INVESTIGATION OF THE ROLE OF T CELLS IN AIRWAY INFLAMMATION USING NOVEL MURINE MODELS OF DISEASE

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SUMMARY

Asthma is a highly prevalent disease characterised by variable airflow obstruction, bronchial hyperresponsiveness and airways inflammation. Studies over the last 20 years have established that an immunological response centred on Th2 lymphocytes and their mediators is critical to the development of the disease, although other elements including structural cells are thought to contribute to the final outcome. In this thesis animal models have been developed to investigate the role of inflammation in the development and progression of allergic airways disease. As $CD4^+$ T cells have been suggested as a possible therapeutic target in asthma, techniques were employed that allowed the identification and quantification of antigen-specific T cells in different anatomically relevant compartments including, draining and peripheral lymph nodes, lungs and bronchoalveolar lavage fluid, providing novel information on the role of T cells in these models. This information produced has important implications for future development of therapeutic agents targeting T cells.

To investigate the role of antigen-specific T cells in airways inflammation, an adoptive transfer model was established. The aim of the model was to allow tracking of antigen-specific T cells as well as inducing airway eosinophilia in conjunction with IgE production and associated Th2 cytokines. Our investigations revealed that a single immunisation with OVA followed by 1-3 intranasal antigen airway challenges did not induce BAL eosinophilia although identification of Ag-specific T cells was possible in the lymph nodes and the lung. However, it was found that a more aggressive regime of three immunisations and one airways challenge was required to induce lung eosinophilia and associated pathology (long model). Manipulation of the single immunisation, single airway challenge with the addition

of LPS to antigen in the airways challenge resulted in lung eosinophilia and pathology (short model) as seen in the three prime, one challenge model, demonstrating that different models can be adapted to produce the same outcome. It was found that when antigen-specific T cells were compared between the two models where pathology or no pathology was see, resulted in different expression of certain cytokines, costimulatory molecules and chemokine receptors. When no pathology was present, it was found that IFNγ was upregulated in both models. In the long model when pathology was present, IL-5 expression was upregulated, and in contrast IL-13 was upregulated in the short model when pathology was induced, with CCR3 upregulated in both models. This data demonstrates that although similar endpoints were observed the route by which the response was initiated differs.

To determine the site of T cell division in airway inflammation, antigen-specific T cells were tracked in different compartments throughout the course of disease and their expression of proliferating cell nuclear antigen (PCNA) was measured as an indication of their recent division. When T cell division was quantified by LSC in the long model it was found that there was an early wave of T cell division seen in the lung, followed by similar in the draining lymph node. Subsequently, another wave of antigen-specific T cell division was seen in the lung tissue. The early T cell division may have been caused by uptake and presentation of antigen by resident dendritic cells, which have been shown to be present in the lung. The location of these dendritic cells allows them to have direct contact with incoming antigens. It is known that there is a dendritic cell network situated immediately above and beneath the basement membrane of the upper and lower airways enabling these cells to sample the epithelium for inhaled antigens. It has also been shown in experimental

models of respiratory infection that a population of memory T cells are established in the lung parenchyma and the lung airways. Airway resident memory cells contribute significantly to recall responses by providing immediate effector activity at the site of antigen entry, showing that there is local antigen presentation in the lung. This data gives an insight into the location and timing of T cell division and activation and may enable more accurate therapeutic intervention in airways inflammation.

Toll-like receptors (TLRs) are a critical part of innate immunity allowing recognition of conserved molecular patterns on microbial products. Both epidemiological and laboratory investigations have shown a complex relationship between TLR ligands and allergic airways inflammation with some studies demonstrating a protective effect and others showing disease exacerbation. In this thesis I investigated the role of LPS and Pam3CSK4 (BLP) in the above mentioned murine models of airways inflammation. Given locally, LPS or BLP resulted in an increased antigen-driven eosinophilic inflammation in the airways, which was accompanied by increased antigen-specific T cell numbers in the airways. When given systemically, LPS or BLP was shown to reduce airways inflammation, although no effect on antigen-specific T cells was observed. The results emphasise the many underlying factors involved in the immune response, and give an insight into the role exogenous factors may play in mediating the immune response.

The effect of the anti-inflammatory agent etanercept (anti-TNFα) was studied in the airways models. It was shown that TNFα blockade during either the induction or effector phases caused no reduction in pathology associated with the disease. However, blockade during the

induction phase prevented lymph node hypertrophy. When TNFα blockade was administered throughout the induction and effector phases of disease, lymph node hypertrophy and lung pathology were reduced, although the reduction in BAL eosinophilia was not significant. This was associated with a reduction in BAL IL-5 production, showing that anti-TNFα treatment is needed throughout the course of the disease to reduce inflammation. When anti-TNFα treatment was administered in a more acute short model of airways inflammation, the result was more pronounced, showing a reduction in BAL eosinophilia, pathology, lymph node hypertrophy, BAL IL-5 production and IgE production. When L-selectin expression was assessed on antigen-specific T cells it was found that the draining lymph nodes and the lung tissue had increased expression in both anti-TNFα and control mice. These data suggest that although anti-TNFα treatment affects the accumulation of T cells in draining lymph nodes, it does not mediate its effects via the expression of L-selectin. Thus anti-TNFα may be useful in severe and acute asthma.

These data have contributed to our understanding of how inflammatory and immunological processes combine to result in eosinophilic airways inflammation and highlight the multifactorial nature of asthma. The findings concerning the role and location of T cells in allergic airways inflammation provide useful novel insights into underlying immune pathologic pathways. This has important implications for future development of therapeutic agents targeting T cells.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AHR	Airway Hyperresponsiveness
ASM	Airway Smooth Muscle
APC	Antigen Presenting Cell
BAL	Bronchoalveolar Lavage
CD	Clusters of Differentiation
CIA	Collagen Induced Arthritis
CFSE	Carboxyfluoroscein Succinimidyl Ester
COPD	Chronic Obstructive Pulmonary Disease
CTL	Cytotoxic T Lymphocytes
DALYs	Disability-adjusted Life Years
DCs	Dendritic cells
DLN	Draining Lymph Node
DNA	Deoxyribonucleic Acid
EB	Eosinophilic Bronchitis
ECM	Extracellular Matrix
ELC	ED11 Ligand Chemokine
ELISA	Enzyme Linked Immunosorbant Assay
FACS	Fluorescence Activated Cell Sorter
FCS	Foetal Calf Serum
FEV	Forced Expiratory Volume
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor

GINA	Global Initiative For Asthma
H&E	Haematoxylin and Eosin
HBSS	Hank's Buffered Salt Solution
ICAM	Intracellular Adhesion Molecule
ICOS	Inducible Co-stimulatory Molecule
IF	Immunoflouresence
IFNγ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
IL1R	Interleukin 1 Receptor
i.n.	Intranasal
i.p.	Intraperitoneal
IRAK	IL1R-associated Kinase
i.v.	Intranvenous
KO	Knock Out
LBP	LPS-binding Protein
LFA	Leukocyte Function Antigen
LPS	Lipopolysaccharide
LSC	Laser Scanning Cytometry
LT	Leukotriene
mAb	Monoclonal Antibody
MC	Mast Cell
MDC	Monocyte-derived Chemokine

mDC	Monocyte-derived Dendritic Cell
MHC	Major Histocompatibility
MyD88	Myeloid Differentiation Factor-88
NBF	Neutral Buffered Formalin
NFκB	Nuclear Factor κB
NK	Natural Killer
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PAR	Protease-activated Receptors
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
pDC	Plasmacytoid Dendritic Cell
PG	Prostaglandin
PLN	Peripheral Lymph Node
PRR	Pattern Recognition Receptors
RA	Rheumatoid Arthritis
RANTES	Regulated Upon Activation, Normal T Cell Expressed And Secreted
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
SA	Streptavidin
SCID	Severe Combined Immunodeficiency
SEM	Standard Error Of The Mean
SLC	Secondary Lymphoid Tissue Chemokine

TACE	Tumour Necrosis Factor Alpha Converting Enzyme
TARC	Thymus And Activation Regulated Chemokine
T-bet	T-box Transcription Factor
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming Growth Factor
Th1	T Helper 1
Th2	T Helper 2
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
TSLP	Thymic Stromal Lymphopoietin
UK	United Kingdom
VCAM	Vascular Cell Adhesion Molecule

CHAPTER 1 - INTRODUCTION

1.1 Introduction

1.1.1 The burden of lung disease

Allergic diseases such as asthma, rhinitis, eczema and those associated with food are reaching epidemic proportions in developed and developing countries [1]. The prevalence of asthma alone has almost doubled over the past twenty years, affecting approximately 300 million people worldwide and an estimated 400 million by 2025 [2]. The rapid increase in asthma has been attributed to aspects common in western culture, including outdoor and indoor pollution, childhood immunizations, and cleaner living conditions, although no single cause has been identified [2, 3].

The profile of both infectious and non-infectious lung diseases in developing countries over the past century reflects prevailing sociopolitical and economic forces. The lung, perhaps more than any other organ system is influenced by poverty, occupation and personal habits. These influences are seen in the association between tuberculosis and pneumoconiosis first described in miners, the increasing prevalence of asthma and smoking-related chronic obstructive pulmonary disease, and the current dual epidemics of tuberculosis and infections associated with the human immunodeficiency virus (HIV). The global prediction for developing countries is that by the year 2020 respiratory diseases (including infections) will account for a large majority of deaths and a considerable burden of disability adjusted life years. The Lung Health Survey 2002 performed in Cape Town provides disease prevalence and has identified complex interactions between causative factors and disease. Consistent and biologically plausible associations between smoking and susceptibility to tuberculosis and pneumonia in HIV-infected patients have been reported.

In 2001, the British Thoracic Society published the report 'The Burden of Lung Disease' which collates for the first time, statistics detailing the size of the problem of respiratory diseases in the United Kingdom [4]. Figures in this report showed that one in four deaths in the UK can be attributed to respiratory disease, and that more people in the UK die from respiratory illness than from coronary heart disease or cancer. Significant morbidity was also commented upon: 7% of adults reported long-term respiratory symptoms or illnesses and in children the most commonly-reported long-term illnesses are those of the respiratory system. This pattern is repeated on a global basis with respiratory infections, malignancies and airways disorders all featuring in the ten leading causes of mortality worldwide and conditions such as chronic obstructive pulmonary disease (COPD) are predicted to increase as a cause of death by 2030 [5]. Reports such as these indicate that respiratory diseases pose one of the major challenges for health strategists, clinicians and researchers alike on both a national and global scale.

1.1.2 The burden of asthma

Asthma mortality is relatively low compared to other respiratory diseases, however awareness has increased over recent years, and it is now recognised that morbidity created by this condition is extremely high. There are an estimated 15 million disability-adjusted life years (DALYs) lost to asthma every year [6] suggesting considerable unmet clinical need. The current high prevalence of asthma is not easily explained by our current understanding of the underlying pathophysiology, a fact highlighted by the Global Initiative for Asthma (GINA) [7], which has identified investigation into the causation of asthma as a key area for research.

1.1.3 Trends in asthma

Is has become apparent that not only is asthma common [6], but its prevalence has increased steadily throughout the latter part of the last century by rates of up to 5% per year [8, 9]. The reasons behind this trend are not clear and increased diagnosis and over-reporting of symptoms do not account for the size of the increase [10]. Some evidence suggests that it may be associated with a concurrent increase in atopy [9], an increase that some researchers have attributed to reduced exposure to microbial insults in industrialised nations – the so-called 'Hygiene Hypothesis' [11] which will be discussed in more detail later. Recent reports would suggest that the rising prevalence of asthma may now be slowing [12] and just as it was unclear what caused the steady increase of the past few decades, it is unclear now why it may be reaching a plateau. This only serves to underline our need to understand more fully both the causes of asthma and the factors that exacerbate or relieve it.

To understand the mechanisms involved in any disease, including asthma, it is necessary first to understand the working of the immune response itself. I will give a brief overview introducing the basics of the immune response, to give a better understanding of how diseases are initiated and controlled.

1.2 Innate and adaptive immunity

The immune response of mammals can be divided into two parts: innate and adaptive immunity. The innate immune response provides a non-specific defence against infection, which is rapid (within a few minutes), and has no immunological memory. The innate system protects against pathogens. This defence extends from physical barriers (for example

skin and epithelia) to the recruitment of inflammatory cells (such as macrophages, mast cells, eosinophils, neutrophils, and dendritic cells (DCs)). Thereafter, the immune system produces a more specific response mediated by T and B lymphocytes. These lymphocytes efficiently eradicate pathogens, and provide immunological memory, which confers increased protection against re-infection. The innate and adaptive immune systems are not mutually exclusive, with many cells of the innate system also participating in the effector mechanism of the adaptive response. A number of cells of both the innate and adaptive immune system have been implicated in the pathogenesis of asthma.

1.2.1 Innate defences

The innate immune system is evolutionarily the most ancient of an organism's defences against pathogens and includes physical barriers, antimicrobial agents and cells. Although the adaptive immune system specialises in generating receptors recognising a wide variety of foreign proteins in the form of T cell receptors or immunoglobulin, the innate response also has its own means of detecting invading pathogens. As there is no mechanism for generating receptor diversity in the way that T and B cells can do, conserved (and therefore usually biologically essential) motifs on the pathogen, termed pathogen-associated molecular patterns (or PAMPs), are recognised [13]. The corresponding receptors are pattern recognition receptors (PRRs) and include mannose-binding lectin and the scavenger receptors. PRRs may sense molecular structures present in pathogens or endogenous danger signals which indicate profound cellular damage. Various families of PRRs exist, which are either expressed on the cell surface, reside in extracellular compartments or may be released as secreted molecules in the extracellular space. The intracellular NOD-like receptor (NLR)

family seem to play a pivotal role in the recognition of intracellular PAMPs [570]. NOD-like receptors are cytosolic proteins that contain a central nucleotide-binding oligomerization domain (NACHT), an N-terminal effector-binding domain and C-terminal leucine-rich repeats (LRRs). NOD-like receptors have been implicated as ancient cellular sentinels mediating protective immune responses against intracellular pathogens. Recent studies have described the genetic association of polymorphisms in NOD-like receptor genes with complex chronic inflammatory barrier diseases, such as Crohn's disease and asthma and with rare auto-inflammatory syndromes including familial cold urticaria, Muckle–Wells syndrome and Blau syndrome [571].

Another family of PRRs, which has received considerable interest, is the Toll-like receptors (TLRs). These receptors bind a wide variety of microbial products, but are particularly noteworthy because of their ability to influence the developing adaptive immune response. This link between innate and acquired immune responses suggests a mechanism by which agents such as pollution, cigarette smoke and microbial products could affect immunological processes such as those found in asthma.

1.2.1.1 TLRs

Toll was first discovered when Drosophila fruitfly embryos deficient in Toll displayed aberrant ventral-dorsal development, and diminished protection against fungal infection [14-16]. Subsequent work revealed the presence of a family of mammalian homologues to Toll, termed TLRs [17, 18], which recognize pathogens, or pathogen-derived products (see table 1.1). TLR activation triggers both the early innate and adaptive immune responses.

TLRs recognize PAMPs displayed by various microbial components including gram-positive and -negative bacteria [19-23]; DNA and RNA viruses [24-26]; fungi [27] and parasites and protozoa [28-31]. TLRs are type I transmembrane receptors that possess an extracellular leucine-rich repeat domain and cytoplasmic domain homologous with that of the interleukin 1 receptor (IL1R) family [17, 32]. Upon stimulation, TLRs and IL1R interact with the adapter molecule, myeloid differentiation factor-88 (MyD88), recruiting the IL1R-associated kinase (IRAK), leading to the activation of the transcription factor nuclear factor-κB (NFκB) and inflammatory gene transcription [33]. All TLRs characterised so far, except TLR3, signal through the adaptor protein MyD88. However some TLRs also appear to signal through a MyD88-independent pathway under specific conditions.

TLR2 recognizes a wide variety of PAMPs from bacteria, yeast, fungi, and parasites, and is thought to act as a receptor by forming heterodimers with TLR1 or TLR6 [34, 35]. TLR2/6 heterodimer binds gram positive cell wall components, such as peptidoglycan, and mycoplasmal lipopeptides that are diacylated, whereas TLR2/1heterodimers recognises bacterial lipopeptides that are triacylated [34-37]. TLR4 functions as a homodimer or homo-oligomers and detects lipopolysaccharide (LPS), a major cell wall component of gram negative bacteria [17, 23]. Recognition of LPS also requires the association of LPS-binding protein (LBP) [38], CD14 [39] and MD2 [40]. MyD88-dependent and MyD88-independent pathways both mediate TLR4 signalling [41, 42].

TLR	Exogenous Ligand
TLR1	Mycobacterial lipoprotein, triacylated lipopeptides
TLR2	LPS, zymosan, peptidoglycan, lipoproteins, lipotechoic acid, glycosylphosphatidylinositol (GPI)-anchor proteins, Pam3Cys-Ser-Lys4
TLR3	Poly (I:C) (viral ds RNA)
TLR4	LPS, respiratory syncytial virus protein F
TLR5	Flagellin
TLR6	Mycoplasmatic lipoproteins, lipotechoic acid, peptidoglycan
TLR7	ss RNA (virus), resiquimoid, imiquinoid
TLR8	Resiquimoid, imiquinoid
TLR9	CpG DNA, dsDNA (virus), ISS
TLR10	Unknown
TLR11	Uropathogenic bacterial antigens, profilin (*T. gondii*)

Table 1.1: Toll-like receptors and their exogenous receptors.

1.2.1.2 TLRs and the immune system

TLRs are involved in both innate and adaptive immunity. Initial recognition of the highly conserved PAMPs on pathogens by the innate immune cells causes release of chemokines, recruitment of cells to sites of inflammation, triggering of phagocytosis with degradation and presentation of pathogen-derived peptides. TLRs are highly expressed on antigen presenting cells (APCs), which upon activation release inflammatory cytokines and express co-

stimulatory molecules. TLRs are able to activate the innate system and they also initiate the development of antigen-specific adaptive immune responses.

Pathogen recognition by TLRs induce the expression of selectins, chemokines and chemokine receptors that regulate cell migration to sites of inflammation [43]. Innate cells such as neutrophils, natural killer (NK) cells, mast cells, eosinophils, DCs, macrophages, endothelial and epithelial cells express a variety of TLRs which, upon binding of ligands, will generate pro-inflammatory signals and cytokines that will result in microbial killing [20, 37, 44-48]. In the absence of TLRs, particularly TLR2/TLR4 or MyD88, macrophages show impaired bacterial killing compared with wild type cells [48], suggesting that TLRs are important in the initial recognition, cell migration, phagocytosis, and clearance of pathogens by the innate system.

DCs express a large number of TLRs, however the precise pattern of TLR on different subsets of DCs is unknown. Human myeloid DCs express all 11 TLRs except TLR4 and TLR9 and can recognize bacterial, fungal and viral pathogens [49-52]. Plasmacytoid DCs (pDCs) express TLR7 and TLR9, whereas CD11c+ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6, and TLR8 [49-52]. Mouse DC TLRs are more heterogeneous than human, with most TLRs expressed on most DC subsets [53, 54].

TLRs initiate adaptive immunity mainly through DCs. These immature cells capture microbial antigens in the peripheral tissues, mature, process the antigen into peptides, and then migrate to draining lymph nodes to present processed peptide to T cells. The activation

of TLRs results in downregulation of inflammatory chemokine receptors (such as CCR6) and upregulation of receptors for lymphoid chemokines (such as CCR7), which stimulates DC migration to lymphoid tissue [55, 56]. TLR activation also increases the expression of CCR2, CCR5, and CCR7 on DCs; enhances the production of IL-12, tumour necrosis factor α (TNF-α) and IL-6 by DCs; and up-regulates co-stimulatory molecules such as CD40, CD80 (B7-1), CD86 (B7-2) and major histocompatibility (MHC) class II [55-59]. These changes allow DCs to mature and present antigenic peptides to T cells more effectively.

