*B*, $p < 0.05$) and neutrophils (Figure 6.2*D*, $p < 0.05$), but dexamethasone did not. In contrast, the amount of lymphocytes present in lavage fluid was reduced by dexamethasone (Figure 6.2*C*, $p < 0.05$) but not Tβ4SO.

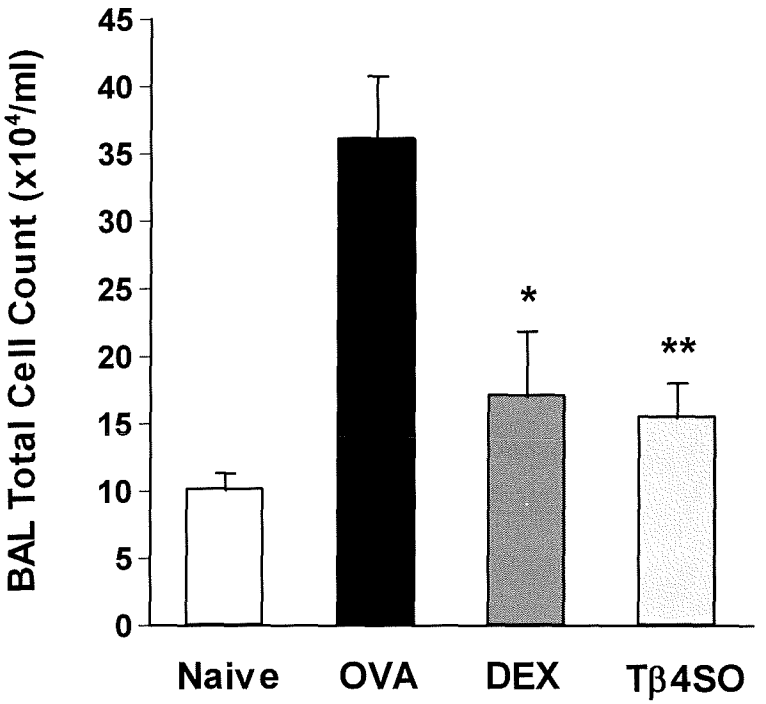


Figure 6.1. Total bronchoalveolar lavage cellularity after treatment with dexamethasone and thymosin β4 sulfoxide.

BALB/c mice were sensitised with OVA and then challenged with OVA intranasally on three consecutive days from day 25 - 27. Dexamethasone (DEX) 3 mg/kg or thymosin β4 sulfoxide (Tβ4SO) 20 μg were given 30 minutes before each antigen challenge. Control mice were given PBS (OVA). Bronchoalveolar (BAL) cell counts were performed on day 28. Total BAL cellularity was significantly reduced by dexamethasone and Tβ4SO treatment. Data are expressed as mean ± SEM. (n = 6 – 11 mice per group). * $p < 0.05$, ** $p < 0.01$. Statistical significance was assessed by ANOVA.

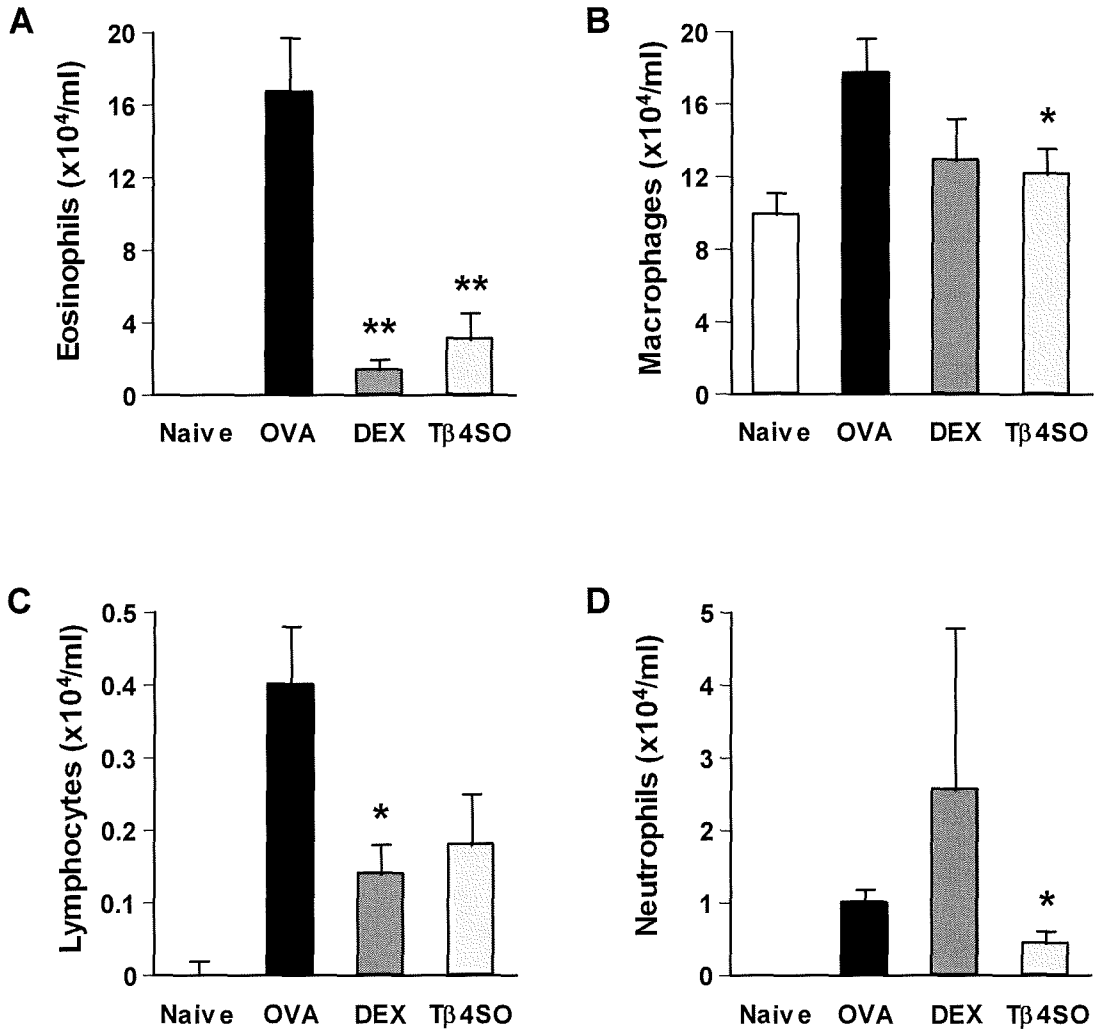


Figure 6.2. Bronchoalveolar lavage differential cell count after treatment with dexamethasone and thymosin β4 sulfoxide.

Differential cell counts were determined on the bronchoalveolar (BAL) cell counts performed on day 28. BAL (**A**) eosinophils were significantly reduced by both dexamethasone (DEX) 3 mg/kg and thymosin β4 sulfoxide (Tβ4SO) 20 μg treatment when compared to PBS-treated controls (OVA). **(B)** Macrophage and **(D)** neutrophil numbers were reduced by thymosin β4 sulfoxide whereas **(C)** BAL lymphocytes were suppressed by dexamethasone treatment. Data are expressed as mean ± SEM. (n = 6 – 11 mice per group). * $p < 0.05$, ** $p < 0.01$. Statistical significance was assessed by ANOVA.

6.3 Histological analysis of lung inflammatory infiltrates

Histological analysis of lung tissue was done to assess if the reduction in BAL cellularity and eosinophilia was representative of a decrease in total lung inflammation. Representative tissue sections from each of the groups are shown in Figure 6.3. OVA-sensitised and challenged mice which received PBS as control treatment (Figure 6.3*B*) had peribronchial and perivascular inflammatory infiltrates containing eosinophils, and associated mucosal hyperplasia in the bronchial walls. Mice treated with dexamethasone (Figure 6.3*C*) or Tβ4SO (Figure 6.3*D*) had a reduction in lung inflammatory infiltrates. However, on histological scoring, although there was a significant decrease in perivascular inflammation with dexamethasone treatment ($p < 0.05$), the reduction observed in peribronchial and total lung inflammatory scores following treatment with either dexamethasone or Tβ4SO did not reach statistical significance (Figure 6.4).

6.4 Bronchoalveolar lavage cytokines

Treatment with thymosin β4 intraperitoneally in a murine model of endotoxic shock reduced serum concentrations of IL-1α and TNFα levels (Badamchian et al., 2003). To determine if the anti-inflammatory effects of Tβ4SO were mediated by alterations in cytokine production, BAL IL-4, IL-5, IL-6 and IFN-γ levels were measured. Treatment with Tβ4SO and dexamethasone significantly reduced IL-4 and IL-5 concentrations in BAL fluid (Figure 6.5*A* and *B*, $p< 0.01$ for both Tβ4SO and dexamethasone). Although a reduction in IL-6 was observed (Figure 6.5*C*), this was not statistically significant. IFN-γ was not detected in the lavage fluid (data not shown).