TLRs may also be involved in determining the T helper 1 (Th1)/ T helper 2 (Th2) balance [60]. The density of antigenic peptide presented, the degree of co-stimulation, and the amount and nature of cytokines and inflammatory mediators produced by DCs at the site of inflammation have all been shown to influence T cell differentiation [61].

1.2.2 Adaptive Immunity

Adaptive immunity is capable of recognising and selectively eliminating specific foreign microbes and molecules (e.g. foreign antigens). Unlike innate immune responses, adaptive immune responses are not the same in all members of the species but are reactions to specific antigenic challenges. The four main characteristics of the adaptive immune response are: antigenic specificity, diversity, immunologic memory and self/nonself recognition [62].

Antigen specificity permits the immune response to distinguish among antigens. The immune system can recognise billions of unique structures on foreign antigens, generating

huge diversity. Once the immune system has recognised and responded to an antigen, it exhibits immunological memory, causing a second encounter with the same antigen to be heightened [63]. This means that there is life-long immunity to most infectious agents after the initial encounter. The adaptive immune response only responds to foreign antigens, making a distinction between self and nonself, reducing inappropriate responses.

An effective immune response involves two major groups of cells: lymphocytes and antigen presenting cells. Because lymphocytes produce and display antigen-binding cell-surface receptors, they mediate the defining immunological attributes of specificity, diversity, memory, and self/nonself recognition. There are two main types of lymphocytes: B lymphocytes and T lymphocytes. As the main focus of this thesis concerns T cells I will briefly discuss B lymphocytes and elaborate more on T lymphocytes.

1.2.2.1 B lymphocytes

B lymphocytes mature in the bone marrow and upon leaving express an antigen-binding receptor on its membrane, which is a membrane-bound antibody molecule. When a naïve B cell first encounters the matching antigen for its membrane-bound antibody, the binding causes the cell to proliferate. The progeny differentiate into memory B cells and effector B cells called plasma cells [64]. Memory B cells have a longer life span than naïve cells and express membrane-bound antibody as their parent did. Plasma cells produce antibody as a form that can be secreted, although they only survive for a few days, secreting large amounts of the antibody in this time. These antibodies are the major effector molecules of humoral immunity.

1.2.2.2 T lymphocytes

$CD4^+$ T cells are probably the most important helper cells involved in the immune response. They are responsible for many effector functions as well as activating other aspects of the immune response, including B lymphocytes, $CD8^+$ T cells, macrophages, DCs and many others [65]. $CD4^+$ T cells are present in high numbers in secondary lymphoid organs (lymph nodes, spleen) and also in the bloodstream, comprising about 60-70% of the T cell population. Mature $CD4^+$ T cells are characterised by CD3 expression, and αβ T cell receptor (TCR). $CD4^+$ T cells are crucial in most immune responses including autoimmune diseases, chronic inflammatory diseases and hypersensitivity diseases. Five to ten percent of T cells in the body express receptors composed of γ and δ chains, which are structurally similar to the αβ TCR but have different specificities. The γδ TCR may recognise a variety of protein and non-protein antigens. T cells expressing γδ TCRs are abundant in epithelia, suggesting that these T cells recognise microbes commonly encountered at epithelial surfaces.

1.2.2.3 T cell activation

Activation of T cells occurs when a professional APC displays the appropriate set of ligands to a naïve T cell. This process is well regulated and has restrictions on the type and location of cells that can present antigen with MHCII, as well as the requisite sets of co-stimulatory molecules, before the T cell can be fully activated (See Figure 1.1). To be recognised by a $CD4^+$ T cell, antigen must be ingested by an APC, degraded into peptides and presented on the surface of the APC, by newly synthesised MHCII molecules (reviewed in [66]) and

finally recognised by a T cell antigen receptor. The TCRs recognise fragments of antigens bound to molecules of the MHC on the surface of an APC. The peptide binding proteins are of two types, MHCI and MHCII, which stimulate cytotoxic T cells (CTLs) and helper T cells respectively. Intracellular antigens bind to MHC I molecules and are recognised by CTLs, which become activated and can directly kill a target cell. Extracellular antigens, are presented by MHC I molecules to helper T cells, which once activated, have immune regulatory functions. The APC must also display a number of accessory molecules, which trigger specific receptors on the T cell to provide co-stimulation via the TCR. These co-stimulatory signals are necessary because the TCR cannot initiate intracellular signalling on its own, as it does not undergo conformational changes after binding antigen and has short cytoplasmic regions. CD4 is a 55kDa single chain molecule, made up of four Ig superfamily domains, which bind to the α_2 domain of the MHCII molecule. The binding of CD4 stabilises the binding of the T cell to the APC and initiates tyrosine kinase mediated signalling via the TCR. This allows the first step in TCR-mediated signalling, allowing the activation of the CD3ζ chain by bringing together the CD4 and CD3-TCR complex.

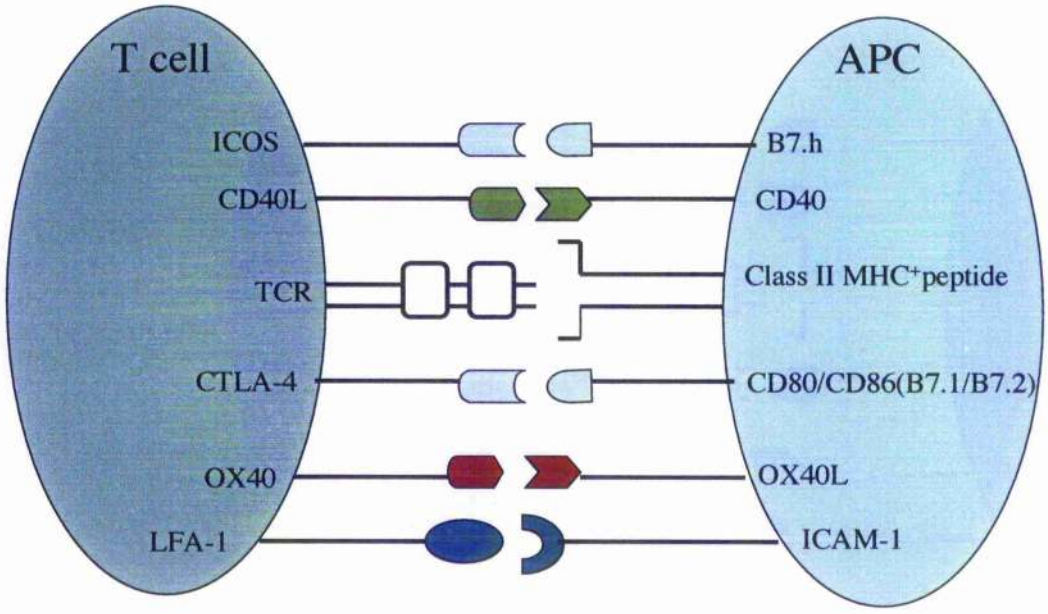

Figure 1.1: T cell and APC co-stimulatory molecules

Major surface molecules of $CD4^+$T cells involved in activation of these cells and the molecules on APCs recognised by the receptors are shown. Abbreviations: Inducible co-stimulatory molecule (ICOS), cytotoxic T lymphocyte associated protein 4 (CTLA-4); lymphocyte function associated antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1).Many of the co-stimulatory molecules belong to the CD28 or TNF/TNF receptor families. CD28 is present on mature $CD4^+$ T cells and binds to CD80 and CD86 on APCs. CD28 is important for $CD4^+$ T cell activation, and is essential for clonal expansion, effector T cell differentiation, germinal centre formation and memory cell generation [67]. CD28 co-stimulation prolongs TCR signalling, causing an increase in IL-2 production and improved $CD4^+$ T cell survival [68]. Inducible co-stimulatory molecule (ICOS) is highly homologous to CD28, but is expressed after CD28-mediated activation of $CD4^+$ T cells. ICOS binds to

B7RP1 on B cells and promotes the differentiation of effector T cells after clonal expansion [69].

Of the TNF family of receptors, CD40 ligand (CD40L) is most important in T cell activation. CD40L is expressed on activated T cells and binds CD40 on APCs. It is important in T cell help for B cell differentiation and the production of IL-12 and interferon (IFN)γ in Th1 cell differentiation. CD40L expression requires the activation of T cell via CD28 and TCR.

There are many other molecules involved in co-stimulation of T cells, including OX40, which binds OX40L on DCs. Many adhesion molecules also act as accessory factors during T cell-APC interaction, such as intracellular adhesion molecule (ICAM) 3 and its ligand DC-SIGN, leukocyte function antigen 1 (LFA-1) and its ligand ICAM-1, which form organised binding complexes and aid the focus of TCR on correct MHCII complexes. These interactions also enhance signalling within the T cell.

1.2.2.4 Dendritic Cells and CD4+ T cell priming

DCs are a sparsely distributed, migratory group of bone-marrow derived leukocytes that are specialised for the uptake, transport, processing and presentation of antigens to T cells [70-72]. As immature cells, DCs continuously circle the peripheral tissues, sampling the environment for antigens. Once they encounter a danger signal, which may be a microbial product or tissue damage, migration of the DCs to lymph nodes is initiated. The antigen is then processed and presented on the APC as described above. DCs also upregulate co-

stimulatory molecules required for effective interaction with T cells. The most important chemokine involved in DC migration is CCR7, which binds the chemokines ED11 ligand chemokine (ELC/CCL19) and secondary lymphoid tissue chemokine (SLC/CCL21) which are produced in lymphoid organs, and are responsible for recruiting both DCs and naïve T cells to this site [73]. Once in the lymph nodes, mature DCs can efficiently trigger an immune response by T cells with a receptor that is specific for the foreign peptide MHC complexes on the DC surface [74]. Quiescent DCs do not express CCR7, however they have receptors for chemokines produced by tissue cells such as CCR6, and chemokines produced during inflammation (CCR1, CCR2, CCR5).

DCs interact with other cells as well as T cells, including B cells and NK cells and may also remain in peripheral tissues as inflammatory mediators, instead of migrating to lymph nodes [74]. These different roles for DCs has led to the identification of different subsets of cells (Table 1.2), based on their origin, tissue distribution, differentiation status and function [75]. The relationship between the DC subsets remains unclear, however they are thought to have distinct effects on CD4 T cells, by stimulating Th1 or Th2 responses or T cell tolerance.

Phenotype	Subtype	Location
$CD4^{-}$ $CD8^{hi}$ $CD205^{hi}$ $CD11b^{-}$	Lymphoid	Spleen, Thymus, Mesenteric LN, Skin draining LN
$CD4^{+}$ $CD8^{-}$ $CD205^{-}$ $CD11b^{+}$	Myeloid	Spleen, Mesenteric LN, Skin draining LN
$CD4^{-}$ $CD8^{-}$ $CD205^{-}$ $CD11b^{+}$	Myeloid	Spleen, Mesenteric LN, Skin draining LN
$CD4^{-}$ $CD8^{-}$ $CD205^{-}$ $CD11b^{+}$	Myeloid	Spleen, Mesenteric LN, Skin draining LN
$CD4^{-}$ $CD8^{lo}$ $CD205^{hi}$ $CD11b^{+}$	Langerhans	Spleen, Mesenteric LN, Skin draining LN

Table 1.2: DC subsets

1.2.2.5 Differentiation

After engagement of the TCR by the appropriate MHC complex as described above, clonal expansion of T helper cells is triggered, followed by rapid programmed differentiation [76, 77]. Naïve T helper cells can differentiate into at least two functional classes of cell during an immune response: Th1 cells, which secrete IFNγ, and Th2 cells, which secrete IL-4 [78]. Th1 cells are responsible for cell-mediated immunity, whereas Th2 cells are responsible for extracellular immunity. Both Th subsets have been implicated in pathological responses. For example, Th1 cells can mediate organ-specific autoimmunity and Th2 cells have been implicated in the pathogenesis of asthma and allergy [79].

A number of models have been proposed to explain how naïve T cells differentiate into Th1 or Th2 cells [80, 81]. Many factors influence the generation of Th1 or Th2 cells. The cytokines IL-12 and IL-4 acting through STAT4 and STAT6 respectively are thought to play a major role in the outcome (reviewed in [76, 77]). It has been proposed that certain transcription factors are important in the gene-expression programmes of Th1 and Th2 cells (Figure 1.2). T-box transcription factor (T-bet) has been shown to have a central role in Th1 development [82, 83], and GATA-3 seems to be crucial to induce Th2 cell differentiation [84, 85] (See Figure 1.2). IL-25 (IL-17E), a distinct member of the IL-17 cytokine family, plays important roles in evoking T helper type 2 (Th2) cell–mediated inflammation that features the infiltrations of eosinophils and Th2 memory cells [572]. Recent studies have shown that DCs activated by thymic stromal lymphopoietin (TSLP) are able to prime naïve $CD4^+$ T cells to differentiate into Th2 cells that produce high amounts of TNFα but no IL-10 [86]. It was found that TSLP-induced OX40L on DCs was required for triggering naïve

CD4' T cells to produce IL-4, IL-5 and IL-13. It was also shown that OX40L promoted TNFα but inhibited IL10 production in developing Th2 cells, could not polarise Th2 cells in the presence of IL-12 and exacerbated IL-12-induced Th1 cell inflammation by promoting TNFα and inhibiting IL-10 [86]. IL-33 is a cytokine belonging to the IL-1 superfamily. IL-33 induces helper T cells to produce type 2 cytokines. IL-33 mediates its biological effects by interacting with the receptors ST-2 and IL-1 Receptor Accessory Protein, activating intracellular molecules in the NF-κB and MAP kinase signalling pathways that drive production of type 2 cytokines from polarized Th2 cells [573]. As well as transcription factors that are specific to Th subsets, recent studies have shown that some phenotypic markers are associated with each subset [87]. Th1 cells have been shown to preferentially express the ligands for P and E selectin and facilitate the differential migration of these cells into inflammatory sites such as inflamed joints or sensitised skin [88]. CCR3, an eotaxin receptor, mediates the migration of Th2 cells, eosinophils and basophils to tissues undergoing allergic reactions, and is not expressed on Th1 cells [89].

IL-17-producing $CD4^+$ T cells (Th-17 cells) have been identified as a unique subset of Th cells that develops along a pathway that is distinct from the Th1 and Th2 cell differentiation pathways. One proposed mechanism is IL-23-dependent, but independent of STAT-1, T-bet, STAT4, and STAT6. The hallmark effector molecules of Th1 and Th2 cells, e.g., IFN-γ and IL-4, have each been found to negatively regulate the generation of these Th-17 cells. By a second proposed mechanism, de novo differentiation of Th-17 cells in the absence of IL-23 has been demonstrated by treatment of naïve CD4 cells with TGF-β1 and IL-6, an effect heightened by IL-β and TNF-α.

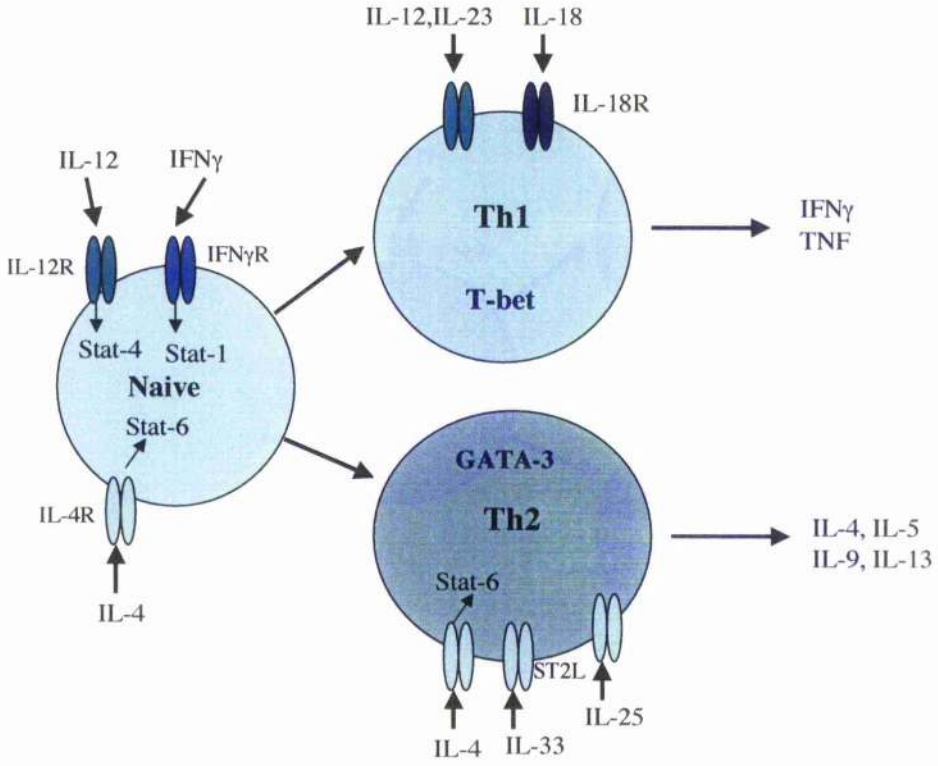

Figure 1.2: Factors involved in T cell differentiation.

1.3 Asthma Pathophysiology

1.3.1 Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) is a characteristic feature of asthma and refers to the ability of airways to narrow after exposure to constrictor agonists [90]. Airway responsiveness is measured using inhalation challenges with airway constrictor agonists, such as anticholinergics, in both clinical and research laboratories. The severity of AHR correlates with the severity of asthma and the amount of treatment needed to control the symptoms [91, 92]. The degree of AHR is also associated with other features of asthma

including variation in peak flow rates, the improvement in (forced expiratory volume) FEV_1 after inhalation of bronchodilators, and the degree of airway constriction caused by exercise [90].

Many different factors contribute to AHR found in asthmatic patients, and numerous inflammatory processes are thought to be important. Eosinophils are thought to play a major role in the airways by producing changes in airways tissue, including epithelial damage, thickening of the basement membrane and release of mediators that cause bronchial smooth muscle contraction [90]. Many of these processes are involved in interacting to produce these different responses and different mechanisms are involved in different components of AHR. Although bronchial wall eosinophilic infiltration is a prominent feature of asthma it is not solely responsible for AHR, since corticosteroid treatment reduces eosinophils but does not prevent AHR. However, the changing levels of eosinophilia may affect the changes in AHR. Many clinical studies have shown that in allergen induced asthma varying degrees of antigen exposure with increased eosinophils also increase AHR in asthmatics [93, 94].

1.3.2 Remodelling

As well as the function of lungs being altered in asthmatics, the structure of the airway is also changed. The term remodelling has been used to describe the deposition of new matrix in the airway walls and is associated with proliferation and activation of myofibroblasts [95]. It has been shown in severe asthmatics that epithelial injury and impaired repair led to the release of profibrogenic growth factors that act on mesenchymal cells to convert them to

myofibroblasts, which show increased ability to synthesize extracellular matrix (ECM) and produce mitogens and differentiation factors for smooth muscle cells [96]. The thickening of the airway wall, which has been shown in patients with varying degrees of asthma, may be one of the main differences between healthy individuals and asthmatic patients [97]. In fatal cases of asthma wall thickening has been shown to be greater compared to patients with mild or non asthmatic patients [98].

An increase in airway smooth muscle (ASM) involving both hyperplasia and hypertrophy are important in AHR [99][[100]. Several studies in patients that have died from severe asthma have revealed an increase in the muscle mass in the airway wall compared to normal controls [101] [102]. These studies have examined lung tissue, which have shown that there are changes in airway morphology and structure but also entails changes in cell-cell interactions and changes in the regulation of cells [103]. The changes in structure include an increase in matrix deposition, which is caused by increased mucus secretion by mesenchymal cells and an imbalance in matrix degrading enzymes and inhibitors of proteases [104] (see figure 1.3). ASM plays an important role in promoting and mediating airway remodelling. However, the physiologic consequences of airway remodelling remain unclear. *In vitro* studies of ASM have shown that they are able to promote airway inflammation through the secretion of cytokines and chemokines and alter its phenotype in response to growth factors, but these responses are still to be proven *in vivo*.