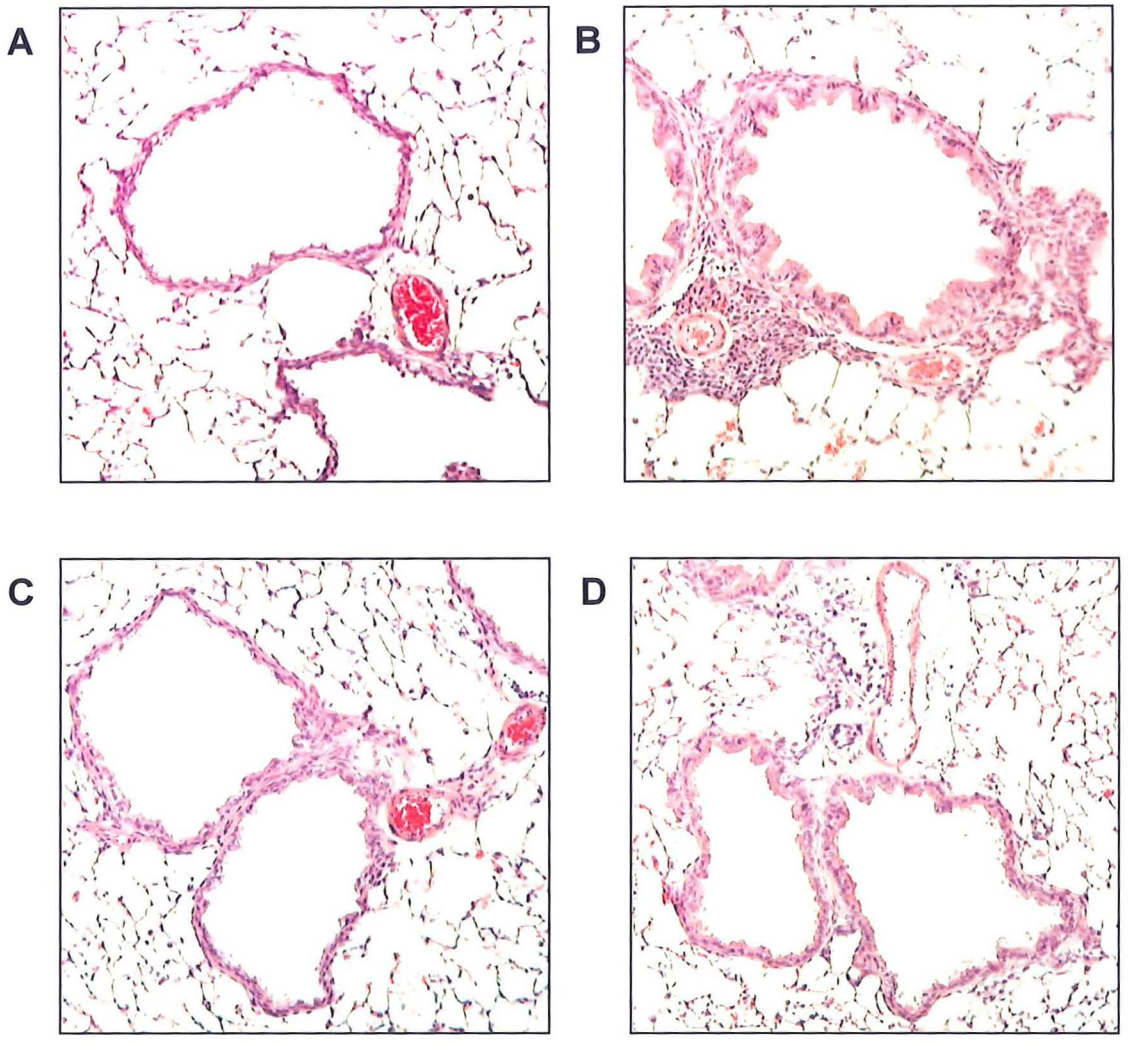


Figure 6.3. Histological evidence of decreased lung inflammation in mice treated with dexamethasone and thymosin β4 sulfoxide.

A representative section is shown from each group of mice (n = 3 mice per group). **(A)** Naïve mouse given a PBS challenge, **(B)** OVA-challenged mouse; peribronchial and perivascular infiltrates are seen, with eosinophils present, and mucosal hyperplasia. **(C)** OVA-challenged mouse plus treatment with dexamethasone 3 mg/kg and **(D)** OVA-challenged mouse plus treatment with thymosin β4 sulfoxide 20 μg; both these groups show a reduction in inflammatory infiltrates compared with **(B).** H&E staining, magnification x200.

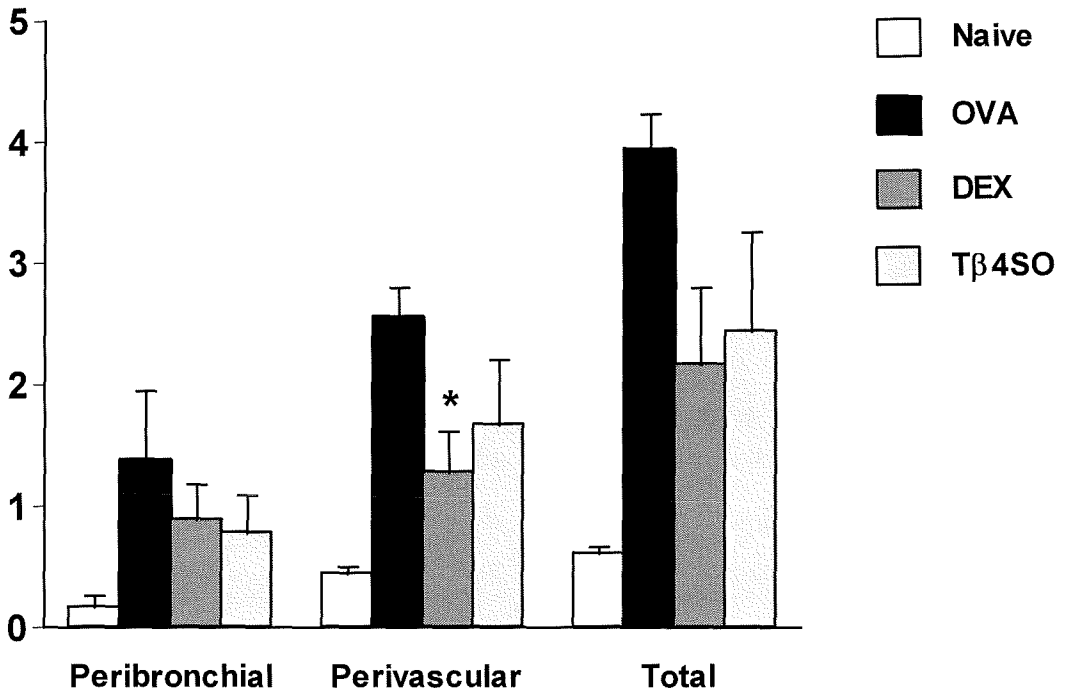


Figure 6.4. Histological score of lung inflammation in mice treated with dexamethasone and thymosin beta 4 sulfoxide.

Total lung inflammation on histological sections was determined as the sum of peribronchial and perivascular scores. Inflammation was graded on the scale 0, none; 1, mild, 2, moderate; 3, marked and 4, severe. An increment of 0.5 was used when severity of inflammation fell between two integers. Intraperitoneal treatment with dexamethasone (DEX) 3 mg/kg, but not thymosin beta 4 sulfoxide (Tβ4SO) 20 μg produced a significant reduction in perivascular when compared to PBS-treated mice (OVA). Peribronchial and total lung inflammatory scores were not significantly different in mice treated with dexamethasone or thymosin β4 sulfoxide from control mice given PBS. Data are expressed as mean ± SEM. * $p < 0.05$. (n = 3 mice per group). Statistical significance was assessed by Student's *t* test.

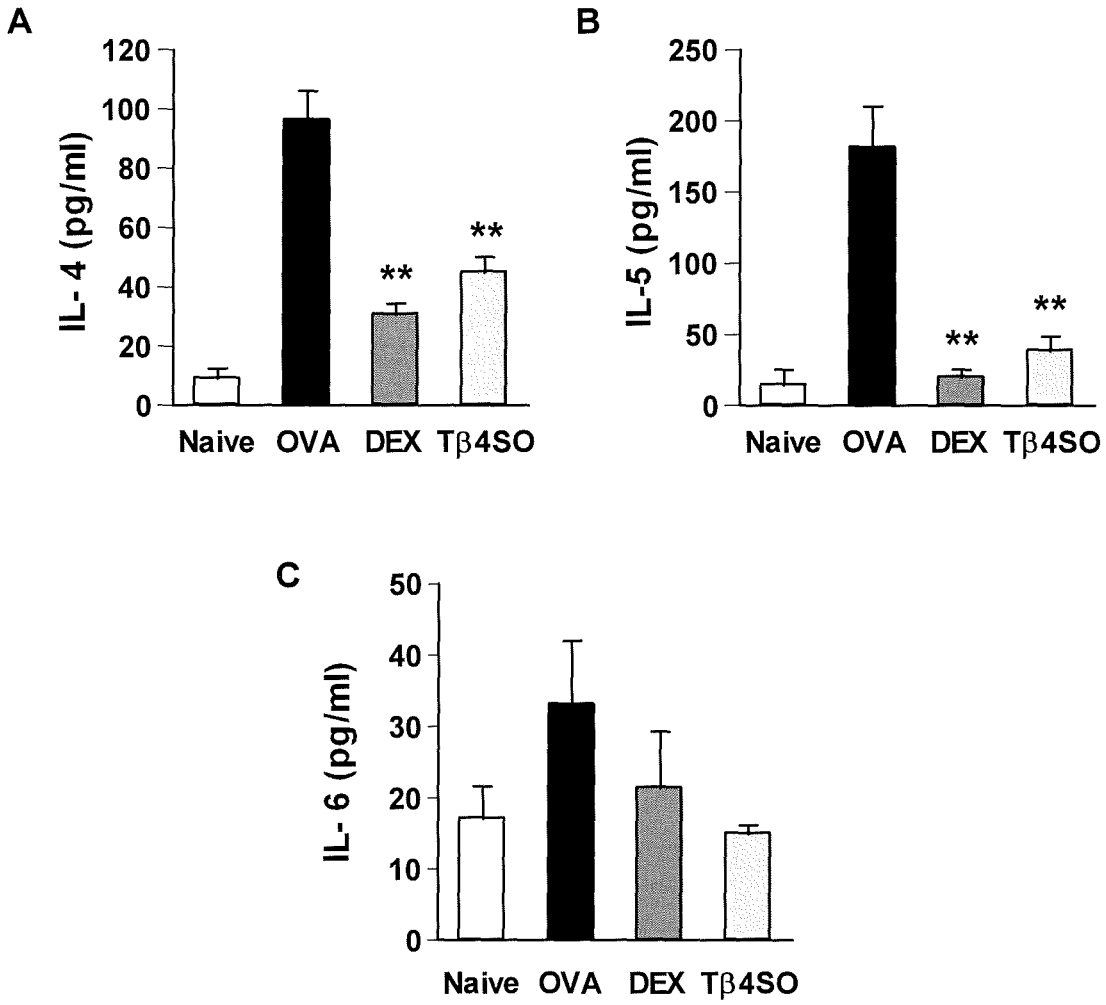


Figure 6.5. Broncholavelolar lavage cytokine levels in mice treated with dexamethasone and thymosin β4 sulfoxide.

Bronchalveolar lavage (BAL) IL-4, IL-5 and IL-6 levels were determined by ELISA. **(A)** IL-4 levels and **(B)** IL-5 levels were significantly reduced by treatment with dexamethasone (DEX) 3mg/kg and thymosin β4 sulfoxide (Tβ4SO) 20 μg compared to PBS-treated controls (OVA). The reduction seen in **(C)** IL-6 levels was not significant. Data are expressed as mean ± SEM. (n = 6 -11 mice per group). ** $p < 0.01$. Statistical significance was assessed by ANOVA.