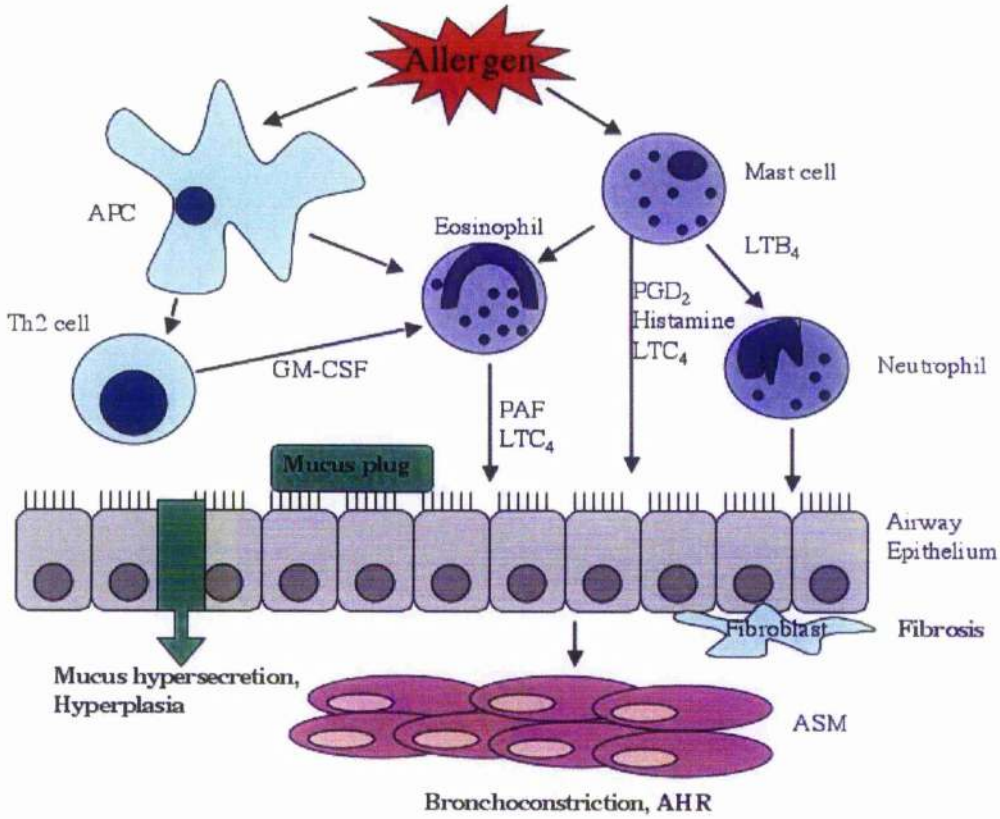

Figure 1.3: The pathophysiology of asthma. Several inflammatory cells are recruited and/or activated in the airways, releasing a variety of inflammatory mediators that have acute effects on the airways, such as bronchoconstriction, mucus hypersecretion, and also structural changes including fibrosis and hyperplasia.

Details of the pathological changes found in the asthmatic lung have been obtained from bronchial biopsies of patients and post-mortem examinations of subjects who have died from asthma. Such studies have shown that an inflammatory infiltrate consisting of T lymphocytes, eosinophils and mast cells is typical and that these changes may be seen in non-allergic ('intrinsic') as well as allergic asthma, suggesting common mechanisms of

disease. T cells appear to be predominantly of a Th2 phenotype [105-107], which will be explored later.

Other characteristic changes are found in the epithelium and airway wall. Epithelial damage and shedding is common and may correlate with severity of AHR although some studies suggest that these findings may be a sampling artefact. Goblet cell hyperplasia is presumed to contribute to mucus hypersecretion and thereby to airflow obstruction in asthma, and has been shown to be present in both mild asthmatics and fatal cases [96, 108]. Further airway narrowing is caused by thickening of the airway wall, much of which can be attributable to an increase in smooth muscle mass.

Mast cells (MCs) are resident in all normal tissues, where they are believed to play an important role in tissue homeostasis, wound healing, and host defence, particularly bacterial infection [109]. Chronic mast cell activation contributes to the pathophysiology of many diverse diseases through the synthesis and release of numerous proinflammatory mediators and cytokines, the pattern of which varies depending on the stimulus [110].

Mast cells secrete histamine, prostaglandin (PG) D2, and leukotriene (LT) C4, which are capable of inducing bronchoconstriction, mucus secretion, and mucosal oedema, all features of asthma. This is evident during experimental allergen challenge, in which blockade of these mediators attenuates the early decrease in lung function [111]. However, mast cells also synthesize and secrete a large number of cytokines (including IL-4, IL-5 TNF, and IL-13), which regulate both immunoglobulin (Ig) E synthesis and the development of eosinophilic inflammation, and several profibrogenic cytokines, including transforming

growth factor (TGF) β and basic fibroblast growth factor [109, 111]. The serine proteases tryptase, chymase, and carboxy-peptidase are major secretory products of human mast cells that can interact with various cell types via protease-activated receptors (PARs) and by other processes to alter their behaviour. Importantly, bronchial mucosal mast cells in subjects with asthma exhibit features of chronic ongoing activation. Although most of these studies have been performed in atopic individuals, similar evidence has been obtained to support a role for ongoing mast cell secretory activity in both nonatopic and occupational asthma [111].

It is generally accepted that the disordered airway physiology and airway wall remodelling characteristic of asthma are consequences of the inflammatory process, but there are examples where this relationship is weak. This is most evident in patients with eosinophilic bronchitis (EB), a condition that accounts for about 15% of cases of cough referred to respiratory specialists. It is characterized by corticosteroid responsive cough and the presence of a sputum eosinophilia occurring in the absence of variable airflow obstruction or AHR [112]. However, despite differing functional effects on the airways, the immunopathology of asthma and EB is virtually identical [113, 114]. Thus, in bronchoalveolar lavage (BAL), induced sputum, and airway biopsies, the extent of T-cell and eosinophil infiltration and activation, mucosal mast cell numbers, IL-4 and IL-5 cytokine expression, epithelial integrity, subbasement membrane collagen deposition, and mediator concentrations including histamine and PGD_2 are almost identical. This suggests that many of the immunopathological features previously attributed to causing asthma may not be fundamental to the development of airflow obstruction and AHR.

These findings illustrate the concept that both immunological inflammatory processes and changes involving structural tissues such as smooth muscle and epithelium, contribute to asthma pathophysiology. The inflammatory changes strongly implicate cells such as T cells and eosinophils as facilitators and effectors respectively, and the evidence for these processes shall be considered in more detail.

1.3.3 Inflammation and asthma

It had been known for decades that asthma is an inflammatory disease of the airways [115] and is characterised by repeated episodes of airway obstruction and wheezing. Inhalation of allergens causes airway inflammation and increased airway responsiveness, which are both important in characteristics of clinical asthma [116]. After allergen inhalation there is a temporary influx of neutrophils [117] followed by a substantial accumulation of eosinophils and basophils [118]. The eosinophilia after allergen inhalation corresponds to levels seen in clinical asthma and is also persistent, which suggests the increased production and importance of these cells. The presence of eosinophils after exposure to allergen has been known for nearly 100 years. However, it was only recently thought that inflammation was the cause of asthma. Studies carried out in the early 1980's using fibre optic bronchoscopy allowed evaluation of asthmatic patients, which showed that even when patients were asymptomatic, airway inflammation was still present. Also in the early 1980's Mosmann *et al* published papers describing that CD4 lymphocytes *in vitro* could be polarised into two subsets known as Th1 and Th2 cells. Subsequent studies showed that Th2 cells were present in the airways of asthmatics [105]. Because Th2 cytokines induce eosinophilia, IgE

production, mucus production, and mast cells and are associated with other allergic diseases, they are thought to be involved in the pathophysiology of asthma.

1.4 T cells in asthma

There are several factors that interact to cause airways hyperresponsiveness, and these can be considered in two broad categories: immunological mechanisms and the accompanying inflammatory changes, and non-immunological mechanisms (which comprise mainly the structural elements of the airways, as discussed above). The immunological mechanisms have been extensively studied over the past twenty years both in human subjects and animal models and current opinion favours a system whereby inflammation dominated by a Th2 pattern of cells and cytokines [2] is key to the development of AHR.

1.4.1 Th2 cells in asthma

In both allergic and intrinsic asthma, the inflammatory response is associated with the recruitment of activated $CD4^+$ T lymphocytes expressing a Th2 cytokine profile [119, 120] and an influx of eosinophils. The interleukins, IL-4, IL-5, IL-9 and IL-13, expressed by activated Th2 cells which are the dominant cell type in bronchoscopic biopsies and bronchoalveolar lavage samples obtained from patients with asthma, are thought to play a central role in the pathophysiology of asthma [105]. In murine models, T cells have also been shown to play a crucial role: transfer of primed, polarised Th2 cells into naïve recipients reproduces features of allergic airways inflammation following antigen challenge [121-123]. Similarly, such features are reduced or abolished if T cells are absent [124, 125].

Asthma is characterised histologically by a predominance of Th2 lymphocytes [126]. Bronchial biopsies taken from atopic and non-atopic asthmatics show elevated expression of transcription factors GATA-3, and STAT-6 [127]. It was demonstrated that STAT-6 and IL-4 receptor alpha were both necessary for the development of murine airways inflammation [128, 129]. It has also been shown that the cytokine IL-13 receptor heterodimer utilises the IL-4 receptor alpha chain, and STAT-6 pathway. In murine models STAT-6 and GATA-3 deficiency or inhibition leads to suppression of airway eosinophilia and AHR. Conversely, the transcription factor T-bet, which is associated with Th1 cell development, is decreased in the airways of asthmatic patients compared to healthy controls and its deletion in mice leads to the development of asthma-like features [130].

Exactly how Th2 cells contribute to an asthmatic phenotype has not yet been fully elucidated but several studies have advanced our knowledge in this area and the roles of individual Th2 cytokines such as IL-4, IL-5 and IL-13 in inducing specific features of asthma, either directly or via other cells or cytokines are becoming clearer.

1.4.2 Th2 cytokines

Multiple cytokines play a critical role in orchestrating and perpetuating inflammation in asthma (figure 1.4) and have been implicated in the pathophysiology of asthma (See table 1.3).

Of the cytokines thought to be important in asthma, IL-4, IL-5 and IL-13 have been studied in greatest detail. In allergic patients IL-4 has been shown to be present in the serum and in

the BAL. Peripheral blood and mononuclear cells from atopic asthmatics show increased IL-4 production in response to dust mite allergen [131] and BAL samples from asthmatics have shown increased IL-4 levels [114, 132]. In a study where nebulised IL-4 was given to patients with mild asthma, a significant increase in AHR associated with elevated sputum eosinophil numbers was seen [133].

In asthma IL-4 stimulates mucus-producing cells and eotaxin and other inflammatory cells from fibroblasts, suggesting that IL-4 is involved in airway remodelling. IL-4 upregulates expression of vascular cell adhesion molecule (VCAM) -1 on vascular endothelium to direct the migration of T lymphocytes, monocytes, basophils and eosinophils to the inflammatory site [134]. By increasing the expression of eotaxin, IL-4 inhibits eosinophil apoptosis and promotes eosinophil chemotaxis and activation [135]. IL-4 is essential in the development of allergic inflammation, as it is able to drive the differentiation of Th0 lymphocytes to Th2 cells [80]. This feature is unique to IL-4 and is not shared by IL13, which has many commonalities with IL-4. IL-4 is also capable of preventing T lymphocyte apoptosis ensuring that activation and proliferation of the T lymphocytes is maintained.

Cytokine	Effects in asthma
IL-1	Induces AHR Induction of eosinophils, T cell and epithelial activation Growth factor for Th2 cells
IL-2	Stimulates growth and differentiation of T cells Induction of eosinophilia
IL-3	Stimulates induction of eosinophilia and mast cells
IL-4	Stimulates eosinophil growth Promotes development of Th2 cells and IgG synthesis Inhibits development of Th1 cells
IL-5	Enhances production, maturation and activation of eosinophils Promotes development of Th2 cells Induces AHR
IL-6	Activation of T cells Inhibits IL-1 release Induces IgE production
IL-9	Stimulates proliferation of activated T cells Enhances production of IgE
IL-10	Decreases eosinophil survival, AHR Inhibits macrophage activation
IL-13	Activates eosinophils Induces production of IgE
IL-15	Stimulates growth and differentiation of T cells
IL-16	Induces eosinophil migration Stimulates growth and chemotaxis of T cells
IL-17	Co-stimulation of T cell proliferation Activates epithelia, endothelial cells and fibrosis
IL-18	Inhibits IL-4 dependent release of IgE Causes release of IFNγ from Th1 cells
IL-21	Increases TNFα production
IL-23	Induces proliferation of memory T cells Causes IFNγ secretion from T cells
IL-25	Induces IL-4, IL-5, IL-13 and eotaxin production in the lung Induces AHR, IgE production and eosinophils
IL-27	Enhances Th2 cytokine release
TNFα	Activates epithelium, endothelium, APCs and macrophages Induces AHR
IFNγ	Decreases eosinophilia after antigen challenge Inhibits Th2 cells and IgE release Induces AHR

Table 1.3: Cytokines involved in asthma

There have been many animal studies conducted that further strengthen the role of IL-4 in allergic inflammation [136-139]. When IL-4 is neutralised with anti-IL-4 antibodies in mice the development of allergen specific IgE is inhibited and eosinophilic inflammation and airway reactivity is reduced. The use of antigen (ovalbumin) specific induced airway models has given significant insight into the functions of IL-4 and many other cytokines. Using these models IL-4 has been shown to be essential for the development of ovalbumin-induced eosinophil influx into the airways, IgE production and an increase in airway responsiveness [122]. Transfer of IL-4 knock-out (KO) Th2 cells reduced airways eosinophilia and mucus hypersecretion but not AHR suggesting that IL-4 has a role in the inflammatory aspects of asthma but not AHR. More recent evidence suggests that IL-4-mediated effects on eosinophilia may act through suppressing the actions of IFN-γ allowing recruitment of eosinophils from the lung interstitium into the airways.

Most studies have proven a role for IL-4 in inflammation rather than AHR. However, a study by Corry and co-workers showed that neutralisation of IL-4 during antigen sensitisation led to decreased AHR but not eosinophils [137]. Another study demonstrated a similar decrease in airway responses after blocking of the IL-4 receptor [128] but this may be because IL-4 and IL-13 share the IL-4Rα receptor component. Thus IL-4 appears to be important in establishing a Th2 response, including IgE production [140] and subsequent inflammation, although effects on AHR may be indirect or attributable to IL-13. Despite these findings in murine models, anti-IL-4 monoclonal antibody, while demonstrating some benefit in patients with severe asthma [141, 142], has so far failed to establish itself in clinical practice.

Despite its homology to IL-4 and sharing the IL-4Rα, IL-13 appears to have a more fundamental role in manifesting the effector features of asthma, particularly AHR [143]. IL-13 is a pleiotropic Th2 cytokine, thought of as a central mediator of asthma. Many studies have demonstrated that administration or overexpression of IL-13 leads to eosinophilia, mucus hypersecretion and AHR and that the neutralisation or knocking out of IL-13 leads to ablation of these key features of asthma. IL-13 appears to be important in both chronic airways inflammation and airway remodelling, although its neutralisation in established disease was ineffective [144]. An increasing number of pathways have been described for IL-13. Studies have shown that it can act directly on epithelial cells to induce mucus production via STAT-6 [145, 146] but can also induce some of its effects on eosinophil recruitment and AHR via both IL-5/eotaxin-dependent and -independent mechanisms. It has also been shown to have effects on DC maturation [148] implying an immunoregulatory role.

Eosinophils are one of the key effector cells in the pathogenesis of allergic inflammation. IL-5 is the key cytokine in the production, differentiation, maturation and activation of eosinophils [149, 150]. In human asthmatics, IL-5 has been isolated from BAL and serum [151, 152] and inhalation of IL-5 leads to eosinophilia and increased AHR. In mice, overexpression of IL-5 leads to eosinophilic pathological changes in the lung characteristic of asthma [153] and when IL-5 production was abolished, either in KO mice or by using neutralising antibodies, there was a corresponding decrease in BAL eosinophilia and AHR [154, 155].

However, the role of eosinophils in asthma remains controversial. It is still unclear whether eosinophils cause any damage in asthma or whether they are just an indicator of the inflammatory process. They may also be part of the protective response against the inflammation. When anti-IL-5 antibody treatment was tried in human clinical trials, there was no improvement in AHR despite an effect on sputum eosinophilia [156]. This suggests that eosinophils may not be causative in asthma although it should be noted that the clearance of eosinophils from the bronchial mucosa by anti-IL-5 was incomplete [157]. Furthermore, in a transgenic mouse overexpressing IL-5 there was a decrease in AHR which was abolished by anti-IL-5 antibodies [158]

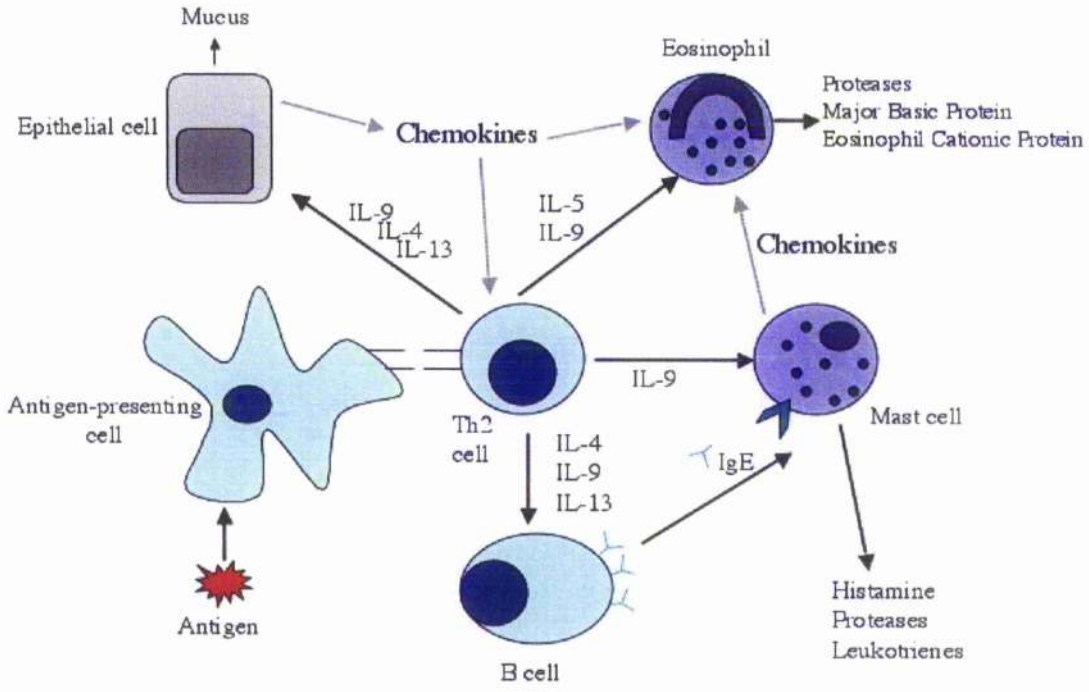

Figure 1.4: Th2 –type cytokine activity in allergic asthma. Upon recognition of the antigen and activation by APCs, naive T cells differentiate into Th2 cells, a process that is promoted by IL-4. Activated Th2 cells stimulate B cells to produce IgE antibodies in response to IL-4, and to a lower extent to IL-13 or IL-9. IgE binds the high affinity IgE receptor at the surface of mast cells, the proliferation and differentiation of which is promoted by IL-9. After contact with antigen, mast cells release the contents of their granules, including histamine, which will induce a bronchospasm, together with newly synthesised prostaglandins and leukotrienes (PGD2 and LTC4). Mast cells also release chemotactic factors that contribute to the recruitment of inflammatory cells, particularly eosinophils, whose proliferation and differentiation from bone marrow progenitors is promoted by IL-5 and IL-9. Finally, epithelial cells upregulate their production of mucus and chemokines in response to Th2 cytokines such as IL-4, IL-13, and IL-9 [159].

1.4.3 Th1 cells in asthma

From the evidence presented above, it seems it is still generally believed that Th2 cells predominantly drive asthma. However, recent data suggests a role for Th1 cells in driving asthma. Th1 and Th2 cells are thought to have counter-regulatory properties, highlighting the importance of maintaining a balance between the two. [78]. Cytokines from Th1 lymphocytes were originally thought to be protective of asthma by skewing the immune response from a Th2 towards a Th1 profile [160]. Furthermore, it was shown that antigen-induced IFNγ production was reduced in peripheral blood lymphocytes of asthmatics [161], and that IFNγ and Tbet-1 were critical for attenuating airways eosinophilia in murine models of asthma [162, 163]. However, it has since been demonstrated that IFNγ is produced by BAL T cells [164] and may enhance airways inflammation [165]. In an adoptive murine transfer model of asthma, it was shown that Th1 cells did not reduce airways inflammation, but actually enhanced established disease through the activation and recruitment of neutrophils into the airways [121, 166, 167]. Randolph *et al* showed that adoptive transfer of either Th1 or Th2 cells followed by antigen challenge was not sufficient to result in eosinophilic airways inflammation whereas co-transfer of Th1 and Th2 cells together was [167]. Furthermore, introduction of a viral infection, typically-viewed as a Th1-dominant inflammatory event, was also able to facilitate eosinophilic inflammation by Th2 cells [168]. The transfer of Th1 clones in a murine model of asthma induced attenuation of the eosinophilic airway inflammation, airway responsiveness, goblet cell hyperplasia and peribronchial fibrosis. These effects are accompanied by an inhibition of Th2 cytokines and an increase of IFNγ in the BAL. However, such treatment augmented noneosinophilic airway inflammation. Th1 cells reduced the expression of eotaxin mRNA and augmented RANTES,

indicating that the adoptive transfer of Th1 clones in asthma model mice diminishes the pathophysiology of asthma but augments noneosinophilic airway inflammation [574]. These results suggest that Th1 cells may be able to attenuate an asthmatic phenotype in murine models through changes in chemokine and cytokine expression and their subsequent effects on the immune response.

Although Th2 responses seem to dominate asthmatic responses, the evidence presented above suggests that Th1 responses may not completely oppose the response as originally thought.

1.4.4 T regulatory cells

As well as mediating the immune response, it is now clear that subset(s) of T cells may also act to regulate immunity. Although the concept of 'suppressor' T cells was largely discredited for many years, the rebranded regulatory T cell (Treg) has been intensively studied in the last decade. Several classifications of Tregs have been proposed, but three main subtypes have been best characterised. 'Naturally-occurring' $CD4^+CD25^+$ T cells, which comprise approximately 5% of all T cells, have been shown to suppress the proliferation and activation of $CD25^-$ T cells in an antigen-independent, cell contact-dependent manner [169, 170] and their absence leads to the development of a variety of autoimmune diseases in mice [169, 171]. Their existence in humans has now been demonstrated [172, 173] and the transcription factor foxp3 has been shown to be critical in their development and function [174-176]. Tr1 cells and Th3 cells differ from $CD4^+CD25^+$ T cells in that they differentiate in the periphery from $CD25^-$ T cells after antigen stimulation.

These Tregs appear to function in a cell contact-independent manner through cytokines such as IL-10 and TGF-β respectively [177-179].

Although mainly studied in relation to autoimmune disease, a role for Tregs in immune dysregulation in asthma is gradually being established. $CD4^+CD25^+$ T cells are able to suppress allergic airways inflammation in murine models [180-182]. The mechanism may be dependent on IL-10 production [180] and although *in vitro* studies of $CD4^+CD25^+$ T cells suggest that their suppressive properties are not cytokine-mediated [169], this can be explained by the finding that IL-10 may be induced by Tregs but produced by alveolar macrophages thereby inhibiting the inflammation [181]. Other experiments have similarly demonstrated protective effects of cells with a phenotype similar to Tr1 cells in experimental asthma models [183, 184]. Interestingly, recent studies have shown that corticosteroid resistant asthma patients may have a defect in IL-10 production from their T cells and that this may be reversed by vitamin D3 treatment [185]. These findings support the hypothesis that Tregs may be an important determinant of the outcome of the asthmatic process.

1.5 Other Cells in asthma

1.5.1 Eosinophils

The presence of increased numbers of activated eosinophils is a main characteristic of asthma and is thought to be central in the pathogenesis of the disease. However, the exact role of eosinophils remains unclear. Airway eosinophilia requires both IL-5 and STAT-6 signalling and it has been shown that CD4 T cells provide signals that promote eosinophil mobilisation, activation and recruitment the respiratory tract. Eosinophils contain many

toxic pro-inflammatory mediators, including granule proteins (major basic protein and eosinophil cationic protein) [186], reactive oxygen species [187], lipid mediators [188] and cytokines, all of which could contribute to airways inflammation. Several pieces of evidence link these cells to asthma: the presence of eosinophils in the airways of asthmatic patients has been recognised for many years [189] and studies have also shown that the degree of eosinophils present in the airways of asthmatics correlates with the severity of the disease [190, 191]. Reduction of AHR by treatment with corticosteroids is associated with a decreased level of eosinophilia in the airways [192].

Animal models have not resolved the issue of the importance of eosinophils in asthma. Most murine models of asthma use eosinophil infiltration in the lung tissue or BAL as indicators of pathology and correlate this with AHR, but several studies have failed to show this correlation [137, 193-195]. AHR has been shown to be either dependent or independent of eosinophils in two different eosinophil-deficient mouse strains [196, 197]. However, the majority of studies are in favour of eosinophils playing an important role in allergic airways inflammation. A study using eosinophil-deficient mice showed that direct intratracheal instillation of eosinophils into the airways restores AHR, perhaps proving a causative link between eosinophils and asthma [198]. Eosinophils can produce both Th1 and Th2 cytokines [199] and may be able to present antigen to T cells [200, 201] implying a potential immunomodulatory role. They have also been implicated in the development of airway remodelling [197].

1.5.2 Mast cells

MCs have been considered to be one of the main effector cells in asthma. These tissue-resident cells are found in the airway submucosa of both healthy subjects and those with asthma but are present in increased numbers in the airway smooth muscle of asthmatic patients [113]. Crosslinking of FcεR1-bound IgE with multivalent antigens initiates the activation of mast cells by promoting the aggregation of FcεRI [202]. The cell activation results in mast cell degranulation, with the secretion of preformed mediators, the *de novo* synthesis of pro-inflammatory lipid mediators and the synthesis and secretion of chemokines and cytokines [203, 204]. MCs may act as effector cells of the early asthmatic response via the release of these inflammatory mediators and granule contents. This concept is supported by increased degranulation of MCs from asthmatic airways [205, 206].

Data regarding the role of MCs in airways inflammation has been inconclusive and depends on the experimental protocol used [207, 208]. This may be due to the fact that several mechanisms interact to produce the features of asthma and that different experimental protocols may emphasise these different mechanisms. Nevertheless, the paradigm of an IgE-mediated, MC-dependent early response in asthma is supported by murine models demonstrating that the IgE receptor FcεRI on MCs is important in the airway response [209, 210] and that activation of MCs using anti-IgE antibody enhances AHR [211]. More recently, the concept that MCs could also play a significant role in the late response and persistence of asthma, has been supported by findings that mast cells may influence the adaptive immune response by production of Th2-type cytokines [114, 212] and chemokines [213] and in numerous other ways [214].

Mast cells are clearly important in the pathogenesis of asthma but it is still unknown exactly how they contribute to the disease outcome. Their role in the immediate response is reasonably well understood, but their influence in ongoing asthmatic disease requires further investigation. These cells provide an interesting link between the innate and adaptive immune response and may be important in gaining an understanding of how innate responses to microbial products, such as lipopolysaccharide, can impact upon the development and persistence of asthma.

1.5.3 B cells and IgE

The ability of B cells to produce IgE means that they may be of importance in asthma pathophysiology. It is probable that their function is in some way regulated by $CD4^+$ T cells and Th2 cytokines have been shown to be important although not crucial for IgE production [140, 215].

IgE has become recognised as a major factor in atopic disease, in particular in allergic asthma where the severity of disease correlates with serum IgE titres [216, 217]. The majority of IgE is bound to the surface of mast cells and basophils by the high affinity IgE receptor FcεRI. This converts FcεRI to an antigen-specific receptor, whose activation leads to mast cell or basophil degranulation and subsequent downstream events associated with the Type I hypersensitivity reaction [209, 210].

The central feature in anaphylactic and immediate hypersensitivity reactions is IgE-dependent activation of mast cells and basophils leading to the release of histamine and other inflammatory mediators, such as prostaglandins and leukotrienes. Furthermore, upon

activation through IgE receptors, mast cells and basophils produce cytokines, such as IL-4 and IL-5, which are potentially important in the recruitment of eosinophils, thus causing chronic allergic inflammation. Second, IgE bound to receptors on antigen-presenting cells, such as CD23 on B cells and to high-affinity IgE receptors (FcRI) on Langerhans' cells and monocytes can enhance antigen internalization and presentation to T cells, resulting in continuous activation of the immune system [575].

The importance of IgE in asthma led to the development of targeted therapy in the form of a monoclonal antibody (mAb) against anti-IgE, omalizumab (Xolair®) [218]. Omalizumab blocks the IgE high affinity receptor, and downregulates DC FcεRI expression [219]. Clinical trials of omalizumab showed improvement in asthma symptoms and steroid usage in asthmatic patients accompanied by a reduction in total IgE levels [220-222]. This study and others have shown that these effects may be due to a reduction in IgE-mediated activation of FcεRI-expressing cells, suppression of sputum and airway wall eosinophilia, reduction in $FcεRI^+$ cells, and a reduction in T and B cells in bronchial biopsies [223].

Although IgE is important in the pathophysiology of asthma, it may not be crucial. AHR and eosinophilic inflammation can still occur in the absence of IgE [224] and in one trial, marked reduction in airway inflammatory cells by anti-IgE treatment was not associated with any change in AHR [223]. Thus, manipulation of IgE is an important addition to the therapeutic armamentarium against asthma, but may not cover all the elements conspiring to create an asthmatic phenotype.

1.5.4 Dendritic Cells

DCs are the most effective APCs, and have been shown to be necessary for disease development in murine models of asthma [225] as previously discussed. They can be identified in both the lung parenchyma and airway compartments [226] and as professional APCs they are responsible for the antigen-specific activation, proliferation and functional polarisation of T cells [227]. The importance of DCs in allergic airways models has been demonstrated by the transfer of Ag-primed DCs into naïve mice leading to an asthma-type phenotype after allergen challenge [228]. Furthermore the absence of DCs results in ablation of these features [225].

Transgenic and inducible knockout murine models have been developed to specifically study the role of DCs in pulmonary allergic responses. These studies have determined that airway DCs take up inhaled antigen and migrate to the draining lymph node (DLN) [226, 229], a process which increases in both speed and numbers of cells when the DCs are activated [230]. Whether an encounter with an aeroallergen results in sensitisation and airway inflammation, or tolerance depends on the phenotypic class [231] and maturation status [232] of the DC. The traditional model of DC-T cell interactions places antigen-presentation in the DLN, but recent evidence suggests that this may also occur in the airway itself [233] and that DCs are important during the secondary response to antigen, not just during sensitisation [234]. Nevertheless, dendritic cells are clearly important in the pathogenesis of asthma, probably in both the sensitisation and effector phases of the disease.

1.5.5 Cell trafficking in asthma

It is also important to consider the trafficking of cells between tissues and organs when considering the mechanisms involved in allergic airways inflammation. Effector T cells are necessary and sufficient to provide the Th2 cytokines needed to induce both histological changes and induce AHR [235]. Although many murine models have been employed to elucidate the mechanisms involved in asthma, none have determined when and where these T cell interactions occur. It is still unclear whether initial interactions occur in the lung itself or in the lymph nodes, and where T cells migrate after antigen challenge. The ability to track T cells of known antigen specificity in different compartments important in airway inflammation could help to resolve these questions. This thesis aims to elucidate some of these unanswered questions using an adoptive transfer model of allergic airways inflammation.

Several stages in leukocyte migration have been studied but it is the role of chemokines and their receptors, particularly in the trafficking of lymphocytes, that has been studied most in regards to asthma [236]. Th2 cells preferentially express the chemokine receptors CRTh2, CCR3, CCR4 and CCR8 [237-239], whereas Th1-related chemokine receptors include CCR5 and CXCR3 [239, 240]. This has been corroborated in asthmatic patients who have been shown to express increased levels of these chemokines receptors [119, 241] and suggest that such molecules may be potential therapeutic targets in asthma.

Inhibition of chemokine receptor signalling in T cells by pertussis toxin reduced allergic airways inflammation in a murine model [242] and in another study, use of STAT6-deficient mice resulted in reduced expression of CCL11, CCL17 and CCL22 (ligands for CCR3 and

CCR4) associated with decreased eosinophilic inflammation [243]. In one study of CCR3-deficient mice, BAL eosinophilia was markedly reduced as was AHR, although T cells were not [244], and another showed reduced BAL eosinophils but increased tracheal mast cells and no reduction in AHR [245]. These results reflect the fact that CCR3 is expressed not only on Th2 cells but also several other cell types associated with asthma including eosinophils [246], mast cells [247] and basophils [248], and also emphasises that elements contributing to eosinophilic airways inflammation and AHR are not exclusive.

Deficiency in, or neutralisation of, CCR4 has not had any effect in experimental allergic airways inflammation although targeting CCR4 ligands (CCL17 and CCL22) does reduce both airways inflammation and hyperresponsiveness suggesting either differences in the effects of blocking the ligand over the receptor, or that additional receptors for CCL17 and CCL22 are contributing [250]. It has been shown in the airways that CCR3 is expressed early, whereas CCR4 appears to be upregulated with repeated or chronic antigen exposure [251]. Thus chemokines and their receptors appear to be temporally regulated with consequent implications for therapeutic intervention. Other studies have shown that CCR8 is not critical for the development of airways inflammation but that blocking chemokines associated with lymphocyte recirculation or memory does result in reduced eosinophilia and AHR .

These results and others suggest that chemokines and their receptors do play an intrinsic role in the pathophysiology of allergic airways inflammation, but that there are additional factors influencing the migration of leukocytes. Gaining an in-depth understanding of these factors and how they function will increase the prospect of being able to target leukocyte trafficking as a potential therapy for asthma.

1.6 Animal models

1.6.1 Humans or animals?

The use of human patients to study diseases is restricted in its nature, in that most procedures need to be of limited invasiveness, ranging from exhaled breath condensate to bronchial biopsies. It is also difficult or impossible to obtain important tissues to study, such as lymph nodes or lungs, as they cannot be removed from live patients. It may be hard to determine the stage of disease progression patients are in, giving only a limited view of the ongoing disease processes. Consequently, intervention in the disease process and measurement of the outcome is largely limited to clinical trials, which are both costly and time-consuming. Using patients does not allow the generation of knock-outs, identification of antigen-specific lymphocytes, or transgenic studies possible, which have been useful when studying animal models of disease. Both practical and ethical constraints need to be taken into consideration where human subjects are concerned. Furthermore, the genetic diversity of human subjects is vast making these studies more challenging. Despite these drawbacks, human studies have been fundamental in elucidating the mechanisms involved in asthma, and used in conjunction with animal models have given greater insight into the immune response involved in asthma.

1.6.2 Advantages of Animal models

Much of our understanding of the response to allergens and the mechanisms involved in airways inflammation and allergic disease comes from the use of animal models, especially mice, rats and guinea pigs. These investigations have given an insight not only into the

immunologic mechanisms underlying allergy, but have also allowed evaluation of potential therapeutic agents. A major advantage of using animal models is that small rodents can be bred cheaply and quickly, and in-breeding eliminates the genetic diversity encountered in human subjects. However, this may be considered a disadvantage, as humans are genetically diverse, thus suggesting that the disease model may not be directly comparable. Manipulation of the murine genome allows the development of transgenic and knockout strains to investigate the role of specific gene products. Using animal models also allows many tissues that are unobtainable in humans, to be analysed. It is possible to image *in vivo* in murine models allowing interactions to be studied in intact animals, providing valuable information that may not have been discovered *in vitro*.

The mouse model has become readily available and easy to adapt because of the emergence of monoclonal antibodies that are specific for murine proteins, and the availability of knockout and transgenic mice. Mice are currently the only species in which this technology is readily available. Also, the lack of selective non-peptide antagonists to many cytokine, chemokine and growth factor receptors has made the models very appealing [252]. Murine models of airway inflammation are deemed to be useful as many of the features of clinical disease found in humans are also characteristic in the mouse models. It has been shown that mice undergo early and late asthmatic responses [253], and show serum specific IgE, recruitment of lymphocytes and eosinophils and airway hyperresponsiveness when exposed to allergens such as Der p1 [254], which are relevant to humans. Murine models have also been used to measure different aspects of airway remodelling, and it has been found that many of the cytokines, chemokines, growth related peptides and their receptors that are

expressed in the human disease are also expressed in the murine equivalent of the inflammatory murine models. This shows that findings in animal models can be translated to human clinical settings.

Importantly, many of the drugs that have been used in human studies of asthma are able to attenuate responses in the murine models. Glucocorticoids have been shown to inhibit the recruitment of eosinophils and inhibit AHR after antigen challenge in a chronic murine model [255]. Furthermore, β_2-adrenoreceptor agonists provide bronchoprotection against antigen challenge [253]. It is also possible that novel anti-inflammatory agents can be tested in these models as they can be modulated by current therapeutic modalities. As has already been shown in some studies, not all positive results discovered through the use of murine models will prove effective at clinical trial level [154, 256]. However, these studies will still add further understanding of the disease pathophysiology.

1.6.3 Disadvantages of animal models

Despite the many benefits of animal models described above, there are also arguments against using animal models to study diseases. Due to the complexity of asthma it may not be possible to have a single animal model that can replicate all aspects of the human disease. It is also not clear how much the anatomy and physiology of animals differs from that of humans, requiring caution when interpreting results. For example, the mouse has fewer divisions of the bronchial tree and fewer mucus-secreting glands in the respiratory epithelium than humans [257]. Furthermore, although eosinophil degranulation has been shown to occur in asthmatic patients, there has been some doubt over whether this occurs in

murine models of allergic airways inflammation [258, 259], although more recent studies would appear to suggest that it does [260].

In human studies of asthma, relevant biomarkers of therapeutics would be changes in pulmonary function tests, symptom scores or medication use, not all of which can be measured in animals and the assessment of the former has been the subject of much debate over recent years [261-264]. Many studies have also shown that animal studies can produce conflicting results when applied to humans. This has been found in conditions such as septic shock where clinical trials of anti-TNF drugs failed to show benefit [265] despite promising results in animal studies [266]. In asthma, although IL-5 neutralisation in animal models proved effective at reducing airway inflammation and AHR [154, 256], clinical trials of anti-IL-5 antibody have proved disappointing [156].

The ethical justification for the use of animals in research converges with the scientific and in some respects depends on it. It is based upon the view that increasing the knowledge base makes it possible to develop therapies that mitigate pain and suffering caused by illness and trauma, and thereby responds to the moral imperative to do good. It assumes that research can be carried out with no or minimal discomfort or distress to the animal research subjects, and that any pain and suffering experienced is compensated for by the alleviation of human pain and suffering caused by disease and injury. Although animal and human studies sometimes contradict each other, many valuable lessons have been gained from the use of animals.