6.4 Cytokine mRNA expression in lung tissue

Since IL-4 and IL-5 levels were reduced in BAL fluid, the levels of the mRNA were assessed by quantitative RT-PCR to assess if alterations in gene expression accompanied the changes the levels of the cytokines detected by ELISA. Since IL-13 is an important cytokine in Th2 responses in asthma, the levels of its mRNA were also assessed. In *in vitro* models of corneal wound healing, Tβ4 treatment produced a rise in IL-18 mRNA levels 6 hours after treatment, but down-regulated its expression after 24 hours (Sosne et al, 2001); IL-18 mRNA levels were therefore measured to determine if Tβ4SO treatment altered its expression. The levels of IL-4 and IL-5 mRNA did not reflect the cytokine levels measured in BAL fluid (Figure 6.6*A* and *B*). There was a trend for the levels of levels of IL-4, IL-5 and IL-13 (Figure 6.6*C*) to be higher in mice which had been OVA-sensitised and OVA-challenged but Tβ4SO and dexamethasone treatment did not alter their levels of expression. IL-18 mRNA (Figure 6.6*D*) was relatively highly expressed in lung tissue and was greatly reduced in OVA-sensitised and OVA-challenged mice when compared to naïve controls (PBS-treated controls, $p < 0.05$, dexamethasone $p < 0.05$ and Tβ4SO $p < 0.01$). Although the expression of IL-18 was increased in the Tβ4SO-treated group, this was not significant when compared to the PBS-treated controls or dexamethasone-treated group.

6.5 Serum immunoglobulins

Figure 6.7 shows the serum total IgE levels and IgG subclass profiles. Treatment with Tβ4SO or dexamethasone did not significantly alter serum total IgE levels, or those of OVA-specific IgG1 or IgG2a. All OVA treated groups had elevated levels of these immunoglobulins when compared to the naïve mice.

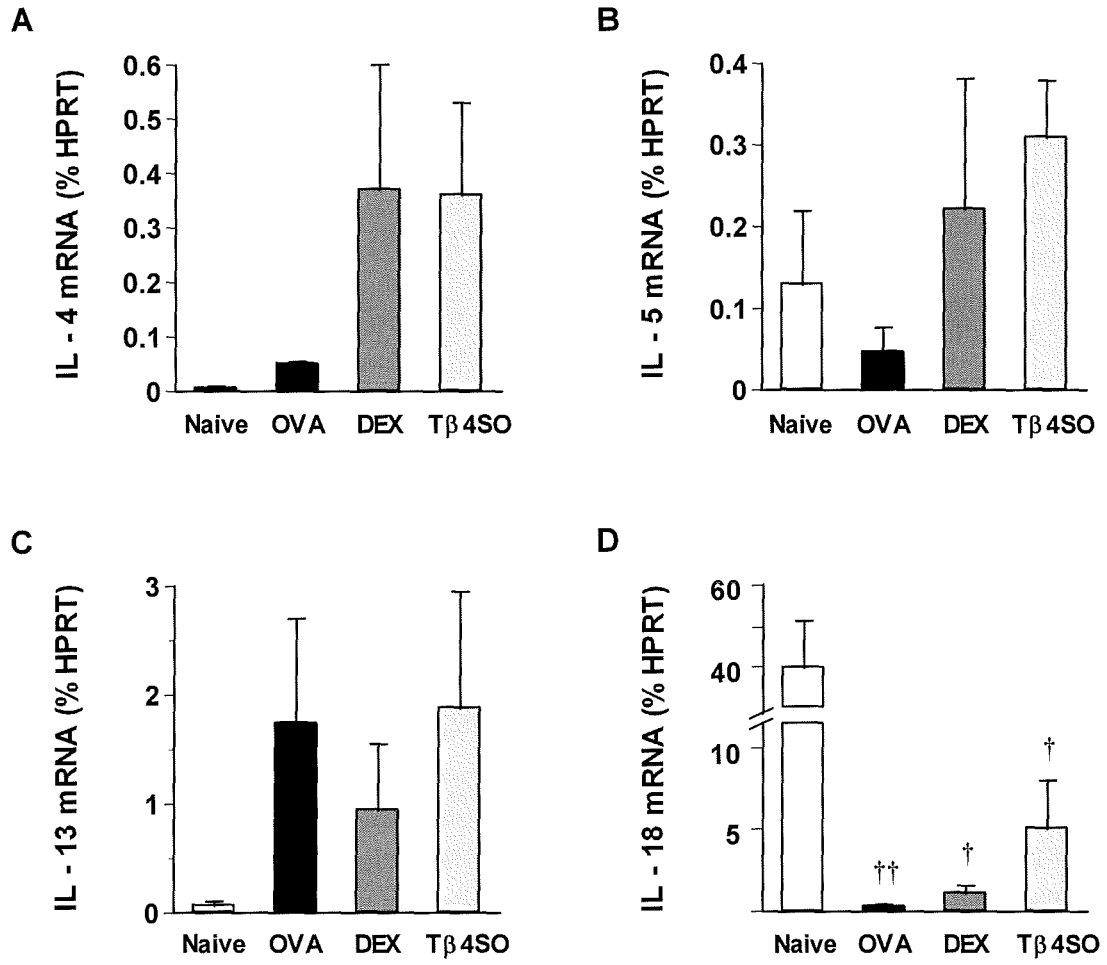


Figure 6.6. Lung cytokine expression in mice treated with dexamethasone and thymosin β4 sulfoxide.

Lung IL-4, IL-5, IL-13 and IL-18 mRNA expression was determined by the use of RT-quantitative PCR on day 28. There was a trend for the levels of **(A)** IL-4, **(B)** IL-5 and **(C)** IL-13 mRNA levels to be increased the groups which had been sensitised and challenged with OVA compared to naïve mice; dexamethasone (DEX) 3 mg/kg or thymosin β4 sulfoxide (Tβ4SO) 20 μg treatment did not alter the mRNA levels for these cytokines compared to mice treated with PBS alone (OVA). **(D)** IL-18 mRNA expression was significantly reduced in OVA-sensistised and challenged mice compared to naïve controls. Data are expressed as mean ± SEM. (n = 3-5 mice per group). † $p < 0.05$, †† $p < 0.01$. Statistical significance was assessed by ANOVA.

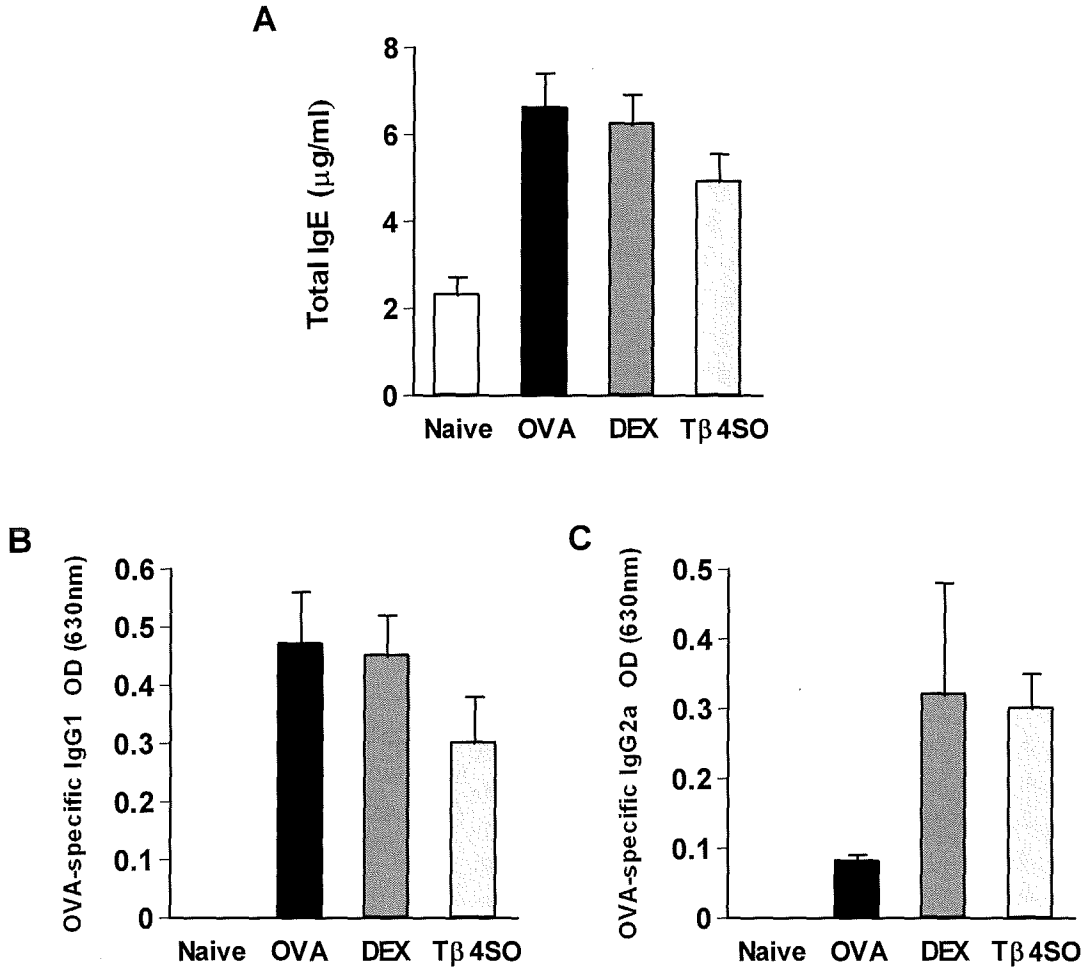


Figure 6.7. Serum immunoglobulin levels in mice treated with dexamethasone and thymosin β4 sulfoxide.

Serum immunoglobulin levels were assessed by ELISA. Treatment with dexamethasone (DEX) 3 mg/kg or thymosin β4 sulfoxide (Tβ4SO) 20 μg did not alter **(A)** serum total IgE levels (n = 6 - 11 mice per group) when compared to PBS-treated controls. **(B)** Serum OVA-specific IgG1 (serum dilution 1/60,000) and **(C)** serum OVA-specific IgG2a (serum dilution 1/400) levels were also unaffected by treatment with dexamethasone or thymosin β4 sulfoxide compared to mice treated with PBS alone (OVA) (n = 6 mice per group). Data are expressed as mean ± SEM. Statistical significance was assessed by Student's *t* test.

6.7 *In vitro* thoracic lymph node cell responses following the *in vivo* administration of thymosin beta 4 sulfoxide and dexamethasone

The reduction IL-4 and IL-5 levels in BAL fluid suggested that Th2 lymphocyte responses were being suppressed. To investigate this further, cells isolated from thoracic lymph nodes of mice previously treated with Tβ4SO or dexamethasone were cultured and *in vitro* OVA-induced cell proliferation and cytokine production assessed.

6.7.1 *In vitro* OVA-induced cell proliferation in thoracic lymph node cells

The OVA-induced cell proliferation responses in thoracic lymph node cultures are illustrated in Figure 6.8*A*. Cell proliferation in response to OVA was reduced in lymph node cells taken from mice treated with Tβ4SO ($p < 0.01$) and dexamethasone ($p < 0.01$) when compared to PBS-treated controls. Prior treatment with Tβ4SO and dexamethasone also reduced spontaneous cell proliferation when compared to that of PBS treated controls (Tβ4SO $p < 0.05$, dexamethasone $p < 0.05$). This response is antigen-specific, since cell proliferation to Concanavalin (Con) A was the same in all groups (Figure 6.8*B).*

6.7.2 *In vitro* OVA-induced cytokine production in thoracic lymph node cells

The OVA-induced cytokine responses, from parallel cultures to the one described in Section 6.7.2, are shown in Figures 6.9. Both Tβ4SO and dexamethasone treatment significantly reduced the production of OVA-induced IL-4 (Figure 6.9*A*: Tβ4SO $p < 0.01$, dexamethasone $p < 0.01$), IL-5 (Figure 6.8*B*: Tβ4SO $p < 0.01$, dexamethasone $p < 0.01$), IFN-γ (Figure 6.8*C*: Tβ4SO $p < 0.05$, dexamethasone $p < 0.01$) and IL-6 (Figure

6.8*D*: Tβ4SO $p < 0.01$, dexamethasone $p < 0.01$) when compared to the response in lymph node cells from OVA-sensitised and challenged mice treated with PBS alone. The reductions in cytokine responses were not OVA-specific since cytokine production induced by Con A (Figure 6.10) was also reduced, although to a proportionately lesser extent than that seen in the OVA-induced cytokine responses.

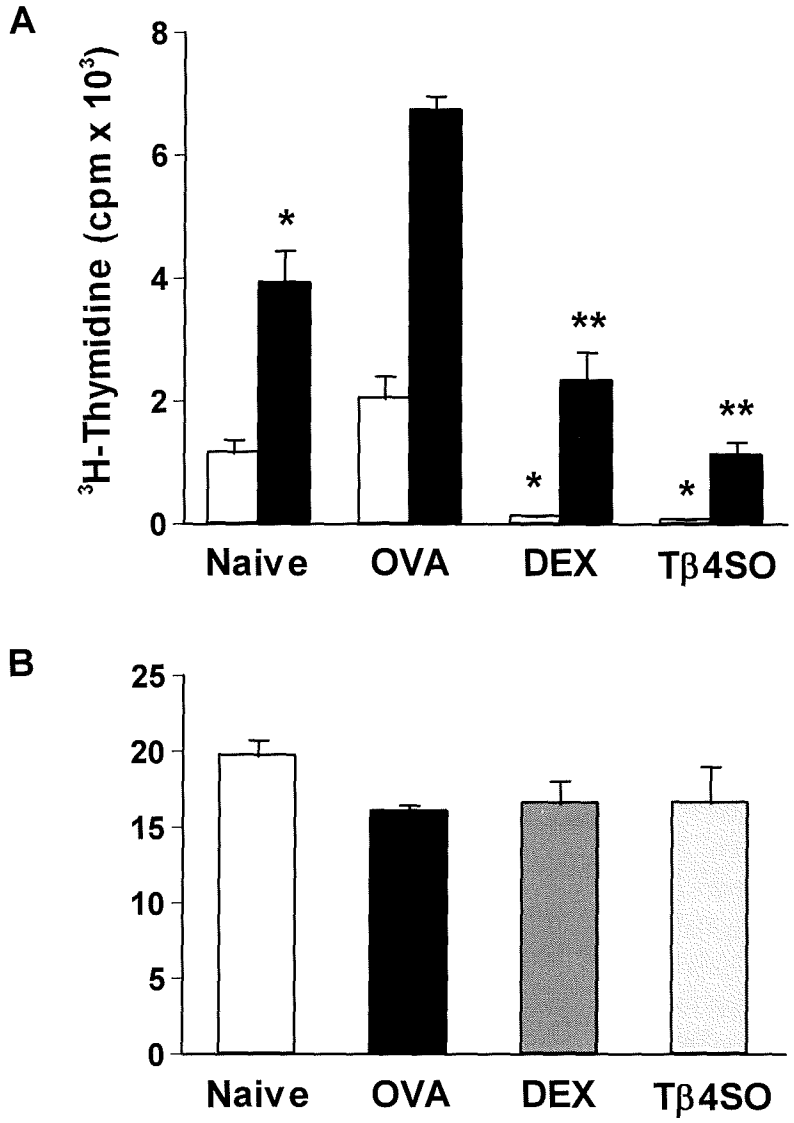


Figure 6.8. Proliferative responses in thoracic lymph nodes cells *in vitro*.

Thoracic lymph node cells (n = 5 mice per group) were harvested from mice on day 28 and cultured for 96 hours with **(A)** medium alone (□) or OVA (■; 500 μg/ml) or **(B)** 5 μg/ml Concanavalin (Con) A. T cell proliferation was assessed by uptake of [^{3}H]-thymidine. **(A)** Mice treated with dexamethasone (DEX) 3 mg/kg or thymosin β4 sulfoxide (Tβ4SO) 20 μg had a significant reduction in OVA-induced cell proliferation and spontaneous cell proliferation when compared to mice treated with PBS alone (OVA). **(B)** The proliferative response to Con A was the same in all groups. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$. Statistical significance was assessed by ANOVA.

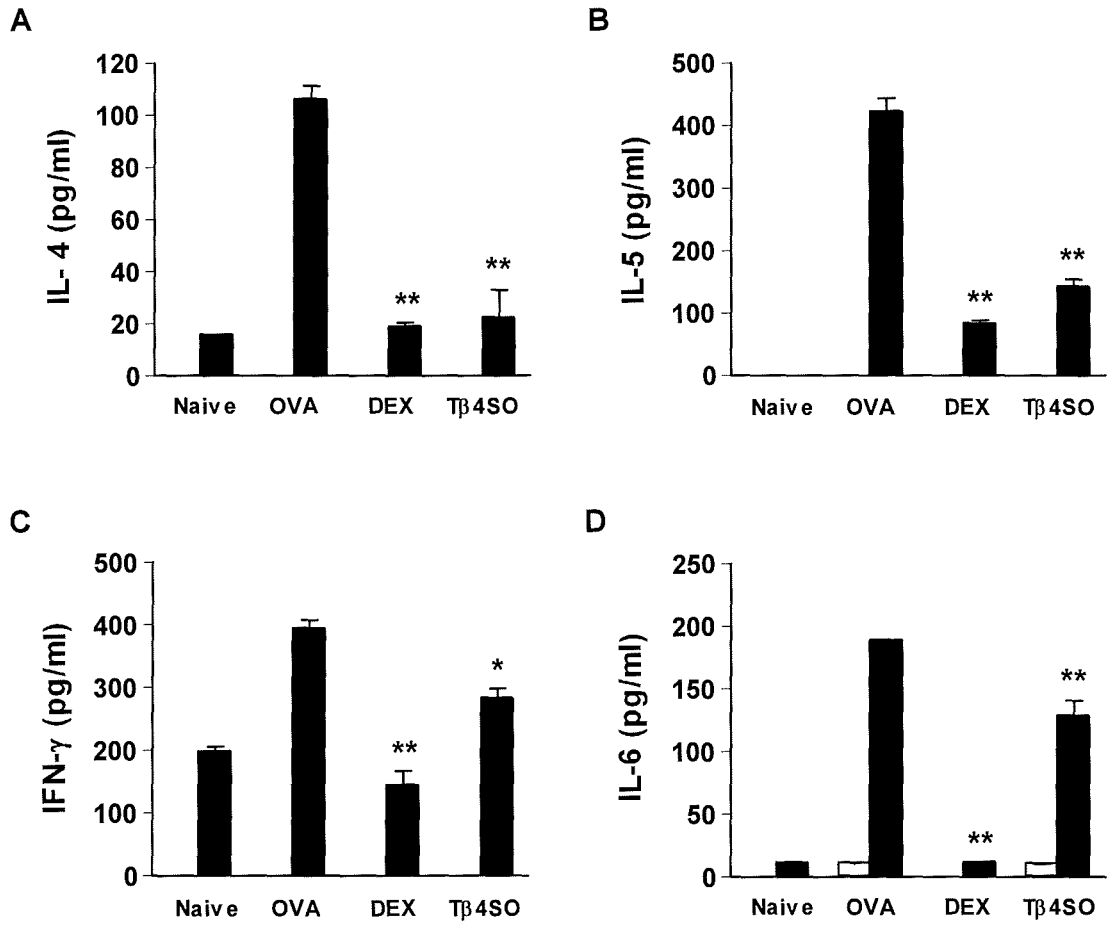


Figure 6.9. OVA-induced cytokine responses in thoracic lymph node cells *in vitro*.

In parallel cultures to those in Figure 6.8, thoracic lymph node cells (n = 5 mice per group) were harvested from mice on day 28 and cultured for 72 hours with medium alone (□) or OVA (■; 500 μg/ml). Cytokine levels in cell culture supernatants were assessed by ELISA. **(A)** IL-4, **(B)** IL-5, **(C)** IFN-γ and **(D)** IL-6 production in response to OVA were all significantly reduced in lymph node cells from mice treated with dexamethasone (DEX) 3 mg/kg and thymosin β4 sulfoxide (Tβ4SO) 20 μg, when compared to cells from mice treated with PBS alone (OVA). Data are expressed as mean ± SEM.* $p < 0.05$, ** $p < 0.01$. Statistical significance was assessed by ANOVA.