1.7 Asthma Therapies

There are two main types of drugs that asthmatic patients use: 'relievers' and preventers' [267]. Patients use relievers when they are symptomatic and preventers are used to keep the disease quiescent. These categorisations also help to determine where potential therapies should be targeted, either at the end-effector mechanisms such as airways hyperresponsiveness to achieve bronchodilation and symptom relief or at underlying processes such as airways inflammation or airway remodelling to try and prevent symptoms developing (See table 1.4).

Drugs	Mechanism of action
β_2 adrenoreceptor agonists	Bronchodilation by inhibiting contraction/inducing relaxation of smooth muscle
Anti-cholinergic agents	Bronchodilation by blocking muscarinic receptors in smooth muscle
Anti-IgE monoclonal antibody	Reduces free IgE levels and so inhibits mast cell/basophil/eosinophil activities
Corticosteroids	Numerous effects on inflammatory mediators
Theophylline	Principally a bronchodilator, but several other actions also proposed
Cromones	Prevents release of histamine and other mediators from mast cells
Leukotriene antagonists	Blocks action of leukotriene mediators

Table 1.4: Current asthma therapies and their mechanisms.

Of these agents, only β_2-agonists and corticosteroids can be truly classified as described above (as reliever and preventer respectively) with the other drugs having a mixture of effects on AHR and/or inflammation. The principal side effect of β_2-agonists is tremor,

caused by direct stimulation of β_2-adrenergic receptors in skeletal muscle [268]. After many years of research these β_2-agonists and corticosteroids are still the conventional asthma treatments, with treatments aimed at novel targets so far failing to make any real difference to the clinical management of asthma [269]. These therapies are mainly used for mild disease, where the inflammatory response of AHR, and variable airflow obstruction are highly responsive to corticosteroids, making these drugs the first choice [95]. In severe and persisting asthma inhaled corticosteroids are only partially effective and patients often require intermittent or continuous oral corticosteroids. Although there are a range of other treatments for these patients, including cytotoxic agents and nebulised bronchodilators, they have variable benefits and have many side effects [270]. Thus, severe corticosteroid-refractory asthma represents a major unmet clinical need. An ideal therapy for asthma would have the anti-inflammatory disease-modifying properties of a preventer but without the damaging side effects of drugs such as steroids.

Much interest has centred recently on 'biologic' therapies i.e. drug treatments mechanistically designed to target a specific biological mediator of a disease, usually as a monoclonal antibody. The theoretical advantages of such agents would be the inhibition of a specific pathway resulting in the desired therapeutic effect with minimal side-effects. Biologic therapies grew out of observations that the cytokine TNFα appeared to have an apical role in an 'inflammatory cascade' of cytokines in rheumatoid arthritis (RA). Anti-TNF treatments such as etanercept and infliximab proved successful in clinical trials and are now established in rheumatoid arthritis [273-275] and other biologic agents for a growing range of conditions are in development [276]. Recently, anti-TNF therapy has shown beneficial effects in severe asthmatics [277]. The main side effects of anti-TNFα therapies are

infections, and although rare, they can be severe and sometimes fatal-opportunistic infections and tuberculosis have been observed in some cases [278]. Anti-IgE mAb therapy has established a place for itself in the clinical management of asthma [220-222]. However, antibodies to IL-5, CD4 and CD11a have so far failed to show any convincing benefit [141, 142, 156, 157, 279].

To be able to develop these drugs, we must continue to refine our understanding of the underlying mechanisms of asthma such as airway inflammation and remodelling. It is also important to understand where cells are interacting, and what with, during disease to be able to specifically target drugs efficiently, thereby reducing any possible side effects. Understanding how these mechanisms work, what exacerbates them and what improves them, should lead to the identification of new therapeutic targets.

1.7.1 TNFα

TNFα was first identified as a factor causing necrosis of tumours in mice [280]. It was subsequently identified as a major proinflammatory agent in conditions such as septic shock [281, 282] and is now recognised as the prototype member of the TNF family of ligands (including lymphotoxins alpha and beta, Fas ligand, CD40 ligand and OX-40 ligand) binding to a corresponding family of receptors, the TNF-receptor family [283, 284]. TNF forms a homotrimer [285], and exists as a 26 kDa membrane-bound protein that may be cleaved by TNF-α converting enzyme (TACE) to form the 17 kDa soluble moiety [286]. Two receptors for TNFα are described [283]. TNFR1 (p55) is widely-expressed, contains the cytoplasmic

death domain found in several members of the TNFα receptor family and mediates many of the proinflammatory actions of TNFα [287, 288]. TNFR2 (p75) does not contain the death domain and its actions are less clear, although it also binds lymphotoxins and has been shown to mediate thymocyte proliferation in response to TNFα and apoptosis of mature activated T lymphocytes [283, 289].

TNFα is a potent pleiotropic cytokine with broad proinflammatory and immunoregulatory activities, and is produced by many cell types including monocytes, macrophages, lymphocytes, neutrophils, eosinophils, and mast cells [290]. It functions as a chemoattractant for neutrophils and monocytes, increases microvascular permeability, and activates T cells, eosinophils, and mast cells [291, 292]. Moreover, TNFα induces endothelial adhesion molecules and thus may initiate the recruitment of circulatory inflammatory cells into the tissue [292, 293]. TNFα mediates direct effects on target tissue cells e.g. keratinocytes, synovial fibroblasts, or osteoclast precursors, all of which may further promote a role in target tissue pathology.

The pro-inflammatory role of TNFα is best described in rheumatoid arthritis. RA synovial membrane contains numerous cytokines including TNFα that may be released upon culture *in vitro*. Neutralisation of the latter suppresses IL-6 and IL-1 secretion, in turn suggesting an apical hierarchical role for TNFα [271, 272, 294]. Over-expression of TNFα in transgenic mice leads to spontaneous erosive polyarthritis [295]. Administration of anti-TNFα antibodies in transgenic arthritis models and in DBA/1 mice with collagen-induced arthritis (CIA) reduced joint inflammation and destruction [295] suggesting that TNFα played a key

role in these disease processes and that neutralising TNFα activity in humans could lead to an improvement in clinical disease. Subsequent clinical trials revealed that anti-TNFα therapy is beneficial in RA although only a proportion of patients respond to treatment [273-275, 296-299]. Thus approximately 40% of patients respond with at least 50% improvement in disease activity, with some achieving remarkable responses that are sustained over time and associated with prevention of articular damage assessed radiographically [283-286]. Considerable residual unmet clinical need is reflected in those partial and non-responders that remain.

1.7.2 Anti-TNFα in asthma

In the lung, TNFα is synthesised and stored mainly in mast cells and alveolar macrophages [300]. It functions as a chemoattractant for neutrophils and monocytes, increases microvascular permeability, and activates T cells, eosinophils, and mast cells. It has been shown that there is increased expression of TNFα in the airway of asthmatic patients compared to normal subjects [300, 301]. Mast cells are unique in being the only tissue-resident cells with granules containing preformed TNFα and consequently during the early stages of inflammation or infection they may be the sole source of this cytokine, and exert their biological effects by releasing preformed and *de novo* synthesised mediators such as histamine, proteases, leukotrienes, prostaglandins, and cytokines on cell activation [302]. TNFα is able to stimulate the production of IL8, RANTES and GM-CSF by airway epithelial cells, which increases the expression of adhesion molecules such as ICAM-1 and VCAM-1, which are involved in the recruitment of inflammatory cells to the airways [303].

This is also important for the recruitment of eosinophils to the airways. TNFα is also thought to be involved in airway remodelling through its effects on fibroblasts and by inducing eosinophils to release matrix metalloprotease 9 [304].

A recent study by McLachlan and colleagues has emphasised the link between mast cells, TNFα and the regulation of inflammation and immunity [305]. They showed in a murine model that injection of *E. coli* into the footpad led to hypertrophy of the popliteal (draining) lymph node attributable to influx of T and B lymphocytes, and was associated with mast cell degranulation in the footpad. Mast cell deficient mice (W/W^v) mice showed significantly reduced lymph node swelling which was reversed by reconstitution of the mice with wild-type mast cells, but not with TNFα-deficient mast cells. Lymph nodes are a central immunological site of the adaptive immune response and are important for the interaction of T cells and APCs and further induction of the immune response.

1.8 Infection and asthma

Many patients presenting with exacerbations of asthma also have a respiratory tract infection (RTI). This has been highlighted in studies showing that between 37-44% of adult patients with acute asthma have evidence of RTI [306, 307]. How infections influence the development and exacerbation of asthma requires further study if we are to provide effective management for patients with asthma. In particular it is essential that the cellular and molecular basis for these interactions are elucidated in order to manipulate them. Three ways

in which infection can interact with asthma can be considered: viral infections, bacterial infections and how microbial products may affect the asthmatic process.

1.8.1 Viral infections and asthma

Up to 85% exacerbations of asthma in children and up to 44% in adults may be associated with viral infections [307, 308] suggesting that viral RTI may be a major trigger of acute asthma. Studies of experimental rhinovirus infection have shown that there is increased histamine release, eosinophilic infiltration and AHR in the lungs of atopic asthmatics compared to healthy subjects [309-311]. Studies of patients with exacerbations of asthma have shown that viral infection, exposure to allergen and sensitisation to allergen appear to have a synergistic effect in triggering an acute attack [312]. Whether viruses can affect the susceptibility of an individual to develop asthma is less clear and studies on respiratory syncytial virus (RSV) have shown that RSV bronchiolitis in infancy can increase the risk of developing atopy and asthma later in childhood [313] but that this risk may have normalised by age thirteen [314]. This difference in effect depending on whether the infection takes place before the onset of asthma or atopy, or after the disease has become established may help to explain the differences between clinical studies showing correlation between RTIs and infection and epidemiological data suggesting a protective effect on a population of exposure to infection.

1.8.2 Bacterial infections and asthma

The studies demonstrating a link between RTI and exacerbations of asthma detected mostly viruses, although some evidence implicating bacterial infection is available [306]. Most studies however, only provide circumstantial rather than causative evidence. For example, patients undergoing an exacerbation of asthma were found to have a higher rate of colonisation with *Branhamella catarrhalis* (now renamed *Moraxella catarrhalis*) than control subjects, although there was a similarly high rate in stable asthmatics [315]. Another study implicated chronic sinusitis in difficult to manage asthma [316]. Although sinusitis has been associated with worsening of airway inflammation [317] it is not known whether or not this is due to the bacterial pathogens that are commonly implicated in sinusitis such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. More convincing is evidence that 'atypical' organisms such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are associated with exacerbation of asthma [318-320] with some studies suggesting that treatment of these organisms may improve asthma control [321]. Although it seems likely that bacterial infection adversely affects asthma, evidence to support this is not as comprehensive as that for viruses, warranting further study in this area.

1.9 Microbial products and asthma

Allergic diseases are typically characterized by predominance of Th2 cytokines, such as IL-4 and IL-5, which promote IgE production and eosinophilia [322]. However, the process by which microbes and their products, such as endotoxin, influence expression of this phenotype remains unknown. Some propose that early exposures result in a skewing of T-cell differentiation toward the Th1 phenotype and away from Th2 [323]. Evidence for this

mutual exclusivity comes from laboratory studies, which show that Th1 cytokines, such as interferon-γ, are capable of suppressing IL-4 and IL-5 production [324], and observational studies, which show that house dust endotoxin levels are positively associated with interferon-γ production by CD4+ T cells but not IL-4, IL-5, or IL-13 [325]. However, the incidence of both Th2 diseases, such as asthma, allergic rhinitis, and atopic dermatitis, and Th1 diseases, such as type 1 diabetes mellitus, multiple sclerosis, and Crohn's disease, has increased, suggesting a more general loss of T-cell suppression [326]. It has been proposed that the lack of intense infections in industrialised countries owing to improved hygiene, vaccination and use of antibiotics may alter the immune system such that it responds inappropriately to innocuous substances [327]. The "hygiene hypothesis" [11] has been given an immunological framework in which the balance between Th1 and Th2 immune responses is pivotal [328]. It has been postulated that limited exposure to bacterial and viral pathogens during early childhood results in an insufficient stimulation of Th1 cells, which in turn cannot counterbalance the expansion of Th2 cells and results in a predisposition to allergy [329]. Although endotoxin exposure can result in IL-12 production and the subsequent expression of interferon-γ [330], a Th1 cytokine, TLR-4 signalling has also been shown to be important for optimal Th2 development [331].

A recent study used explanted nasal mucosa from 22 children and 17 adults and stimulated with allergen and/or LPS [332]. In mucosa taken from children with atopy, stimulation with both allergen and LPS abrogated Th2 cytokine expression and increased Th1 cytokine production when compared with allergen stimulation alone. However, Th2 cytokine expression was restored when these cells were also incubated with neutralizing antibody

against IL-10, IL-12, or interferon-γ, suggesting that the effect of LPS was mediated in part through the production of these cytokines. Stimulation of cells with both allergen and LPS also resulted in an increase in the number of T-cells expressing TLR-4 receptors, an increase in the number of TLR-4–positive cells that contain IL-10, and an increase in the number of cells that express both CD4 and CD25 when compared with allergen stimulation alone. The effect of LPS stimulation on IL-10 transcription and TLR-4 expression appeared diminished in adults when compared with children. Together these findings suggest that LPS may down-regulate allergic response through Th1 skewing and/or the expansion of regulatory T cells, but that these effects are most prominent in children, whose developing immune system is still susceptible.

To date, two cross-sectional studies suggest that in situations of high environmental endotoxin exposure, functional mutations in the TLR-4 gene may protect against bronchial reactivity [326] and atopy. The protective effect of TLR-4 function mutations on airway responsiveness may reflect reduced localized inflammation in the lung. However, the mechanism by which high endotoxin exposure and TLR-4 functional mutations may interact to lower the risk of atopy has yet to be established.

1.10 Objectives

The introduction discusses the current understanding regarding the pathophysiology of asthma. A great deal of research has provided the basis for the current understanding of a Th2-mediated inflammatory disease of the airways, with increasing evidence suggesting that

underlying irregularities in the structural elements of the airways may be of importance. A great deal of this information has been gained from using animal models. However, it has been shown that this can be conflicting with clinical studies and also confusing due to the variability in models, strains and conditions. Many questions remain unanswered in fully understanding then mechanisms underlying airway inflammation, including how asthma therapies really work and how external factors influence disease outcome. The objectives of this thesis are therefore:

1. **To establish and characterise a murine model of allergic airways inflammation**

   Adoptive transfer of transgenic T cells allows antigen-specific cells to be identified in different cellular compartments. Employing this system, the precise locations can be tracked *ex-vivo* in a murine model of allergic airways inflammation, providing novel information on the interactions of antigen-specific T cells in the airways response.

2. **To investigate the effects of TNF blockade on mechanisms of airway inflammation**

   The effects of inhibiting TNF activity in these models will be examined and the results compared with findings in emerging clinical trials of anti-TNF therapies in asthma.

3. **To investigate the impact of microbial products, specifically LPS, on allergic airways inflammation**

Using the model established above, the effect of exogenous inflammation on airways inflammation will be examined and characterised.

4. **To elucidate where and when T cell interactions take place in allergic airways inflammation**

Tracking $CD4^+$ T cells will help to define the behaviour of antigen-specific lymphocytes *in vivo* and aim to clarify important issues involving the location of effector and memory T cell populations in allergic airways inflammation.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Animals

2.1.1 Animals

Wild-type BALB/c and C57BL/6 mice were purchased from Harlan Olac (Bicester, Oxon, UK). DO11.10 mice are homozygous for the chicken ovalbumin (OVA) peptide$_{323-339}$/I-A^{d}-specific DO11.10 TCR transgenes (detected using the clonotypic mAb KJ1.26) on the BALB/c background [333], and were bred in-house by Biological Services, University of Glasgow.

2.1.2 Animal welfare

All mice were housed in pathogen-free conditions in facilities run by Biological Services, University of Glasgow and University of Strathclyde in accordance with United Kingdom Home Office regulations under the Animals (Scientific Procedures) Act 1986. Severe immuno-compromised) SCID mice were housed in micro-isolator cages with filtered air and were fed sterilised feed. All procedures were carried out under Project Licence Number 60/3046.

2.2 Preparation of cell suspensions for adoptive transfer

Peripheral lymph nodes, mesenteric lymph nodes, and spleens from DO11.10 BALB/c mice were pooled and forced through Nitex (Cadisch Precision Meshes, London, U.K.) using a syringe plunger. The suspensions were washed in sterile RPMI 1640 (Invitrogen Life Technologies, Paisley, U.K.). Cells were washed by adding 1 ml of FACS buffer (See table

2.3) before the suspensions were centrifuged at 450xg for 5 min and the supernatant discarded. The percentage of KJ1.26^{+}CD4^{+} DO11.10 T cells in these preparations was determined by flow cytometric analysis as described below. Cell suspensions containing 3 x 10^{6} Tg T cells in 200 μl were injected i.v. into age-matched BALB/c recipients.

2.3 Adoptive transfer model of allergic airways inflammation

T cell receptor Transgenic (Tg) Ag-specific T cells were adoptively transferred into recipient mice before induction of airways inflammation [334]. In these experiments 3x10^{6} transgenic cells were adoptively transferred into naïve BALB/c recipients before immunisation with 100μg OVA (chicken OVA, fraction V was obtained from Sigma-Aldrich) in a 1% alum suspension (Brenntag Biosectar, Frederikssund, Denmark) by i.p. injection on up to three occasions a week apart, followed by an intranasal challenge of 50μg OVA from 1 to 3 times. The mice were culled on subsequent days. Samples collected included BAL fluid for cytospin and flow cytometry analysis of BAL cells and enzyme linked immunosorbant assay (ELISA) analysis of cytokines; serum for immunoglobulin assays; draining lymph nodes (DLN: mediastinal) and peripheral lymph nodes (PLN: axillary, brachial, inguinal, cervical) for flow cytometry analysis for Ag-specific Tg cells, immunoflourescence (IF)-staining for Ag-specific cells and *ex vivo* cell culture with Ag-restimulation; and lungs for IF-staining for Ag-specific cells and histological analysis.

2.4 CFSE-labelling of cells

In some experiments, T cells were labelled with the green-fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen Ltd, formerly Molecular Probes). Single cell suspensions were prepared as below and washed in HBSS (Invitrogen Life Technologies, Paisley, U.K) before being resuspended in a suitable volume to give a concentration of $5x10^7$ cells/ml. 0.5µl/ml 10mM CFSE was added before incubation at 37°C for 10 minutes. The cells were then diluted in at least 10x volume complete RPMI (cRPMI) (see table 2.3) before centrifugation at 400x*g* for 5 minutes at 4°C and resuspended in a medium and volume appropriate for subsequent use.

2.5 Intranasal dosing

Mice were anaesthetised by intraperitoneal injection of 250µl avertin (1:1 w/v solution of 2,2,2-tribromoethanol in tert-amyl alcohol (Sigma Aldrich, UK). 30µl of reagent was then administered to the nostril using a 200µl pipette. Mice recovered from the anaesthesia in a warmed environment before being returned to standard housing conditions.

2.6 Intratracheal dosing

Mice were anaesthetised as for intranasal (i.n.) dosing. Once unconscious, the mouse was positioned vertically by their upper incisors on a custom-made support (Institute of Biological and Life Sciences (IBLS) Workshop, University of Glasgow). This allowed direct and atraumatic access to the trachea via the mouth using a blunt-tipped 25G needle (needles

purchased from Becton Dickinson (BD), Franklin Lakes, USA) and blunted by the IBLS Workshop). The reagent was then administered via the needle using a 1ml syringe. Mice were allowed to recover from the anaesthesia in a warmed environment before being returned to standard housing.