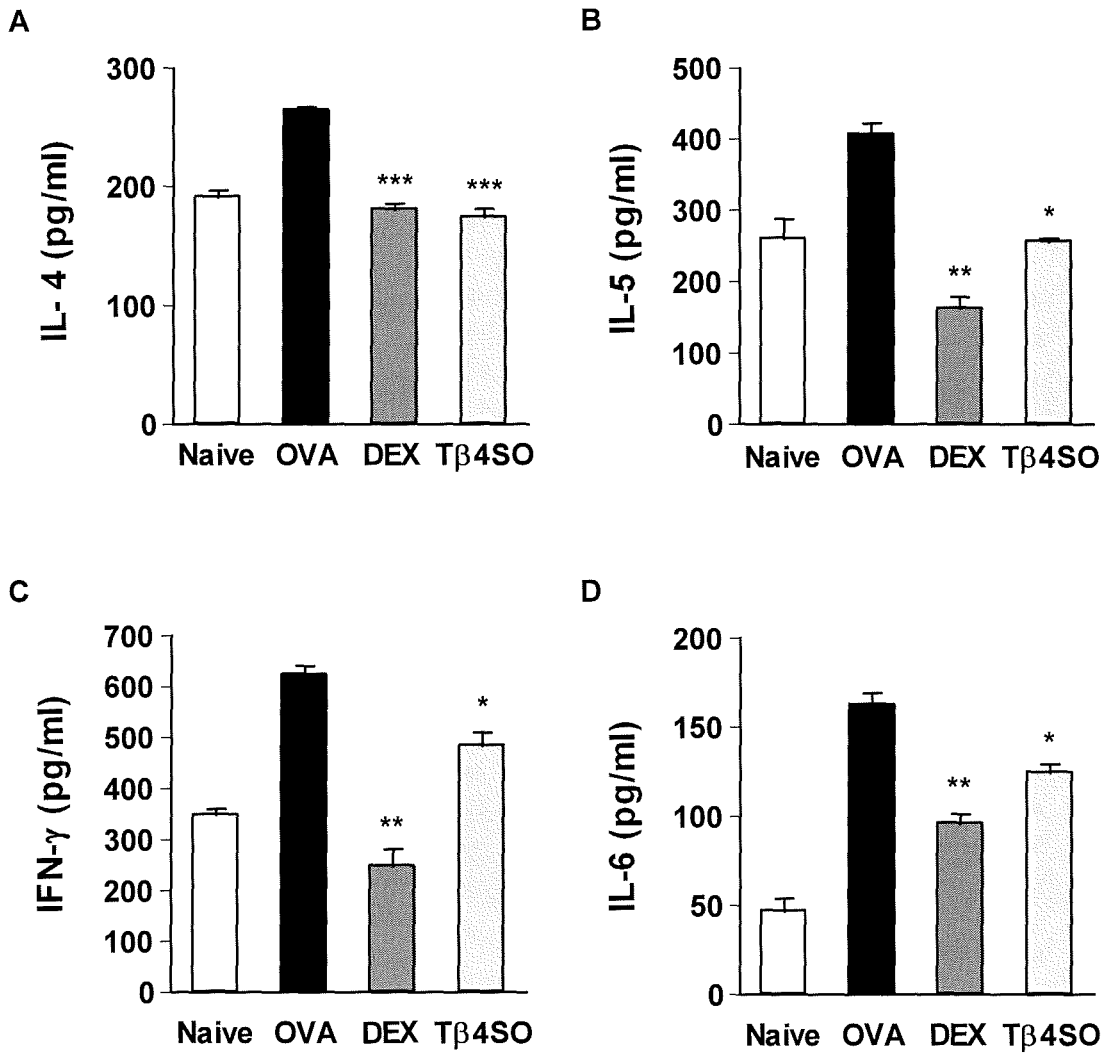


Figure 6.10. Concanavalin A-induced cytokine responses in thoracic lymph node cells *in vitro*.

In parallel cultures to those in Figure 6.9, thoracic lymph node cells (n = 5 mice per group) cultured for 72 hours with Concanavalin (Con) A 5 μg/ml. Cytokine levels in cell culture supernatants were assessed by ELISA. **(A)** IL-4, **(B)** IL-5, **(C)** IFN-γ and **(D)** IL-6 production in responses to Con A were all significantly reduced in lymph node cells from mice treated with dexamethasone (DEX) 3 mg/kg and thymosin β4 sulfoxide (Tβ4SO) 20 μg, when compared to cells from mice treated with PBS alone (OVA). Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistical significance was assessed by ANOVA.

6.8 Comparison of the anti-inflammatory activities of thymosin beta 4 and thymosin β4 sulfoxide

In the study of Young et al. (1999) it was proposed that Tβ4SO was the active anti-inflammatory component of thymosin β4 and that any anti-inflammatory action of the non-oxidised thymosin β4 was due to contamination of the preparation with Tβ4SO, either during preparation of the compound or spontaneous oxidation during storage. A provisional experiment was done to assess if Tβ4SO reduced inflammation in comparison to Tβ4.

These results are presented in Figure 6.11. Total absolute BAL cell count numbers were reduced in mice treated with Tβ4SO ($p < 0.05$). The percentage of eosinophils was also significantly lower in Tβ4SO treated mice ($p < 0.01$). There was a reciprocal rise in the percentage of BAL macrophages in the group of mice which received Tβ4SO. There was no difference in percentages of neutrophils or lymphocytes between the two groups (data not shown). Although not strictly comparable, the total cell count in the Tβ4-treated group (mean ± SEM: 30.6 ± 3.1 x 10^4) was similar to that of the PBS-treated control group in the initial experiment in section 6.2 (30.1 ± 4 x 10^4 cells per lavage) while that of Tβ4SO mice was lower (22.5 ± 2 x 10^4 cells per lavage). The percentage eosinophils was also comparable (Tβ4-treated 43.1 ± 4.29 % *vs* PBS-treated controls 48 ± 3.1 %) with the Tβ4SO-treated group in this experiment having a lower percentage eosinophil count (33.5 ± 3.9%). This would suggest that Tβ4SO has anti-inflammatory activity while Tβ4 does not.

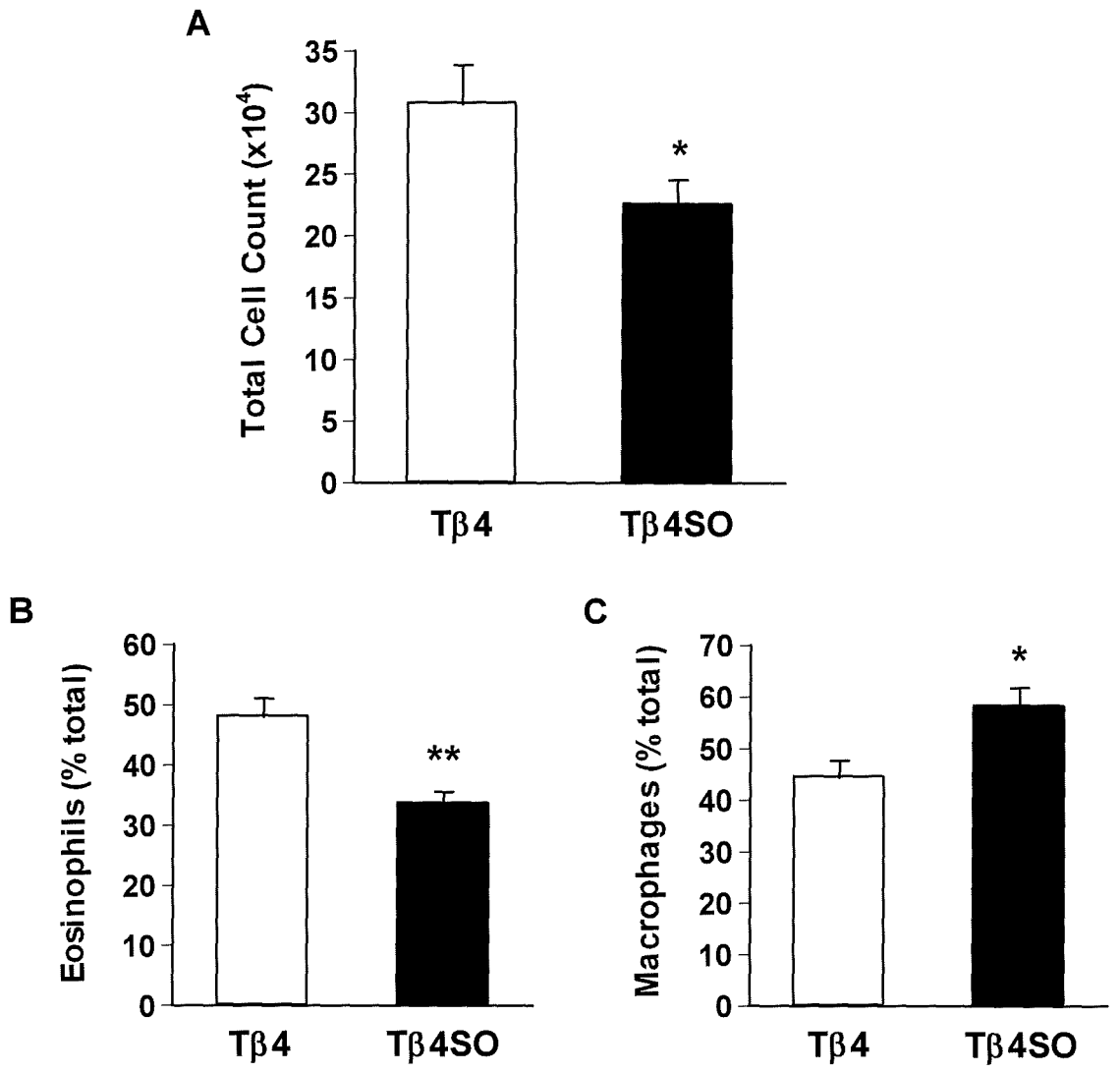


Figure 6.11. Bronchoalveolar lavage total cellularity, and eosinophil and macrophage proportions in mice treated with thymosin β4 or thymosin β4 sulfoxide.

BALB/c mice were sensitised with OVA and then challenged with OVA i.n. on three consecutive days from day 25-27. Thymosin β4 (Tβ4) 20 μg (n = 10) or thymosin β4 sulfoxide (Tβ4SO) 20 μg (n = 11) were given 30 minutes before each antigen challenge. BAL total cell counts and differential counts were performed on day 28. **(A)** Total BAL cellularity and **(B)** percentage eosinophilia were significantly reduced in mice treated with thymosin β4 sulfoxide (**C**) The percentage of BAL macrophages is higher in mice treated with thymosin β4 sulfoxide. Data is expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$. Statistical significance was assessed by ANOVA.

6.9 Chapter discussion

The above data confirm that thymosin β4 sulfoxide (Tβ4SO) has anti-inflammatory properties. In this murine model of asthma, Tβ4SO and dexamethasone reduced BAL total cell count and eosinophil numbers to a similar extent. This was not associated with a significant reduction in inflammatory infiltrates in tissue histology. Prior treatment with both Tβ4SO and dexamethasone significantly reduced levels of IL-4 and IL-5 in the bronchoalveolar lavage fluid of treated mice and also reduced OVA-induced production of these cytokines in cultures of thoracic lymph node cells. Tβ4SO treatment reduced BAL total cellularity and eosinophilia when compared to thymosin β4.

In the above experiments, treatment with both Tβ4SO and dexamethasone suppressed BAL eosinophil numbers in this mouse asthma model. This was not associated with a significant reduction in inflammatory infiltrates in lung histology. This may reflect the fact that only low numbers of tissue sections (n = 3 mice per group) were taken from each group of mice; to detect a difference in histological scoring between Tβ4SO-treated mice and mice treated with PBS alone would require 7 - 14 mice per group, for α level 0.05 and a β level of 0.8, for the standard deviations observed for these groups in this study. Higher doses of Tβ4SO and dexamethasone, or a longer duration of treatment, may be required to suppress tissue inflammatory infiltrates.

In addition to decreased eosinophilia in the BAL fluid, a reduction in BAL macrophage and neutrophil numbers was observed in Tβ4SO-treated mice but not those treated with dexamethasone. In human asthma, neutrophilic airway inflammation is relatively steroid-resistant and corticosteroids have been demonstrated to be ineffective in reducing airway neutrophilia in severe asthmatics (Wenzel et al., 1997). Corticosteroids

prevent neutrophil apoptosis while enhancing that of eosinophils (Meagher et al., 1996). This may explain why neutrophil levels are not reduced by dexamethasone treatment in the murine model of asthma used above. Infection may also produce a neutrophilia which would be unresponsive to steroids, but infection seldom developed in experiments with this murine asthma model. Treatment with Tβ4SO, but not dexamethasone, also led to a significant decrease in BAL macrophage numbers. In contrast, lymphocyte numbers were decreased by dexamethasone, but not by Tβ4SO. Corticosteroids have several suppressive actions on lymphocytes, including the inhibition of cell migration (Pitzalis et al., 1997), down-regulation of activation markers (Almawi et al., 1996) and the enhancement of apoptosis (Zacharchuk et al., 1990). All the mechanisms may contribute to the decreased level of lymphocytes seen in dexamethasone treated mice.

Although Tβ4SO treatment did not significantly reduce lymphocyte numbers in BAL fluid, the reduction in BAL IL-4 and IL-5 levels observed in the above study suggested that Tβ4SO may be having an effect on T lymphocyte function in this murine asthma model. This is consistent with the observation that LTβ4, a compound related to Tβ4SO, has been shown to reduce inflammatory responses in animal models of the T cell-mediated conditions of irritant contact dermatitis and allergic contact dermatitis (Girardi et al., 2003). A suppressive action for Tβ4SO on lymphocyte activity was confirmed by the *in vitro* studies on thoracic lymph node cells. Tβ4SO and dexamethasone both reduced OVA-induced cell proliferation in thoracic lymph node cultures. Their anti-proliferative response was antigen-specific since it did not alter the proliferative response to the T cell mitogen Concanavalin (Con) A. Both Tβ4SO and dexamethasone also suppressed spontaneous lymph node cell proliferation, indicating that this suppressive action on lymphocytes is also occurring *in vivo*. Thymosin beta 4

sulfoxide and dexamethasone were also able to suppress OVA-induced IL-4, IL-5, IFN-γ and IL-6 cytokine production. This was part of a more general immunosuppressive action on cytokine production since cytokine secretion in response to Con A was also reduced, but to a proportionately lesser extent. For dexamethasone, this is consistent with the fact that glucocorticoid-mediated down-regulation of cytokine production does not involve signalling via T-cell receptor activated pathways, but requires direct binding to promoter regions of cytokine genes (Almawi et al., 1996). Since Tβ4SO reduces cytokine production and cell proliferation in a similar pattern to that of dexamethasone, it may activate similar signalling mechanisms. The ability of Tβ4SO to reduce OVA-induced cell proliferation indicates that it is able to influence antigen specific-responses in lymphocytes, but whether this is a direct action or a consequence of alteration of down-stream signalling processes remains to be elucidated.

The above results indicate that divergent pathways are involved in the effects of dexamethasone and Tβ4SO on lymphocyte proliferation and cytokine production, since Con A can overcome the suppressive action of these agents on cell proliferation but not cytokine secretion. Since treatment with Tβ4SO reduces IL-4 and IL-5 levels in BAL fluid and OVA-induced production of these cytokines in thoracic lymph node cultures, it is likely that a reduction in Th2 lymphocyte activity and cytokine secretion contributes to the reduction in eosinophil levels observed in BAL fluid. The reduction in OVA-induced IFN-γ secretion in thoracic lymph node cultures also suggests that Tβ4SO may also suppress Th1 lymphocyte activity.

Serum total IgE levels or OVA-specific IgG1 or IgG2a levels were not altered by the administration of either Tβ4SO or dexamethasone. As serum was collected 96 hours after the first antigen challenge, the same time as the initial administration of

dexamethasone or Tβ4SO, and this is likely to be too short a period to observe any changes in serum antibody levels.

The current studies also demonstrated that Tβ4SO was able to reduce BAL total cell count and eosinophilia in comparison to Tβ4. The rise in macrophage proportions in this experiment is most likely to reflect a compensatory rise for the decline in relative proportions of eosinophils present. Young et al. (1999) reported that Tβ4 preparations can be contaminated with approximately 5% Tβ4SO, and can oxidise spontaneously in air. Therefore, the Tβ4 used may have some anti-inflammatory activity due to contaminating Tβ4SO and this could explain why the reduction in eosinophilia was not as pronounced as that observed in the first experiment.

Thymsosin β4 sulfoxide has been observed to have definite anti-inflammatory properties, both in the murine model of allergic airway inflammation used in these present studies, and previously in other inflammatory models. The exact mechanisms by which Tβ4SO exerts its extracellular actions are unknown. Most studies concerning the extracellular actions of thymosins have used thymosin β4, the immunomodulatory activity of which might be due to the presence of contaminating Tβ4SO. It is unclear how thymosin β4 is released from cells, since it lacks a signal peptide. It is likely that thymosin beta 4 is oxidised to Tβ4SO locally at sites of inflammation by free oxygen species, such as H_2O_2, which are produced by neutrophils and other inflammatory cells. It is possible that Tβ4SO may have an anti-oxidant role, since this peptide can be subsequently reduced by the activity of methionine sulfoxide reductase (Moskovitz et al., 1998). By inhibiting further influx of neutrophils, Tβ4SO acts in a negative feedback loop to prevent further oxidative damage (Young et al., 1999). Thymosin β4

can be bound to collagen and fibrin by the activity of Factor XIIIa, a transglutaminase released from activated platelets (Huff et al., 2002), which may help maintain high concentrations of Tβ4SO in inflamed areas. In addition to promoting the release of Tβ4SO from monocytes (Chettibi et al., 1994), corticosteroids may further augment the activity of Tβ4SO by promoting the secretion of anti-proteases such as leukocyte protease inhibitor 2 (Abbinante-Nissen et al., 1995). In a wound healing model, a synthetic peptide analogue of part of the actin-binding region also had anti-inflammatory activity (Philp et al, 2003), indicating that other regions of Tβ4SO in addition to the methionine sulfoxide residue at position 6, might also contribute to the immunomodulatory actions of Tβ4SO. Thymosin β4 has been shown to inhibit the production of granulo-monocytic bone-marrow progenitor cells which would then differentiate to form eosinophils or neutrophils (Bonnet et al., 1996). As yet, it is unknown whether Tβ4SO binds to cell surface receptors to alter intracellular signalling pathways. Although thymosins have been localised to the nucleus (Watts et al., 1990), there is no evidence that they act as transcription factors, or bind to them in a similar manner to the action of glucocorticoids.

In conclusion, the above data confirm that Tβ4SO has anti-inflammatory activity in this murine model of allergic asthma. At the doses given, it reduced BAL total cellularity and eosinophilia to a similar extent as dexamethasone. In this study, Tβ4SO was more effective than dexamethasone in reducing BAL macrophage and neutrophil numbers, but less effective in suppressing lymphocyte levels. The decrease in BAL cellularity was not accompanied by a reduction in inflammatory infiltrates in lung histology, but was associated with a reduction in IL-4 and IL-5 levels in BAL fluid. In thoracic lymph node cell cultures, Tβ4SO reduced OVA-induced proliferation of lymph node cell cultures and OVA-induced cytokine secretion, indicating that at least part of its anti-

inflammatory activity in this experimental model is mediated by its actions on lymphocytes. Tβ4SO was effective in reducing BAL total cell count and cellularity when compared to the non-oxidised Tβ4. Further investigations are needed to determine if Tβ4SO has direct inhibitory effects on eosinophils and other cell types, and to elucidate its mechanisms of action. Thymosin beta 4 sulfoxide may have therapeutic potential in allergic asthma.

Chapter 7

General Discussion

7.1 The role of IL-18 in allergic airway inflammation

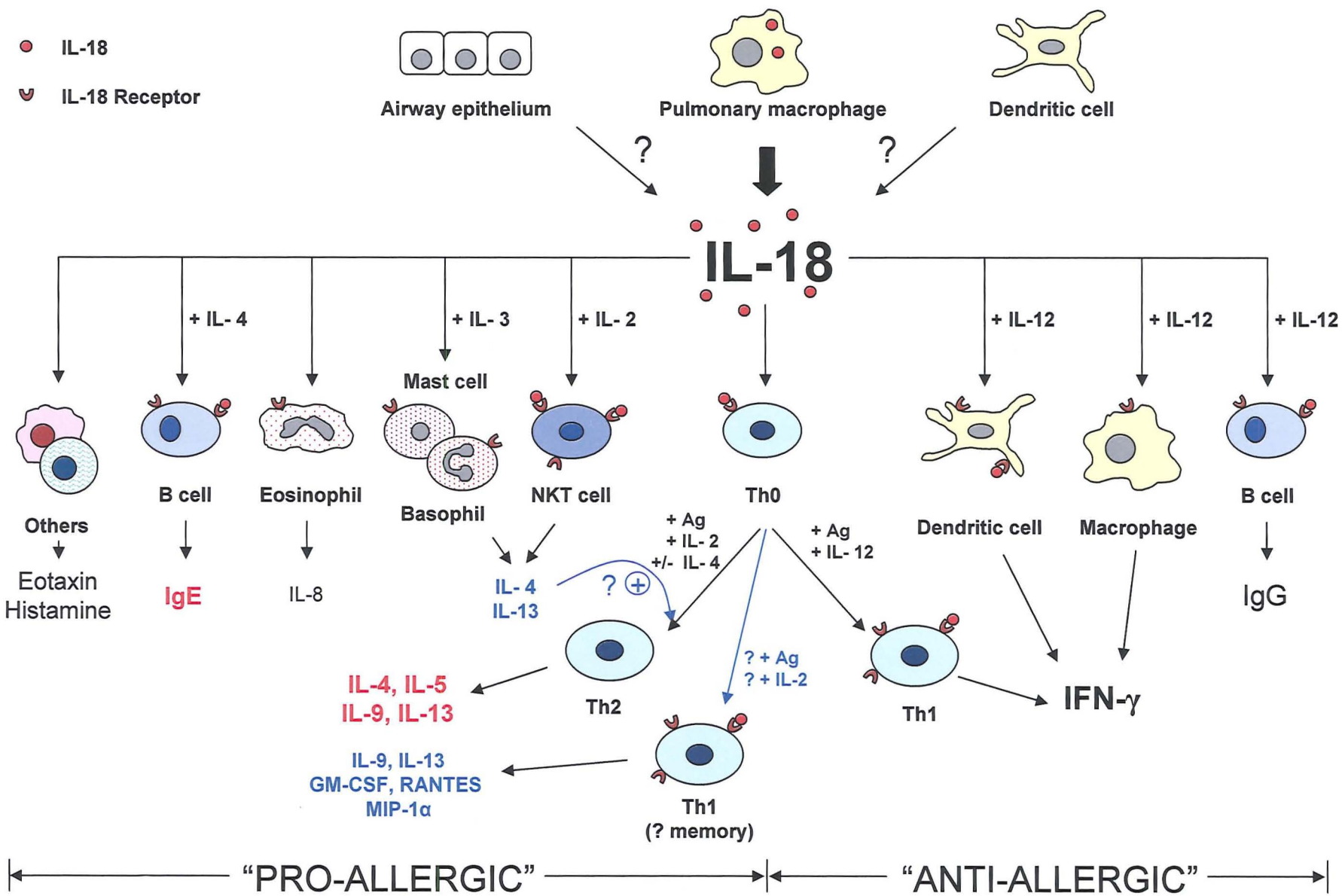
The inflammatory response in asthma involves the complex interaction of many cell types, cytokines, chemokines, intercellular adhesion molecules, enzymes and other small pro-inflammatory molecules. Th2 lymphocytes, secreting the cytokines IL-4, IL-5 and IL-13, are thought to have an important central role in the modulation of the asthmatic response. In contrast, Th1 lymphocytes which secrete IFN-γ may have anti-inflammatory activity, since Th1 and Th2 cells were initially shown to mutually antagonise the development of each other in animal models of parasitic infection (Mosmann and Coffman, 1989). Since the prevalence of asthma is increasing, it was proposed, in the "Hygiene Hypothesis", that a "cleaner" modern lifestyle alongside decreased viral and bacterial infections in childhood, reduced development of Th1 responses leading to a predisposition to Th2-driven conditions (Umetsu et al., 2002). However, Th1 cells have been shown to enhance airway inflammation in animal models of asthma (Hansen et al., 1999, Randolph et al., 1999a, Randolph et al., 1999b), and alternative cell types, such as regulatory T cells, may be required for effective down-regulation of the asthmatic response (Ling et al., 2004).