2.6.1 Intranasal vs Intratracheal

Intranasal administration of antigen to the airways has been criticised for the inconsistency of the dose of antigen given due to the variability in anaesthesia and also the effect of antigen on the nasal mucosa. However, this technique is easier to administer than intratracheal dosing, which is a favoured method of antigen administration and is more invasive. When intratracheal and intranasal administration was compared in this airway model, no significant differences were seen in the level of eosinophilia induced (data not shown). Intranasal immunisation has also been proven to cause a response in the lower airway, suggesting that its administration is effective [335]. Another method employed to administer antigen to the airways is nebulisation. This method allows the dosing of several mice at one time, without the need for anaesthetic and multiple doses are safe. However, there are also disadvantages to this protocol. Specialised equipment is needed and although a measured dose of antigen is given to each chamber, there is no guarantee that each mouse will inhale an equal amount. As nebulisation is administered in the air, there is a chance that antigen may be present on the fur and ingested by mice during grooming, leading to induction of oral tolerance [336, 337]. Despite the advantages and disadvantages of all three methods, different research groups all still routinely use them, and have all been shown to induce allergic inflammation of the lungs. Our studies have chosen to use intranasal antigen

administration; due to ease of delivery and using intratracheal dosing saw no significant advantage.

2.7 TLR ligands

TLR4 ligand LPS (E. coli 055:B5, Sigma Aldrich, UK) or TLR2 ligand Pam3CSK4 (BLP) (EMC Microcollections GmBH, Germany) was given intranasally or intraperitoneally at varying doses.

2.8 Etanercept

Etanercept (Wyeth Pharmaceuticals, Taplow, UK) a TNFRII-human FC fusion protein, was given at a dose of i.p. (50μg/0.5mg/kg) or s.c. (25μg/0.5mg/kg) injection. Control animals were given human IgG (Sigma Aldrich, UK) by the same route.

2.9 Cardiac puncture

Following an overdose of anaesthetic (500μl avertin i.p.) the heart was exposed and blood was withdrawn using a 23G needle and 1ml syringe inserted into the right ventricle. This method of anaesthetic overdose followed by exsanguination conformed to a Schedule 1 termination according to the Animals (Scientific Procedures) Act 1986.

2.10 Bronchoalveolar lavage

Bronchoalveolar lavage was performed following termination by cardiac puncture. The trachea was exposed and a horizontal incision made between the cartilage rings. The trachea

was then cannulated using polythene tubing (0.58mm internal diameter, 0.78mm outer diameter, Portex-Smiths Medical, Hythe, UK) over a 23G needle attached to a 1ml syringe. The needle was held in place using forceps while a 0.8ml aliquot of PBS (Invitrogen Life Technologies, Paisley, U.K) was injected and the lungs could be seen to inflate. After 10s the lavage fluid was withdrawn using the same syringe and collected in a 1.5ml microcentrifuge tube. This was repeated with a second 0.8ml aliquot, which was pooled with the first. Samples were stored on ice until processing.

2.11 Harvesting of lymphoid organs

Peripheral lymph nodes comprised inguinal, popliteal, axillary and brachial lymph nodes (LNs). Cervical, mesenteric and para-aortic lymph nodes were also included if maximal cell numbers were required. Draining lymph nodes refer to thoracic lymph nodes. In experiments where LNs were weighed or were required for immunohistochemistry (IHC) or immunofluorescent staining the same node site was sampled on each occasion (left inguinal LN for PLN and central mediastinal LN for DLN). For IHC and IF, nodes were snap frozen in liquid nitrogen in OCT embedding medium (Bayer, Newbury, UK) before being stored at –70°C. For cellular processing, LNs and spleens were collected into incomplete RPMI (iRPMI).

2.12 Lung samples

After opening the thoracic cage, the lungs were carefully separated from surrounding tissue by blunt dissection and removed *en bloc* with the heart. For histological analysis, lungs were inflated with 1ml 10% neutral buffered formalin (NBF) via the trachea, which was then tied

off with thread. The lungs were immersed in NBF for at least 72 hours before processing. For IHC or IF, lungs were inflated with 1ml 50% OCT in PBS and immersed in OCT in an aluminium foil mould before freezing at –20°C with final storage at –70°C.

2.13 Peritoneal lavage

Mice were killed by cervical dislocation and the peritoneum exposed. 5ml of ice-cold PBS was injected into the peritoneal cavity using a 23G needle and 5ml syringe. The fluid within the cavity was manipulated for 15 seconds to allow adequate mixing and mobilisation of cells before aspiration using the same syringe. Generally 4-5ml of fluid was recovered. The volume of peritoneal lavage fluid obtained was recorded and the sample was kept on ice until further processing.

2.14 Serum samples

Blood was collected into 1.5ml microcentrifuge tubes and allowed to clot at room temperature for 2-3 hours. Clotted samples were then centrifuged at 18400xg for 30 minutes at 4°C. Serum was carefully aspirated from the samples and collected in 0.5ml microcentrifuge tubes and stored at –70°C.

2.15 Preparation of blood cells for flow cytometry

Blood was initially collected into heparinised capillary tubes before being expelled into 5 ml round-bottom polystyrene test tubes (FACS tubes, BD) by flushing with 1ml FACS buffer. Cells were washed by centrifugation at 400g for 5 minutes, resuspended in 1ml FACS buffer

and centrifuged again as above. The cell pellet was incubated in 200µl Fc block (anti-CD16/32 hybridoma supernatant from Clone 2.4G2) for 10 minutes at 4°C before staining. Samples were subjected to red cell lysis by incubation in FACS Lysing Solution (BD) for 10 minutes at room temperature and then washed in 1ml FACS buffer as above. Cell pellets were resuspended in 200-1000µl FACSFlow (BD) and filtered through 100µm nylon monofilament gauze (Cadisch Precision Meshes Ltd., London) before analysis using a FACSCalibur flow cytometer (BD). Data was analysed using either Cellquest (BD) or FlowJo (Tree Star Inc, Ashland, USA). An aliquot of cells could be removed after the red cell lysis stage for cytospin analysis if required.

2.16 Lymphoid organs

Lymph node or spleen cells were dispersed by crushing under 100xm nylon gauze using the end of a syringe plunger in iRPMI. Cells were washed twice in 1-25ml iRPMI according to the number of organs used by centrifugation at 400xg for 5 minutes at 4°C, then resuspended in an appropriate volume of either incomplete or complete RPMI. If red cell lysis was required it was performed before the final wash by resuspending spleen cell pellets in 2ml Red Cell Lysis buffer for 1 minute at room temperature before addition of 10x volume iRPMI. The number of viable cells was determined using a Neubauer haemocytometer (Weber Scientific International Ltd, Teddington, UK) diluted in 0.1% w/v trypan blue.

2.17 Cell culture

Cells derived from lymphoid organs were routinely cultured in complete RPMI at 37°C in a humidified atmosphere enriched with 5% CO_2. Cells were cultured at a concentration of $1x10^6$ cells/ml in 2ml/well. Response to antigen stimulation was performed by adding sterile OVA to a final concentration of 1mg/ml to cultures with control cultures supplemented with medium alone. For measurement of cytokine production, cultures were performed in 24 well plates and supernatants harvested after 96 hours and stored for further analysis. Remaining cells could be collected at this stage for FACS analysis by washing the wells with 1ml iRPMI or FACS buffer. For assessment of the proliferative response, cultures were performed in triplicate in round-bottom 96 well plates ($2x10^5$ cells in 200µl per well) and assayed at 72 hours. For the last 8 hours of culture, cells were pulsed with 1Ci/well tritiated ([^{3}H]-labelled) thymidine (West of Scotland Radionuclide Dispensary, Glasgow, UK) before being harvested onto glass fibre filter mats (PerkinElmer LAS (UK) Ltd., UK) using a Wallac 1295-004 96 Well Harvester (PerkinElmer) and [3^H]-thymidine incorporation measured using a Wallac 1205 Betaplate Liquid Scintillation Counter (PerkinElmer).

2.18 Cytospin preparations

Cytospin preparations were used to perform differential cell counts from BAL samples, peritoneal washes and blood cells. For BAL samples, the volume of sample was first estimated by weighing the sample against an empty 1.5ml microcentrifuge tube. The cells were then centrifuged at 400g for 5 minutes at 4°C and the supernatant removed and stored at –20° C for later analysis. Cell pellets were resuspended in 1ml PBS and the cells counted, allowing the calculation of total cells per ml of BAL fluid. $5x10^4$ cells were then spun onto

glass slides using a Shandon Cytospin3 (ThermoShandon, Runcorn, UK) at 350rpm for 6 minutes. The slides were allowed to air-dry for 10 minutes before being fixed in methanol at room temperature for 10 minutes. Fixed cytospin slides were stained with Rapi-Diff II (a rapid Romanowsky stain) and then had coverslips affixed using DPX mountant (BDH Laboratory Supplies, Poole, UK). Differential cell counts were performed by counting at least 400 cells by light microscopy in consecutive high power fields (x1000 magnification), identifying different cell types by staining and morphological characteristics. Experimental conditions were blinded to the observer during counting. Peritoneal lavage and blood samples were treated in a similar fashion.

2.19 Flow cytometry

Lymphoid cells were analysed for the presence of DO11.10 Tg T cells by staining for surface expression of CD4 and the Tg TCR [338]. Single cell suspensions were prepared from lymphoid organs as described above, resuspended in 200µl Fc block and incubated for 10 minutes at 4°C. 1µl each of phycoerythrin (PE)-labelled anti-CD4 mAb and biotin-labelled KJ1-26 mAb (recognising the transgenic TCR) or appropriately-labelled isotype controls (all antibodies from BD Pharmingen unless otherwise stated) was added to each sample which was then incubated for 20 minutes at 4°C. Cells were washed by addition of 1ml FACS buffer followed by centrifugation at 400g for 5 minutes at 4°C. The cell pellets were resuspended in 50µl FACS buffer with 1µl streptavidin-conjugated fluorescein (Vector Laboratories Inc, Burlingame CA, USA) and incubated for a further 10 minutes at 4°C. The cells were washed FACS buffer as above and resuspended in 1ml FACSflow.

In some experiments, the expression of memory markers was examined using antibodies against CD45RB and CD62L labelled with the appropriate fluorochromes. In these cases, the staining procedure was the same with the appropriate antibodies or isotype controls added after the addition of Fc block. In experiments where CFSE labelled cells were transferred, three-colour analysis was performed using appropriate flourochromes and isotype controls. Analysis was performed using Cellquest or FlowJo software. Antibodies used for flow cytometry are summarised in Table 2.1.

Antigen	Isotype	Supplier (Clone)	Label
CD4	Rat IgG2a	BD (RM4-5)	PE, PerCP,APC
CD45RB	Rat IgG2a	BD (16A)	FITC
CD62L	Rat IgG2a	BD (MEL-14)	PE
OVA-TCR	Mouse IgG2a	(KJ1-26)	Biotin
Sterptavidin-PE	N/A	BD	PE
Streptavidin-FITC	N/A	Vector Laboratories	FITC
Streptavidin-APC	N/A	BD	APC

Table 2.1: Flow cytometry reagents

2.20 Lung histology

Lung specimens collected in 10% neutral buffered formalin were transferred into 70%, 90% and 100% ethanol successively to dehydrate. After embedding in paraffin wax, sections were cut at a thickness of 6μm and mounted on microscope slides. Sections were stained with

haematoxylin and eosin (H&E). For allergic airways inflammation experiments the degree of inflammation was assessed on lung sections using the scoring system discussed in Chapter 3 (Table 3.1). All sections were scored by the same observer who was blinded to experimental conditions at the time of counting.

2.21 Immunofluorescence staining for KJ1-26$^+$ T cells, proliferating cell nuclear antigen (PCNA) and B220

KJ1-26$^+$ (OVA-specific) T cells were identified on tissue sections using tyramide signal amplification (TSATM) [339]. 6µm sections from lung or lymph node samples frozen in OCT were cut using a cryostat (ThermoShandon) and mounted onto SuperFrost slides (BHD, Poole, UK) before being allowed to air-dry and stored at –20°C until further processing. Slides were brought to room temperature and fixed in acetone and the sections outlined with a wax pen to allow addition of solutions without cross contamination. The remainder of the staining process was carried out in a humidified darkened chamber. Sections were rehydrated in PBS for 10 minutes before incubating in 0.1% azide/2% H_2O_2 for 45 minutes, changing the solution three times. After washing in PBS, endogenous biotin was blocked by sequential 15 minute incubations with Avidin then Biotin solutions (Avidin-Biotin blocking kit from Vector Laboratories), each followed by washes in TNT buffer. The primary antibody, biotinylated KJ1-26, was diluted 1:250 in 1% blocking reagent (BR, from TSATM Biotin system, Perkin Elmer Life Sciences, Boston, USA) and added to sections which were incubated for 40 minutes. Control sections were incubated with 1% blocking reagent (BR) only. Sections were washed three times in TNT buffer between the remainder of the steps. Streptavidin-horseradish peroxidise (HRP, from TSATM kit, Invitrogen Ltd)

was diluted 1:100 in 1% BR and added to sections which were incubated for 30 minutes. Sections were then incubated in biotinylated tyramide diluted 1:50 in amplification diluent (both from TSATM kit) for 10 minutes. Sections were washed three times in TNT buffer. Streptavidin-AF647 (Molecular Probes) diluted 1:500 in BR was added to KJ1.26 stained sections for 30min, then sections were washed three times in TNT buffer. Anti-PCNA (Santa Cruz,UK) diluted 1:250 in BR (isotype rabbit-IgG, BD) was added to sections and incubated for 30 minutes, then sections were washed three times in TNT buffer. Then anti-rabbit IgG-HRP (BD) diluted 1:500 in BR and B220-FITC (BD) diluted 1:250was added to slides and incubate for 30 minutes. Sections were washed three times in TNT buffer. Finally, the fluorochrome Pacific tyramide blue (Invitrogen Ltd) was added, diluted 1:100 in amplification buffer (from TSATM kit) was incubated for 30 minutes before being washed three times in TNT. The slides were left to dry briefly and mounted in Vectashield (Vector Laboratories) with a glass coverlsip and sealed with nailvarnish.

Sections were imaged using a Laser Scanning Cytometer equipped with Argon, Helium-Neon and Ultraviolet lasers (Compucyte, Cambridge, USA) and data analysed using the WinCyte software (Improvision, Coventry, UK). Laser scanning cytometry combines the identification of fluorescently-labelled cells in tissue sections with the ability to quantify these cells within anatomical regions within the section [340]. A computer-controlled microscope stage allows the precise localisation and quantification of cells identified by their fluorescence following laser excitation. By setting a threshold intensity of KJ1-26 staining, KJ1-26+ T cells can be identified and enumerated within a tissue section or within pre-defined regions within the section.

2.22 Immunohistochemistry staining for KJ1-26$^+$ cells

Lung and LN sections were cut, mounted and fixed as for IF staining then stained as previously described [338]. Briefly, sections were rehydrated in PBS with 2% goat serum (GS) for 15 minutes before incubating for 30 minutes with biotinylated KJ1-26 (diluted 1:500 in 2% GS or 2% GS alone for control sections). Sections were washed three times in PBS before addition of ABC-AP solution (all IHC reagents from Vector Laboratories unless otherwise stated) for 30 minutes. After washing, BCIP/NBT was added and sections incubated for 45 minutes or until blue staining was evident.

If staining for B220 was required, sections were washed in PBS before biotinylated anti-B220 antibody was added (1:400 in 2% GS, or just GS for control sections). Sections were washed in PBS after 30 minutes before addition of ABC-HRP and further incubation for 30 minutes. DAB substrate was added for 10 minutes followed by DAB enhancing solution if brown staining was not evident. Slides were washed in water, then dehydrated by sequential washes in 75%, 95% and 100% ethanol followed by Histoclear (BS&S Ltd, Edinburgh, UK) before mounting with glass coverslips using Vectormount and sealing with nail varnish.

2.23 Enzyme-linked immunosorbent assay (ELISA)

2.23.1 Cytokine analysis

Immulon 4 HBX plates (ThermoShandon) were coated with capture antibody in ELISA coating buffer (details of antibodies in Table 2.2) and incubated overnight. Plates were

washed with ELISA wash buffer before incubating in 200µl/well ELISA blocking buffer for 37°C for 1 hour. After washing, standards at known concentrations and samples were added in duplicate and incubated for 1.5-2 hours. Samples were BAL supernatants, serum or culture supernatants; standards were diluted in a suitable medium to match the sample. The plates were washed again and detection (biotinylated) antibodies added and incubated for a further 1-1.5 hours. Streptavidin-HRP conjugate (Extravidin, Sigma) was then added after further washes and incubated for 30 minutes before washing and addition of TMB substrate (KPL, Gaithersburg, USA) for up to 30 minutes or until sufficient blue colour had developed. Optical density was then read on an MRX II microplate reader (Dynex Technologies, Worthing, UK) at 630nm or at 405nm if the reaction was stopped by addition of an equal volume of Stop solution (KPL). A standard curve was generated from the standard data allowing the calculation of cytokine concentrations in samples.

2.23.2 Multiplex analysis of cytokines

In some experiments, cytokines were analysed by multiplex analysis. This system uses biotinylated detection antibodies for the desired analytes conjugated to beads of defined spectral properties. These beads can be used in a solid phase sandwich immunoassay whereby analytes within a sample bind to the beads via the detection antibody. Addition of the streptavidin-conjugated fluorescent protein R-phycoerythrin (RPE) means that laser excitation of the beads within the detection instrument allows identification of analyte-specific beads with the fluorescence intensity of each bead being proportional to the amount of analyte in the sample. The advantage of this system is that several analytes can be

analysed in a small volume of sample by using a combination of beads thus saving time and sample volume.

Biosource Multiplex kits LMC0002 (Th1/Th2 6-plex), LMC0003 (Inflammatory 4-plex), LMC0131 (IL-13) and LMC0006 (Cytokine 20-plex) were used according to the manufacturer's instructions. All reagents and buffers were sourced from the kits. Briefly, the supplied 96 well filter plate was pre-wetted with Working Wash solution before aspiration of well contents using vacuum apparatus. If more than one kit was being used, beads were premixed. Otherwise beads were resuspended and 25µl bead mixture added per well and washed twice by the addition and aspiration of Working Wash solution from the wells. 50µl/well Incubation Buffer was added followed by 100µl/well standard or 50µl Assay Diluent plus 50µl sample. Plates were covered in aluminium foil and incubated on shaking apparatus at room temperature for 2 hours. The wells were then washed twice and 100µl/well biotinylated detection antibody added and incubated for 1 hour as above. Following two more washes, 100µl/well Steptavidin-RPE was added and incubated for 30 minutes as above. The wells were washed three final times before resuspending the beads in 100µl/well Working Wash solution and acquiring data on a Luminex 100TM. Data was analysed using Luminex software and provided standard curves and sample data for each analyte.

2.23.3 Immunoglobulins

OVA-specific immunoglobulins were analysed from serum samples by first coating Immulon 4 HBX plates with 10µg/ml OVA in OptEIA coating buffer and incubating overnight at 4°C. Plates were washed in ELISA wash buffer and incubated with ELISA

blocking buffer for 1 hour at 37°C before being washed again. Serum samples were diluted to 1:50 (IgE and IgG2a) or 1:500 (IgG1) or a suitable dilution determined by previous experiments and added to plates with serial dilutions down the plate. Test serum from naïve mice was included to allow comparison between plates. After incubation for 2 hours, plates were washed and detection antibodies added (Table 2.2) and incubated for 1 hour. Plates were washed again and streptavidin-HRP conjugate added and incubated for 30 minutes. For OVA-specific IgE, streptavidin-HRP conjugate was added at the same time as the detection antibody for a total incubation of 1.5 hours. After washing, TMB substrate was added for 30 minutes or until sufficient blue colour had developed. Optical densities were read on a microplate reader at 630nm or 405nm if the reaction was stopped with Stop solution. Plotting OD against sample dilution created dilution curves for each sample allowing comparison between samples at an appropriate dilution.

Analyte	Supplier	Capture (µg/ml)	Detection (µg/ml)
Murine			
IL-4	BD	2	2
IL-5	BD	4	4
IL-13	R&D	4	4
IFN-γ	BD	2	1
TNF	BD (OptEIA kit)	Kit	Kit
IgE	BD	N/A	2
IgG1	BD	N/A	0.5
IgG2a	BD	N/A	0.5

Table 2.2: ELISA reagents

2.24 RNA Extraction

RNA extraction was carried out according to the Qiagen RNeasy Kit protocol. Briefly, cells stored at -80°C in RLT buffer were thawed and the sample was homogenised by passing through a 25 gauge needle on a 1ml sterile (RNase free) syringe. 350µl 70% ethanol was added and pipetted to mix. Up to 700µl sample was added to an RNeasy mini column with 2mnl collection tube and centrifuged for 15 seconds at 8000g. The flowthrough is discarded. 350µl buffer RWI is added to the column and centrifuged as before discarding the flowthrough. 80µl Dnase solution (10µl Dnase, 70µl RDD buffer) is added to the column and incubated for 15 minutes at room temperature. Then 350µl buffer RWI is added to the

column and centrifuged as above, discarding the flowthrough and the collection tube. The column is transferred to a new collection tube and 500µl buffer RPE is added to the column and centrifuged as above discarding the flowthrough. Another 500µl buffer RPE is added to column and centrifuged for 2 minutes at 8000g then transferred to a new collection tube. The column is centrifuged at 8000g for 1 minute then transferred to a new collection tube. 30µl RNase free water is added and centrifuged for 1 minute at 8000g, the step is repeated. The RNA levels were measured using a spectrophotometer then the sample is then stored at -20°C until needed.

2.25 Production of complementary DNA (cDNA)

cDNA synthesis was carried out according to Invitrogen Superscript III Reverse Transcriptase kit. Complementary DNA (cDNA) was produced from the total RNA sample by adding 2 µg of RNA, 1µl random primers, 1µl dNTPS and the appropriate amount of Dnase/Rnase free water (Sigma) to give a final volume of 13μl to a Dnase/Rnase free thermo tube (Abgene, Surrey, UK). The resulting suspension was mixed and spun for 1 min at 12,000rpm on a bench top microfuge. The solution was then heated to 65°C for 5 minutes then incubated on ice for at least 1 minute. Samples were centrifuged briefly, then the following was added: 4µl 5X First Strand Buffer, 1µl 0.1M DTT, 1µl RNase OUT, and 1µl Superscript III RT. Samples were mixed by pipetting. The following cycle was used to make cDNA: 25°C for 5 minutes, 50°C for 60 minutes and 70°C for 5 minutes. The samples were then stored at -20°C until required.

2.26 Quantitative Real Time PCR

Quantitative real time PCR was carried out using a Statagene Mx™ machine and analysed using MxPro™ 3000 QPCR software. Primers for genes were designed using the following conditions: Tm: 58°C to 61°C, not bigger difference than 2°C in the primer pair, primer lengths of 19-24 base pairs, Guanine cytosine (GC) contents: 45-55%, PCR amplicon lengths: 100-200 base pairs. Reactions were set up containing 5µl cDNA, 5µM forward and reverse primers, 10µM probe (Invitrogen), master mix (Brilliant® SYBR® Green QPCR master mix), and H_20 make each reaction to 25µl. Solution was plated into 96 well plates. Plates were capped and centrifuged. The plate was then placed in the thermal block of the Mx3000P® system. The assay was run using the SYBR green module in the MxPro analysis software. The assay was run for 35 cycles then analysed accordingly.

2.27 Statistics

Results are expressed as mean ± standard error of the mean (SEM). To test significance Student's unpaired t test was performed. A value of $p < 0.05$ was regarded as significant.

2.28 Buffers

Buffer	Constituents
Phosphate buffered saline (PBS)	8g NaCl, 1.16g Na_2HPO_4, 0.2g KCl, 0.2g KH_2PO_4 in 1 litre distilled water, pH 7.4
FACS buffer	2% FCS, 0.05% sodium azide in PBS
ELISA Coating buffer	0.1M $NaHCO_3$ pH 8.4
ELISA Wash buffer	0.05% Tween-20 in PBS pH 7.4
ELISA Blocking buffer	10% FCS in PBS
OptEIA Coating Buffer	8.4g $NaHCO_3$, 3.56g Na_2CO_3 in 1 litre distilled water, pH 9.4
TNT wash buffer	10ml 0.1M Trizma base, 10ml 0.15M NaCl, 50µl Tween-20, 80ml distilled water
TNB buffer	5ml 0.1M Trizma base, 5ml 0.15M NaCl, 50µl Blocking Reagent (TSA™ kit)
Binding buffer	10mM Hepes/NaOH, 140mM NaCl, 2.5mM $CaCl_2$, pH 7.4
Incomplete RPMI	RPMI-1640 medium
Complete RPMI	RPMI-1640 medium, 10% FCS, 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin

Table 2.3: Buffers

CHAPTER 3CHARACTERISATION OF MURINE MODELS OF ALLERGIC AIRWAYS INFLAMMATION

3.1 Introduction

The most commonly employed animal model of asthma is the murine allergic airways inflammation model [341]. In this model the mouse is sensitised by systemic vaccination with antigen, followed by an airways antigen challenge. This protocol induces inflammation with associated eosinophilia, IgE production and airways hyperresponsiveness. This model was set up to emulate the immunological sequelae of asthma, allowing the underlying immunological and physiological mechanisms to be studied, and potential therapeutic interventions and their outcomes investigated.

Over the years, many variations of the airways model have been established. However, no particular system has been established as standard. Therefore many different mouse strains and immunisation and challenge protocols have been used to study airways inflammation (reviewed in [342]). There is also a great deal of variation in the readouts employed as measurement of disease. These range from bronchoalveolar lavage cell measurements, cytokine and antibody production and controversial AHR readings [261-264]. Thus although animal models are regularly used, these factors should be considered when comparison between different studies are made. It is therefore important that researchers are consistent with their models and subsequent readouts.

The use of animal models has given a wealth of information about many mechanisms involved in the inflammatory process of asthma including cytokines, chemokines, transcription factors and much more [343-347]. The DO11.10 system is well-established in studies of T cell interactions [339, 348] and has also been used in models of experimental

asthma although in most cases the transgenic cells were used principally to investigate the different effects of transferring Th1 or Th2 cells rather than to examine compartment-specific contributions by these cells [194, 242, 349, 350]. Where Ag-specific T cells have been tracked into different tissues following transfer, some studies have identified but not enumerated the transgenic T cells [121]. The remainder of studies have almost exclusively used T cells which have been polarised and activated *in vitro* to either a Th1 or Th2 phenotype before transfer [168, 195, 251, 351-354].

However, not as much attention has been given to when and where the interaction between these and other cells take place. This may be in part due to their low precursor frequency, making them hard to detect [355]. To comprehend how a disease works, it is important to know if antigen-specific T cells play a role in the disease, and therefore could be targeted as a therapy. Furthermore, an unanswered question is the location of activation and maintenance of naïve and memory T cells specific for the eliciting antigen. It is still unknown where antigen presentation takes place, whether it is in the draining lymph node or the lung itself. The use of antigen-specific T cells will enable us to gain a clearer understanding of these problems, and be able to design and target therapeutics specifically and rationally.

The use of the DO11.10 system in this study enables us to track T cells of a known specificity *in vivo* in a model of airways inflammation. The aim of this model was to allow tracking of Ag-specific T cells as well as inducing airway eosinophilia in conjunction with IgE production and associated Th2 cytokines. Our investigations revealed that a single immunisation with OVA followed by 1-3 intranasal antigen airway challenges did not

induce BAL eosinophilia although identification of Ag-specific T cells was possible in the lymph nodes and the lung. However, it was found that a more aggressive regime of three immunisations and one airways challenge was required to induce lung eosinophilia and associated pathology. Manipulation of the single immunisation, single airway challenge model with the addition of LPS to antigen in the airways challenge resulted in lung eosinophilia and pathology as found in the three prime, one challenge model. It was found that when antigen-specific T cells were compared between the long and short models the expression of cytokines, co-stimulatory molecules and adhesion molecules examined differed between the two disease models, suggesting that although similar endpoints were observed the route by which the response was initiated differs.

3.2 Results

To establish a robust murine model of airways inflammation in which lung eosinophilia and associated pathology were induced and antigen-specific T cells could be tracked, an adoptive transfer model was established and optimised. The aim was to induce a relatively low level of eosinophilia so as to allow future investigation of interventions that may exacerbate or ameliorate the inflammation. Initial experiments carried out using a single systemic prime with OVA/alum followed by a single OVA airway challenge (Figure 3.1 A). This protocol did not result in eosinophilia (Figure 3.1 B), although it was possible to identify antigen-specific T cells in the draining lymph nodes of the lungs (Figure 3.1 D). As the single prime of antigen and single challenge of antigen did not induce lung eosinophilia, the protocol was modified to include three OVA airway challenges instead of one, to determine if this was

rigorous enough to induce a response. However, this more aggressive approach did not result in lung eosinophilia (Figure 3.1 C).

3.2.1 Assessment of lung inflammation

An important readout in the assessment of the airway model is the level of lung inflammation present. The assessment of BAL eosinophilia from cytospins is a useful readout but does not give a complete overview of the whole situation. It is important to see what is happening in an intact lung. For this reason a histological scoring system of lung sections, to be used in conjunction with other readouts, was developed.

Histological examination of lung sections for inflammation show that changes in pathology can be heterogeneous andthat some areas may show inflammation, whereas another area on the same section may show no changes. This can be seen in figure 3.2 where two views from the same slide tell a different story. Figure 3.2 A shows perivascular and peribronchiolar eosinophilic inflammatory infiltrates, and figure 3.2 B shows no inflammation. This is representative of human conditions, where it is rare that the whole lung is involved and inflammation is likely to be patchy.

Because of the differences seen in the same lung sections, an objective scoring system was needed to allow fair and reproducible scoring of sections. A system previously optimised by Professor Sarah Howie, (University of Edinburgh) was adopted to overcome this problem [356]. The scoring system is shown in Table 3.1. Briefly, H&E stained sections of whole lungs were scored over 10 consecutive fields at x200 magnification. In order to be included,

each field had to contain a complete transection of at least one bronchiole less than half a field width in diameter, a blood vessel and an alveolar airway. Inflammatory cell infiltrate, i.e. the number of immune cells present, was evaluated around the blood vessel walls (perivascular compartment), the bronchiolar epithelium and the peri-bronchiolar alveolar tissue. Inflammation was also scored on the basis of increased alveolar wall thickness. Figure 3.3 shows these criteria in an inflamed lung section.

Area scored [1]	1	2	3	4
Perivascular compartment[2]	No infiltration	< 20 cells	21-100 cells	>100 cells
Bronchiolar epithelium	No infiltration	<5 cells	6-10 cells	>10 cells
Peribronchiolar alveolar tissue[3]	No infiltration	<20 cells	21-100 cells	>100 cells
Alveolar walls[4]	No infiltration	2-3 cells	4-5 cells	> 5 cells

[1] Each field is viewed at x200 magnification and must contain a complete transaction of at least one bronchiole less than half the field in diameter, a blood vessel and an alveolar airway, in an area of well-inflated tissue.
[2] Cells around blood vessel walls
[3] Sub-bronchiolar tissue: beneath the basement membrane and smooth muscle, not immediately adjacent to a blood vessel.
[4] Focal expansion of alveolar wall by that number of cells.

Table 3.1: Lung inflammation scores

3.2.2 Increasing the number of OVA immunisations produces BAL eosinophilia

As increasing the number of airway challenges did not lead to BAL eosinophilia, a model was set up with three OVA/alum immunisations and one OVA airway challenge (Figure 3.4 A). When BAL fluid was analysed for cell infiltrates it was found that this protocol induced significant BAL eosinophilia (Figure 3.4 B), which was accompanied by an increase in lung inflammation (Figure 3.4 C) compared to the control mice. The early peak of inflammation

seen at day 1 post airways challenge in the lung sections compared to the sustained eosinophilia seen in the BAL fluid suggest that airway and lung inflammation may peak at different times. It may also be due to an influx of inflammatory cells such as neutrophils and macrophages in the lung section that have not been measured in the BAL fluid.

Antibody and cytokine production

Analysis of serum from the same mice showed that three immunisations and one airway challenge could induce an increase in OVA-specific IgE and IgG1 (Figure 3.5 A and B). These results are consistent with data suggesting that IgE production is important in airways inflammation [217], and IgG1 production confirming that the model has a Th2 phenotype. An increase in IL-5 levels was detected in the BAL fluid (Figure 3.5 C), consistent with data showing that IL-5 production is important in airways inflammation and drives eosinophil maturation and activation [149].

Detection of antigen specific T cells

Using flow cytometry, transgenic OVA-specific T cells were detected in the draining lymph nodes of the lung and in the BAL fluid (Figure 3.6 A and B). To examine the distribution and orientation of these cells *in situ*, laser scanning cytometry (LSC) was employed, using the KJ1.26 mAb to analyse lung sections (described in chapter 2). Figure 3.6 C shows the detection of transgenic T cells in sections of lung tissue. There is no significant difference between the groups in the DLN, BAL or lung tissue. Figure 3.6 D shows a flow cytometry plot of %KJ1.26 cells in the DLN at day 3 post airways challenge.

These experiments define a protocol that can be used to induce allergic airways inflammation with eosinophilia as part of the outcome measure with the ability to track and quantify antigen-specific T cells within lymph nodes, lung and airways throughout this process, employing LSC as a novel technique, for the first time in this model. The protocol using three OVA/alum immunisations, seven days apart followed by one airways challenge will be called the "long model" throughout this thesis, and the protocol using one OVA/alum immunisation and one airways challenge ten days later will be called the "short model".

The literature demonstrates that there are many variations on the murine airways inflammation model, many using different protocols to reach similar endpoints. To investigate whether it was possible to manipulate the initial model of one OVA prime and one OVA airway challenge, which did not induce eosinophilia, LPS was added to the OVA challenge.

3.2.3 Effect of LPS addition to OVA airways challenge in the short model

The short adoptive transfer model (Figure 3.1 A) did not produce any BAL eosinophilia when one OVA immunisation and one airway challenge was administered. This is in contrast to the long model where one OVA challenge results in recruitment of eosinophils to the airway. Lung inflammation caused by LPS has been used as a model of acute lung injury, mirroring some features of acute respiratory distress syndrome in humans [357]. To determine if LPS would be enough to induce eosinophilia it was added to OVA and given as the airways challenge in the short model (Figure 3.7 A). Only days 0, 1 and 4 were analysed as endpoints in the short model, due to an increase in animal numbers per group limited the

number of days possible to cull. When OVA plus LPS was introduced into this model as the airway challenge, instead of OVA alone, a significant BAL eosinophilia was induced by day 4 after airways challenge (Figure 3.7 B) plus significant neutrophil influx at day one post airways challenge (Figure 3.7 C). Analysis of inflammation in lung histological sections also showed a trend towards increased inflammation in animals challenged with OVA plus LPS although this was not statistically significant (Figure 3.7 D).

Cytokine and antibody production

The addition of LPS to OVA in the airway challenge resulted in a significant increase in BAL IL-5 production compared with OVA alone (Figure 3.8 A), as seen previously in the long model. The level of IFNγ was undetectable in both groups (Figure 3.8 B). The increase in IL-5 production with the addition of LPS to OVA in the airways challenge may help to induce more eosinophilia in the short model.

The levels of OVA-specific IgE and IgG1 were measured in serum. No difference was seen between antibody levels when OVA and OVA+LPS were compared. (Figures 3.8 C and D).

LPS-induced BAL eosinophilia in the short model is associated with recruitment of antigen-specific T cells in the airway.

Antigen-specific T cells were measured in the DLN and BAL fluid by flow cytometry. No difference was observed between groups in the DLN (Figure 3.9 A). When compared with the OVA alone treated mice, a significant increase in T cells was seen in the BAL fluid in mice treated with OVA+LPS (Figure 3.9 B). This was supported by a similar increase in

transgenic cells in lung sections analysed by LSC for OVA+LPS compared with OVA (Figure 3.9 C), although this data was not significant.

Overall, these results suggest that the addition of LPS to a model that previously showed no airway inflammation or associated pathology has been switched to a model with pathology. This is associated with an increase in IL-5 production and recruitment of antigen-specific T cells to the BAL. This recruitment of T cells also suggests a role for them in the effector phase of the response, after the initial sensitisation. The influence of LPS on airways inflammation will be studied in greater detail in chapter four.

3.2.4 Investigation into the differences between the two models

As shown above, two different models have been manipulated to produce similar end results. The long and the short models produced differing results when OVA immunisation and OVA airway challenge were administered. Only when OVA+LPS airway challenge was given in the short model was eosinophilia and associated lung inflammation observed, although this was accompanied by a transient neutrophil influx not present in the long model. To investigate the differences between the two models, and to determine if we were looking at two different routes to the same end point, or different pathologies, an initial screening was carried out to assess co-stimulatory molecules, cytokines and chemokines (Figure 3.10). Antigen-specific T cells were purified from the draining lymph nodes and lung tissue and analysed by quantitative RT-PCR as described in chapter 2.

The RT-PCR analysis showed that there were differences between the two models where pathology is present (i.e. long model: OVA immunisation with OVA airway challenge, and short model: OVA immunisation and OVA+LPS airway challenge) and where no pathology was seen (i.e. long model: OVA immunisation with PBS airway challenge, and short model: OVA immunisation and OVA airway challenge).

Where no pathology was observed, it was found that in both the short model IFNγ showed a slight increase in expression in both the lung and the DLN, and an increase was seen in the DLN in the long model. IFNγ is known to decrease eosinophilia after antigen challenge, and inhibits Th2 cells and IgE release [344]. It may be possible that pathology in these models is unable to develop due to IFNγ skewing the response towards a Th1 phenotype. In the DLN of the long model the costimulatory molecules were upregulated in the PBS challenged group and slightly increased in the OVA treated mice.. This may be due to the three immunisations given in the long model compared to the one in the short model, although the PBS airway challenge was not sufficient to drive the T cells to produce a response.

Comparison of the two models when pathology was present showed that in the long model the lung tissue shows that none of the factors examined are upregulated. In contrast, in the short model IL-13 together with all of the costimulatory molecules and chemokines were upregulated. When the DLN were analysed from the long model, it was found that IL-5 and CCR3 expression were upregulated, as were the costimulatory molecules ICOS, OX40, and CD40L. However, when the antigen-specific T cells from the draining lymph nodes were analysed from the short model, nothing was upregulated.

This suggests that although there are similarities in the end result (increased eosinophilia, lung inflammation and increased cytokine and antibody responses) the manner by which they were reached is different. Comparison of the two models where no pathology was observed shows that the presence of IFNγ may be enough to inhibit the development of a Th2 response in both models, even though costimulatory molecules are upregulated in the DLN of the long model. When pathology has developed the response seen in the long and short models differs. In the long model the response is greater on the DLN compared to the lung tissue, with increased Th2 cytokine IL-5 and CCR3 which have both been shown to be important in eosinophil development and activation. However, in the short model the response seems to be driven by both costimulatory molecules and chemokines and the Th2 cytokine IL-13. In contrast to the long model the response is shown to be stronger in the lung tissue compared to the DLN.

This initial screening of a limited number of factors potentially deemed important in the pathogenesis of asthma has given an interesting overview into the role of antigen-specific T cells in the disease. Although these results imply that the two models have different mechanisms that lead to a similar endpoint, this is only a snapshot 72 hours after airways challenge (where eosinophilia is at its peak) and may reflect a kinetic difference between the models. To obtain a more precise understanding of the role of Ag-specific T cells in the long and short model, it would be beneficial to carry out a timecourse across the duration of the disease. More rigorous controls are also needed to ensure comparisons between the two models can be carried out accurately. Although antigen specific T cells are important to

track responses in this model of airways inflammation, it may also have been beneficial to analyse the lymph node as a whole as many other cells are involved in the response that will upregulate or express different receptors (e.g. dendritic cells), and this may give a clearer indication of what is driving the response.