IL-18 was initially identified as interferon gamma inducing factor, with the ability to promote Th1 responses in association with IL-12 (Okamura et al., 1995). However, it is now known to promote Th2 responses and, as summarised in Figure 7.1, it can also act directly on other cell types, including eosinophils, mast cells and NKT cells, to promote allergic responses (reviewed by Nakanishi et al., 2001). IL-18 has recently been shown to stimulate the production of IL-9, IL-13, GM-CSF and chemokines, which are also able to enhance allergic responses, from IFN-γ-positive Th1 cells *in vitro* and Th1

Figure 7.1. The actions of IL-18 on cell types involved in allergic responses.

This figure illustrates the cells which may produce IL-18 in the lung, and the cell types on which IL-18 may act to influence the development of allergic airway inflammation. IL-18 has been shown to promote Th2 development and act on several other cell types to augment allergic responses. In the presence of IL-12, IL-18 will assist the maturation of Th1 lymphocytes and have potential anti-allergic actions by stimulating IFN-γ production.

"Others": IL-18 enhances histamine release from human PBMC cultures *in vitro* and eotaxin production in murine lung *in vivo*.


IL-18
IL-18 Receptor
Airway epithelium
Pulmonary macrophage
Dendritic cell
?
?
IL-18
+ IL- 4
+ IL- 3
+ IL- 2
+ IL-12
+ IL-12
+ IL-12
Mast cell
Others
B cell
Eosinophil
Basophil
NKT cell
Th0
Dendritic cell
Macrophage
B cell
Eotaxin
Histamine
IgE
IL-8
IL- 4
IL-13
? ⊕
+ Ag
+ IL- 2
+/- IL- 4
+ Ag
+ IL- 12
IgG
IL-4, IL-5
IL-9, IL-13
Th2
? + Ag
? + IL-2
Th1
IFN-γ
IL-9, IL-13
GM-CSF, RANTES
MIP-1α
Th1
(? memory)
"PRO-ALLERGIC"
"ANTI-ALLERGIC"

memory cells augmented airway inflammation in a murine model of asthma (Sugimoto et al., 2004). In contrast, IL-18-induced production of IFN-γ from Th1 cells, and potentially from pulmonary macrophages and dendritic cells, may down-regulate allergic reactions in the lung. Although the exact circumstances in which IL-18 will promote Th1 or Th2 responses remain to be defined, it is thought that the surrounding cytokine environment is important in influencing this. In particular, the presence of IL-12 clearly causes IL-18 to have a predominant Th1-promoting effect. Conversely, when IL-12 is absent, IL-18 will enhance Th2 responses. Consistent with the ability of IL-18 to promote both Th1 and Th2 development, there is conflicting evidence for its role in both clinical asthma and animal models of allergic airway inflammation.

IL-18 has previously been shown to be expressed in the bronchial epithelium (Cameron et al., 1999) and present in alveolar macrophages (Shigehara et al., 2001). IL-18 therefore has the potential to modulate lung immunity, including the asthmatic response. In the clinical study described in Chapter 3, IL-18 was detected in induced sputum fluid from asthmatic and normal subjects, and was expressed at relatively high levels in induced sputum cells. Its production was localised to sputum macrophages. There was no significant difference in IL-18 levels in induced sputum fluid between asthmatic and normal subjects, in contrast to previous studies where IL-18 was reduced in BAL fluid from asthmatics compared to normal controls (Ho et al., 2002). Cigarette smoking significantly reduced IL-18 levels in induced sputum fluid in both asthmatic and normal smokers. This effect was most pronounced in asthmatic smokers when compared to asthmatic non-smokers where decreased levels of IL-18 protein in induced sputum fluid were associated with reduced IL-18 mRNA expression in induced sputum cells. The reduction in mRNA levels was proportionately less than that in IL-18 protein production, which is consistent with the understanding that the activity of IL-18 is

mainly regulated at a post-translational level by enzymatic cleavage of pro-IL-18 into mature IL-18 by caspase-1, and subsequent proteolytic processing by other enzymes to inactive peptides (Akita et al., 1997). All smokers had higher proportions of neutrophils in their induced sputum samples. The significance of the reduction of IL-18 levels in cigarette smokers is unclear. It may contribute to the increased susceptibility to respiratory tract infections seen in smokers (Aronson et al., 1982) and may also lead to an alteration of the nature of airway inflammation in asthmatic subjects by influencing the balance of Th1 and Th2 cytokine secretion.

The studies performed in a murine model of allergic asthma in BALB/c IL-18 gene knockout mice sensitised and challenged with ovalbumin (OVA), supported the hypothesis that IL-18 may have a pro-inflammatory role in allergic airway inflammation (Chapter 4). The observed effects of IL-18 gene deficiency on allergic airway inflammation are summarised in Table 7.1. Airway eosinophilia was decreased in IL-18 deficient mice when compared to wild-type controls, but this was not associated with a significant reduction in tissue inflammatory infiltrates lung histology. There was a trend for IL-4 levels to be lower in the BAL fluid of IL-18 deficient mice, associated with decreased OVA-induced IL-4 production in thoracic lymph node cultures and IL-4 mRNA expression in thoracic lymph nodes. Serum total and OVA-specific IgE levels did not differ significantly between IL-18 deficient and wild-type mice. In the subsequent studies using the murine model of allergic asthma in wild-type mice (Chapter 5 and Chapter 6), levels of IL-18 levels in BAL fluid were not significantly different between wild-type naïve mice and those sensitised and challenged with OVA (Figure 5.7*C*). Surprisingly, the expression of IL-18 mRNA in total lung tissue was much lower OVA-treated mice in than in naïve mice (Figure 5.16 and Figure 6.6*D*); it is

	The effect of IL-18 deficiency in a murine model of allergic asthma
In vivo	
BAL total cellularity	↓
BAL eosinophilia	↓
Inflammatory infiltrates in lung histology	→
IL-4 in BAL fluid	→
IL-5 in BAL fluid	→
Serum total IgE	→
Serum OVA-specific IgE	→
Thoracic lymph node cultures	
IL-4 (OVA-induced)	↓
IL-5 (OVA-induced)	→
IFN-γ (OVA-induced)	↓
IL-4 (Con A-induced)	→
IL-5 (Con A-induced)	→
IFN-γ (Con A-induced)	↓
Spontaneous cell proliferation	→
Cell proliferation (OVA-induced)	→
Cell proliferation (Con A-induced)	→

Table 7.1. The effect of IL-18 deficiency in a murine model of allergic asthma.

The responses observed in IL-18 gene knockout mice in comparison to wild-type mice, sensitised and challenged with ovalbumin, are summarised in the table above.

not evident from these studies whether IL-18 mRNA expression in the lung is down-regulated at the time of sensitisation with OVA or after antigen challenge.

Although the above murine studies suggest that the pro-inflammatory role of IL-18 in allergic airway inflammation is, at least in part, related to its ability to augment IL-4 production, it is not clear if this effect is most important at the time of sensitisation to antigen or at the time of antigen challenge. In murine models of allergic asthma, it has previously been demonstrated that exogenous IL-18 administered at the time of antigen sensitisation increased the allergic response (Wild et al., 2000) while levels of IL-18 in whole lung tissue increased after antigen challenge and intra-tracheal administration of IL-18 enhanced eosinophil accumulation in the lungs (Campbell et al., 2000). Therefore IL-18 is likely to have pro-inflammatory actions at both these stages through the intra-pulmonary induction of cytokines and chemokines, including IL-4 and eotaxin, which enhance the allergic response.

As stated above, the magnitude of the pro-allergic actions of IL-18 will be influenced by the presence or absence of IL-12. A higher relative amount of IFN-γ to IL-4 will predispose to down-regulation of airway inflammation. Systemic administration of IL-18, especially in association with IL-12, tends to have an anti-inflammatory action in murine models of asthma most probably though the induction of high levels of systemic IFN-γ which suppresses pulmonary eosinophilia (Hofstra et al., 1998). In murine models of asthma where *Aspergillus fumigatus* (Blease et al., 2001) or respiratory syncitial virus (Zhang et al., 2003) were used to induce eosinophilic airway inflammation, the actions of IL-18 were thought to be anti-inflammatory but this again may related to the IL-12 secretion induced by the fungus and virus enhancing the IFN-γ-inducing properties of IL-18.

Genetic factors are likely to be important in determining the ability of IL-18 to enhance or suppress allergic responses. BALB/c mice are genetically predisposed to develop Th2 responses. They have been shown to produce lower serum levels of IL-18 in response to LPS when compared to the more Th1-biased C57BL/6 mice (Habu et al., 2001). However, spleen cells from BALB/c mice produce higher levels of IL-4, and lower levels of IFN-γ, in response to IL-18 and αCD3 stimulation than those from C57BL/6 mice (Xu et al., 2000). Such inherent differences in the propensity to secrete differing relative amounts of IL-4 and IFN-γ could explain the results of Kodama et al. (2000) where a lack of IFN-γ and its suppressive action was thought to a key factor in causing increased airway inflammation after antigen sensitisation and challenge in C57BL/6 IL-18 gene knockout mice. In the BALB/c mice used in Chapter 4, although IFN-γ was also reduced in IL-18 deficient mice, the effect of this may be outweighed by the lack of IL-4, leading to decreased total inflammatory cell numbers and eosinophils in BAL fluid.

Several genetic polymorphisms have been linked to the development of human asthma (Cookson, 2002). Genetic polymorphisms also exist in the human IL-18 gene and these have been associated with allergic disease (Higa et al., 2003a, Kruse et al., 2003). It is therefore possible that IL-18 may also have differing actions in human asthma depending on the genetic influences of each asthmatic subject.

7.2 The anti-inflammatory actions of simvastatin and thymosin beta 4 sulfoxide

Through its ability to induce IFN-γ, IL-18 was initially postulated as a possible anti-inflammatory therapy for asthma. However, the studies in IL-18 gene knockout mice demonstrated that it has probable pro-inflammatory actions in a murine model of allergic airway inflammation. In contrast, both simvastatin and thymosin beta 4 sulfoxide had definite anti-inflammatory properties in this animal model. These are summarised in Table 7.2, together with the effects of dexamethasone.

Simvastatin is a commonly used cholesterol lowering agent in clinical practice. It has been shown to have anti-inflammatory properties independent of its lipid lowering properties in clinical atherosclerotic disease (Albert et al., 2001) and in experimental models of multiple sclerosis (Youssef et al., 2002, Aktas et al., 2003) and rheumatoid arthritis (Leung et al., 2003). The lymphocytes involved in the inflammatory infiltrate in these conditions are thought to be of a predominantly Th1 phenotype. In the murine model of allergic asthma used in this study, it shown for the first time that simvastatin can also have anti-inflammatory actions in Th2-mediated inflammation. The suppressive action of simvastatin on IL-4 and IL-5 levels in BAL fluid and in OVA-stimulated thoracic lymph node cultures implies that that there is a direct suppressive action on Th2 lymphocytes. IFN-γ levels were also reduced suggesting there may be an accompanied suppression of Th1 responses.

	Simvastatin	Tβ4SO	Dexamethasone
In vivo			
BAL total cellularity	↓	↓	↓
BAL eosinophilia	↓	↓	↓
Inflammatory infiltrates in lung histology	↓	→	→
IL-4 in BAL fluid	↓	↓	↓
IL-5 in BAL fluid	↓	↓	↓
Serum total IgE levels	→	→	→
Thoracic lymph node cultures			
IL-4 (OVA-induced)	↓	↓	↓
IL-5 (OVA-induced)	↓	↓	↓
IL-4 (Con A-induced)	→	↓	↓
IL-5 (Con A-induced)	→	↓	↓
Spontaneous cell proliferation	→	↓	↓
Cell proliferation (OVA-induced)	→	↓	↓
Cell proliferation (Con A-induced)	→	→	→

Table 7.2. The anti-inflammatory actions of simvastatin, thymosin beta 4 sulfoxide and dexamethasone in a murine model of allergic asthma.

The effects of treatment with simvastatin, thymosin beta 4 sulfoxide and dexamethasone, when compared to PBS-treated controls, in ovalbumin-sensitised and challenged mice.

Thymosin beta 4 sulfoxide (Tβ4SO) had previously been shown to have anti-inflammatory activity in carrageenan-induced footpad inflammation in mice, in which neutrophils are the predominant inflammatory cell type observed (Young et al., 1999). Peptides related to Tβ4SO have been shown to have immunosuppressive activity in models of irritant and allergic contact dermatitis, where lymphocytes have a regulatory role (Girardi et al., 2003). The data presented in Chapter 6, demonstrate that Tβ4SO can also suppress eosinophilic inflammation in a murine model of allergic asthma. Comparable to simvastatin, the inhibition of airway inflammation is at least partly dependent on the suppression of Th2 lymphocyte activity, since IL-4 and IL-5 levels were again reduced in BAL fluid and thoracic lymph node cultures. Also, IFN-γ levels were reduced by Tβ4SO in thoracic lymph node cultures, suggesting that Th1 lymphocyte responses may also be reduced.

Although both simvastatin and Tβ4SO both appear to inhibit lymphocyte activity, there are likely to be divergent mechanisms through which this occurs. Simvastatin treatment decreased antigen-induced cytokine production from lymph node cells and did not affect cell proliferation, whereas Tβ4SO appears to have a more general immunosuppressive action, inhibiting both OVA-induced cytokine production and OVA-induced cell proliferation. The immunosuppressive action of Tβ4SO more closely resembles that of dexamethasone than simvastatin; since steroids promote the release of Tβ4SO from monocytes (Chettibi et al., 1994), it is possible that Tβ4SO may mediate some of the anti-inflammatory actions of corticosteroids. Only treatment with high dose simvastatin (40 mg/kg) resulted in a significant reduction in inflammatory infiltrates in lung histology, but it maybe that a higher dose of Tβ4SO is required to produce a similar effect on histological inflammation. Neither compound altered the serum total

IgE or OVA-specific IgG1 and IgG2a levels, most probably as they were given at the time of antigen challenge and not with sensitisation.

7.3 Future work

The clinical study using induced sputum from asthmatic and normal subjects did not show any significant difference in IL-18 levels in induced sputum fluid between asthmatics and normal subjects, nor between asthmatics treated with salbutamol alone or those treated with inhaled corticosteroid. The study performed was a cross-sectional study and a longitudinal study would be necessary to assess fully if IL-18 production could be altered by steroid treatment. The ELISA used to determine IL-18 concentrations only detected total IL-18, and so a comparison of the relative proportions of pro-IL-18 and mature IL-18 between asthmatics and normal individuals may reveal differences. This would require Western blotting which would be technically difficult with induced sputum samples because of the small volume of sample obtained. A study using BAL samples may therefore be needed. Such a study could involve a comparison with total IL-18 levels in induced sputum to attempt to explain the difference in the results in this study from previous reports, and permit a more detailed study on the effect of cigarette smoking on cytokine production in the airways and from pulmonary inflammatory cells such as alveolar macrophages. An assessment of changes in IL-18 levels after antigen challenge would also be of interest.

In the studies in the murine model of allergic asthma, the levels of IL-18 in BAL fluid were not altered by OVA-sensitisation and challenge when compared to naïve mice.

Also, the levels of IL-18 mRNA expression in murine lung did not reflect BAL IL-18 levels 24 hours after the last antigen challenge. More detailed investigation of the alterations in IL-18 mRNA expression and protein levels, around the periods of sensitisation and challenge are required, including quantification of the relative amounts of pro-IL-18 and active IL-18 present in the lungs and BAL fluid. The experiments in IL-18 gene knockout mice suggest that IL-18 contributes to the inflammatory response in the murine model of asthma used by augmenting the production of IL-4. Further experiments are needed to confirm these findings and to establish whether levels of other Th2 cytokines, including IL-9, IL-13 and GM-CSF, are also reduced. There was no difference in the ratio of CD4:CD8 lymphocytes between OVA-treated IL-18 ko mice and WT mice. However, since IL-18 can enhance both Th1 and Th2 development, the relative ratios of Th1:Th2 lymphocytes in thoracic lymph nodes and infiltrating into the lung could be assessed. IL-18 has been shown to stimulate IL-4 production from Th2 lymphocytes, NKT cells, mast cells and basophils *in vitro,* and it would be of interest to study how IL-4 production differs in these cells *in vivo* in IL-18 ko mice during antigen sensitisation and challenge. The importance of the activity of IL-18 during sensitisation and challenge could be further assessed by replacing IL-18 at each of these times in IL-18 knockout mice.

Both simvastatin and Tβ4SO have been shown to have anti-inflammatory properties in a murine model of allergic asthma. These effects are at least in part T cell mediated, as demonstrated by the ability of both simvastatin and Tβ4SO to suppress antigen-induced IL-4 and IL-5 production from lymphocytes in culture. Further *in vitro* studies to determine if either of these compounds have a direct suppressive action on eosinophil migration, activation and survival are needed. Also, the specific ability of both

simvastatin and Tβ4SO to inhibit Th1 or Th2 lymphocyte activity could be more fully assessed. It would also be important to investigate if these agents have anti-inflammatory actions if administered before antigen sensitisation. This is particularly relevant for simvastatin, since related statins have been shown to inhibit IFN-γ-induced MHC-II up-regulation on macrophages and subsequent T cell activation (Kwak et al., 2000).

The molecular mechanisms by which both simvastatin and Tβ4SO inhibit airway inflammation require elucidation. It is thought that the anti-inflammatory effects of simvastatin are likely to be independent of reductions in cholesterol lowering, but this could be confirmed by assessing serum cholesterol levels and comparing the effect of simvastatin with that of a different class of cholesterol-lowering agent, such as a fibrate. If the administration of mevalonate, the product of uninhibited HMG-CoA reductase, reversed the anti-inflammatory activity of simvastatin this would indicate that inhibition of signal transduction proteins which require mevalonate-dependent isoprenylation was an important process for the anti-inflammatory action of statins in this animal model. Statins also allosterically inhibit the interaction of the adhesion molecule LFA-1 with ICAM-1 (Weitz-Schmidt et al., 2001) and the expression and binding activity of LFA-1 could be studied *ex vivo* on lymphocytes from mice previously treated with simvastatin.

The intracellular mechanisms of action of Tβ4SO are as yet unknown. It is unclear if Tβ4SO binds to a cell surface receptor and this needs to be established, as does the action of Tβ4SO on intracellular signalling pathways, including those which result in the activation of pro-inflammatory transcription factors such as NF-κB.

Since airway hyper-responsiveness is a feature of clinical asthma, future studies in the murine model of allergic asthma, whether it be to determine the effect of IL-18 deficiency or the outcome of treatment with simvastain or Tβ4SO, should include an assessment of this.

7.4 Concluding remarks

The increasing prevelance of asthma, and the failure of some patients to respond to corticosteroid treatment, means that there is a continual search for new therapeutic agents. IL-18 was initially proposed as cytokine which could have anti-inflammatory activity in asthma, but is now thought to have pro-inflammatory actions in this condition. Its role in allergic airway inflammation is therefore complex and a more complete understanding of its actions still required before any treatment can be targeted at altering its activity. In contrast, both simvastatin and thymosin beta 4 sulfoxide have shown definite anti-inflammatory properties in as murine model of allergic asthma, and these, or related compounds, may therefore have therapeutic potential in clinical asthma.

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