3.3 Discussion

The role of the Th2 cell has been extensively studied in animal models of asthma. However, the importance of antigen-specific T cells and the locations of their cellular interactions are less well understood. To investigate this, an adoptive transfer model of allergic airways inflammation was devised where OVA-specific transgenic T cells (from the DO11.10 mouse) could be identified by use of the KJ1.26 monoclonal antibody. The DO11.10 system is well-established in studies of T cell interactions [339, 348] and has also been used in models of experimental asthma although in most cases the transgenic cells were used principally to investigate the different effects of transferring Th1 or Th2 cells rather than to examine compartment-specific contributions by these cells [194, 242, 349, 350]. Where Ag-specific T cells have been tracked into different tissues following transfer, some studies have identified but not quantified the transgenic T cells [121]. The remainder of studies have almost exclusively used T cells which have been polarised and activated *in vitro* to either a Th1 or Th2 phenotype before transfer [168, 195, 251, 351-354]. Although this gives the advantage of a shorter *in vivo* protocol as the activated cells are able to respond to airways challenge without any requirement for further immunisation, the driving of cells *in vitro* with recombinant cytokines and neutralising antibodies prior to transfer is non-physiological.

The model established here differs in that the development of eosinophilic airways inflammation is combined with the transfer of naïve antigen (OVA)-specific T cells. This enables *in vivo* T cell responses to be analysed through the ability both to identify and quantify Ag-specific T cells in different anatomical locations (lymph nodes, lungs and BAL

fluid). To achieve adequate sensitisation to Ag, systemic immunisation with OVA was required using aluminium hydroxide as an adjuvant. The immunisation leads to expansion of endogenous OVA-specific cells as well as transgenic cells, facilitated by aluminium hydroxide which also biases the development of T cells towards a Th2 phenotype [358]. Thus the transition from naïve to memory T cell with consequent maturation and activation takes place *in vivo*, rather than *in vitro*. The advantage of 'polarising' the T cells in this way is that the associated response to Ag by the animals' endogenous immune cells means that the Tg T cells are more likely to be representative of the natural immune response, rather than causative as in the case of adoptive transfer of previously polarised cells. Thus we have established a novel model of allergic airways inflammation where the Ag-specific T cell response can be analysed in different cellular compartments.

Many established airways models use aerosol OVA to challenge animals. This method allows the dosing of several mice at one time, without the need for anaesthetic and multiple doses are safe. However, there are also disadvantages to this protocol. Specialised equipment is needed and although a measured dose of antigen is given to each chamber, there is no guarantee that each mouse will inhale an equal amount. As nebulisation is administered in the air, there is a chance that antigen may be present on the fur and ingested by mice during grooming, leading to induction of oral tolerance [336, 337]. The intranasal route was chosen in this study due to ease of administration and through comparison of published studies of airways inflammation.

Analysis of BAL fluid content allows the evaluation of cell infiltrates in the airways. However, it is important to analyse lung inflammation in intact samples of lung. Histological analysis of the lungs showed that inflammation was variable over sections, with

some areas displaying severe inflammation and other areas of the same lung showing no sign of inflammation. To overcome this problem, a previously established scoring system was adapted [356]. This scoring system allows objective analysis in a number of ways. Ten fields per section are scored limiting the impact of heterogeneity of pathology. Inflammation is scored in four separate cellular compartments (perivascular, peribronchiolar, bronchiole walls and alveolar walls) giving a more accurate assessment of the disease. Other scoring systems used in lung inflammation models do not look at as many parameters, including only peribronchiolar and perivascular infiltrates in the score [359, 360].

These parameters described above allow an objective score of lung pathology to be established. The histological score and differential cell counts from the BAL may be used together to give a clearer perspective of the inflammatory response. For example, if an inflammatory stimulus is not sufficient to induce BAL eosinophilia, it may have produced changes in cellular compartments, which should be detected in histological sections. This had been proven in clinical trials where IL-5 reduced BAL eosinophilia but had no effect on AHR [156]. However, in a subsequent study it was shown that although BAL eosinophilia was reduced, there was only a 50% reduction in eosinophilia in the airway wall [157], highlighting the need to take both measurements into account.

The experiments in this chapter have shown that the adaptation of the well established OVA induced airways inflammation model [341] used by many groups to study airways inflammation resulted in pathology as previously shown. This model, consisting of three OVA immunisation followed by an OVA airways challenge produces BAL eosinophilia,

lung inflammation, increased OVA-specific IgE and IgG1, and increased IL-5 production. The response to antigen challenge in this model is similar to the response seen in humans, which consists of an IgE-mediated inflammatory cascade in the airways, characterised by the influx of Th2 lymphocytes and eosinophils and chronic pulmonary inflammation [361]. The pathology in the long model was associated with recruitment of antigen-specific T cells to the airways. Several studies have been published that show the importance of T cells in asthma, from their ability to migrate to the lung in response to antigen challenge, and their role in the development of eosinophils and IL-5 production [362-368].

As well as the long model, a shorter model was also developed consisting of one OVA immunisation followed by one airways challenge of OVA plus LPS. This model also resulted in BAL eosinophilia, lung pathology and increased BAL IL-5 production accompanied by increased antigen-specific T cell recruitment to the lungs. However, this model was not associated with an increase in IgE or IgG1 antibody production. This data is supported by several studies showing that there are at least three pathways that lead to airways inflammation in murine models. One is dependent on IgE and MCs, one is dependent on eosinophils and IL-5 and the other is dependent on IL-13 (reviewed in [369]). The addition of LPS to the short model may contribute to the asthma like response as it has been shown that LPS administered at low doses in murine models of airways inflammation causes a Th2 type response [370].

The data presented in this chapter have shown that the antigen-specific T cells involved in the development of the two models of airways inflammation express different levels of

cytokines, co-stimulatory molecules and adhesion molecules. The development of several models of airways inflammation based upon sensitisation by systemic administration of protein antigens such as OVA and subsequent inhalation challenge have gained widespread acceptance. In such models, concomitant development of both eosinophil dominated inflammation and AHR to cholinergic stimuli have been repeatedly demonstrated [154, 371-373]. Although there are many different models in use, it has not been determined why the different models give similar results. Many studies in murine models have shown that the expression of chemokine receptors on Th2 cells are important for the recruitment of these cells to the lungs and in initiating an immune response [236, 345-347, 374]. The role of costimulatory molecules and cytokines have also been studied in airways inflammation [344, 375-378]. However, these factors have not been compared between the different studies.

The two models characterised in this chapter have shown as other studies that there are different routes to the same endpoint. This raised the question of whether we were looking at two different routes to the same endpoint or whether they had different pathologies. To address this question an initial screen of co-stimulatory molecules, cytokine and chemokine expression on antigen-specific T cells was carried out by quantitative RT-PCR to phenotype the two models.

It was found that when the two models were compared when no pathology was present that there was an increase in IFNγ expression in the DLN and lung in the short model and in the DLN in the long model. IFNγ is known to promote a Th1 phenotype and has been shown to suppress the development of airways inflammation [195, 379, 380]. There was also an

increase in costimulatory molecules in the long model, although this may be accounted for by the three immunisations given, which may stimulate the T cells, but the PBS airway challenge may not have been enough of a boost to activate the T cells to mount a response.

Comparison of the two models where pathology was induced showed that both the long and short model has increased expression of all costimulatory molecules examined (CD28, ICOS, OX40 and CD40L). When cytokines were analysed it was found that the long model had increased expression of IL-5, which correlates to the BAL IL-5 measured earlier in the model. IL-5 production has been shown to be important in the maturation and activation of eosinophils in airways inflammation [253, 381-383]. This is in contrast to the results seen in the long model when no pathology was present no IL-5 was expressed, suggesting that IL-5 is important in the induction of airways inflammation. In the short model it was found that IL-5 was not needed to induce inflammation but that the expression of IL-13 was upregulated. Several studies have shown the importance of IL-13 inducing airways inflammation [144, 384-387]. Again this is in contrast to the short model where no pathology was seen. These results also add to the hypothesis that there are several routes to the same endpoint and that many different factors are involved in the induction of airways inflammation. In both the long and the short models CCR3 is upregulated. This chemokine has been implicated in the accumulation of eosinophils in the airways of asthmatics and for the development of AHR [244, 245, 347, 388]. From these results it is clear that although the two models have similar outcomes there are major differences in the way in which the responses were mounted. These preliminary results emphasize the need for caution when comparing different studies from different groups, even if the end results seem comparable.

The experiments in this chapter have optimised and characterised robust models of murine airway inflammation. The use of adoptively transferred OVA-specific T cells allows the identification and quantification of antigen-specific cells in the lymph nodes, lungs and BAL over a timecourse. Using these models I aim to examine the effects of exogenous inflammation (LPS), an anti-inflammatory therapy (anti-TNF alpha) and to track cell divisions and interactions in cellular compartments over the course of the disease.

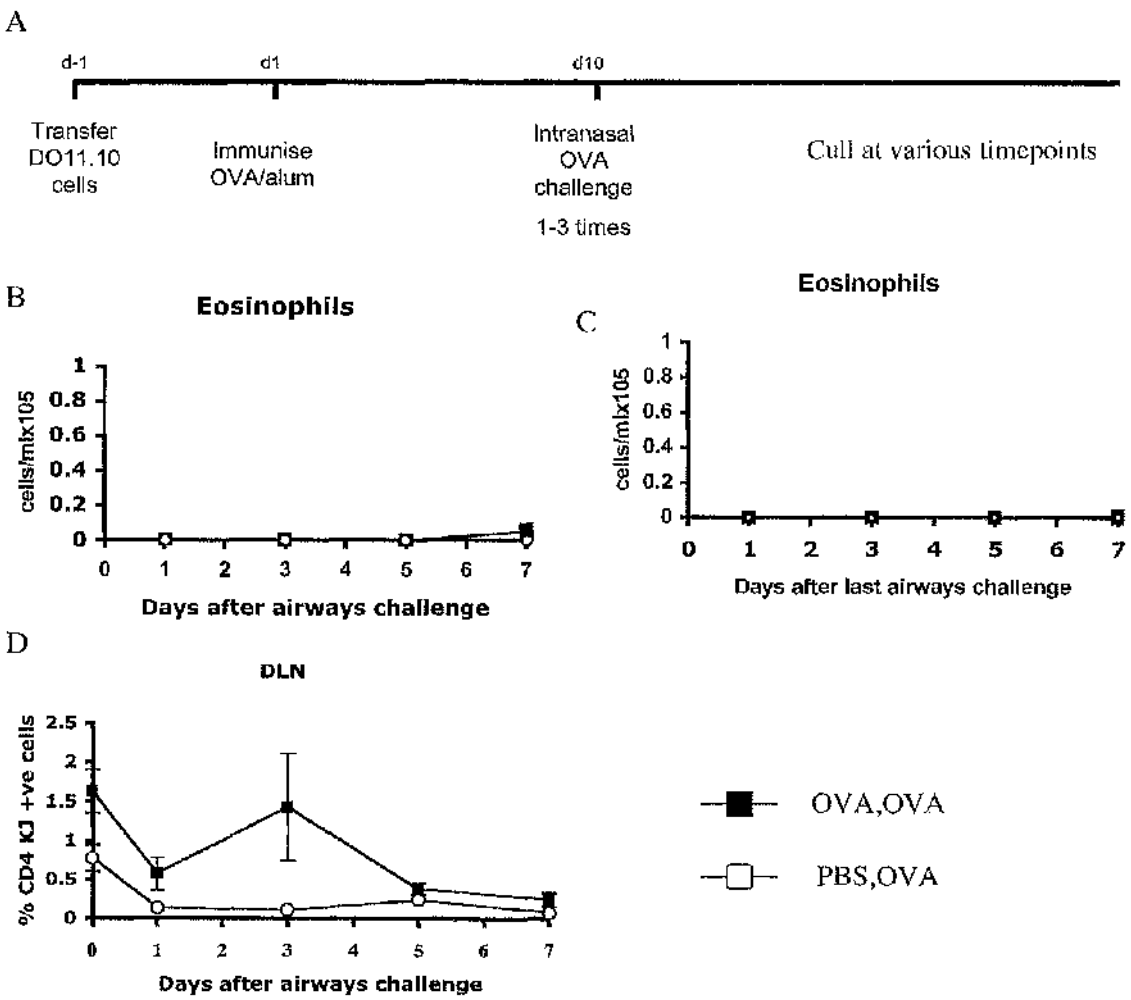

Figure 3.1: Adoptive transfer model of allergic airways inflammation

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients by i.v. injection. Mice received 100μg OVA and 1% alum by i.p. injection followed by 1 or 3 intranasal challenges with 50μg OVA 10 days later or on days 10,11 and 12 (A). Control mice were immunised with PBS/alum and challenged with intranasal OVA. Mice were sacrificed at various timepoints. BAL preparations were sampled and analysed for eosinophil content from cytospins (B and C). Draining lymph nodes were stained with fluorescent antibodies against CD4 and the transgenic T cell receptor (mAb KJ1.26). Cells were

analysed by flow cytometry to identify CD4 and KJ1.26 antigen specific T cells (D). Values are represented as mean ± standard error of the mean (SEM), n= 3 per group, n= 2 repeats.

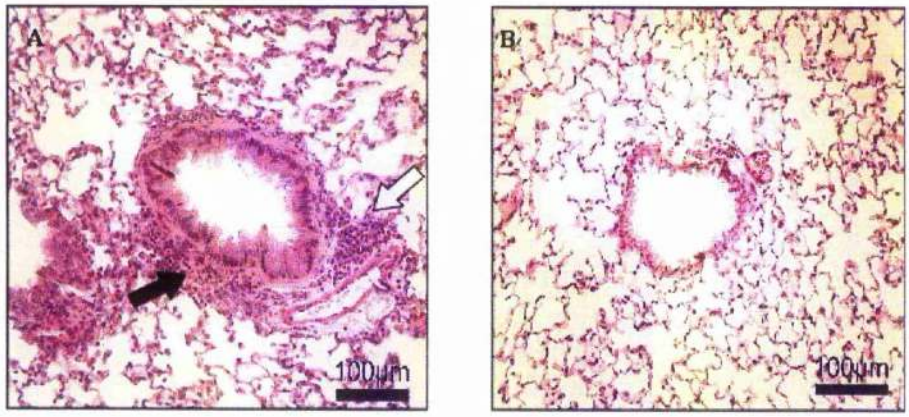

Figure 3.2: Inflammation in lung sections.

Lung sections were taken for histological analysis and stained with H&E. Perivascular and peribronchiolar eosinophilic infiltrates were seen (black and white arrow respectively) in OVA challenged mice (A). Panel B shows a different view from the same lung section with no inflammation.

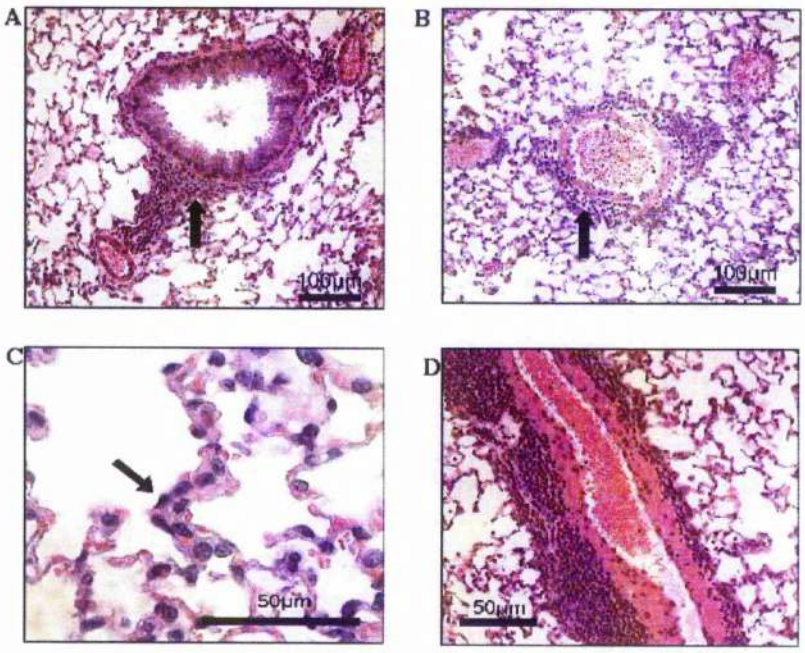

Figure 3.3: Lung inflammation.

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients by i.v. injection. Mice were then immunised with 100µg OVA/alum by i.p. injection 3 times, one week apart, followed by 1 intranasal challenge with 50µg OVA one week later. Mice were sacrificed at various timepoints thereafter. Lung sections were taken for histological analysis and stained with H&E. Figures show peribronchiolar (A), perivascular (B) and alveolar wall (C) inflammation. Longitudinal section of perivascular inflammation (D).

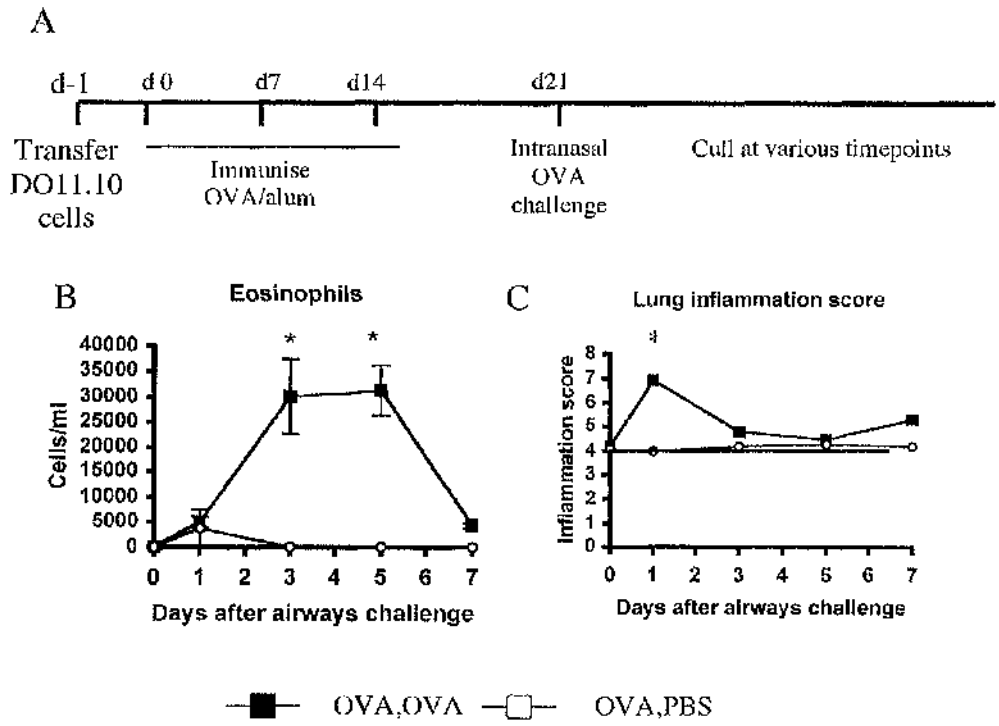

Figure 3.4: 3 immunisations with OVA/alum and 1 airway challenge with OVA induces BAL eosinophilia and associated pathology

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients by i.v. injection. Mice were then immunised with 100μg OVA/alum by i.p. injection 3 times, one week apart, followed by 1 intranasal challenge with 50μg OVA or PBS one week later (A). Mice were sacrificed at various timepoints thereafter. This resulted in a significant increase in eosinophilia (B) and lung pathology (C). Values are represented as mean ± SEM, n= 3 per groupn= 2 repeats (*$p<0.05$).

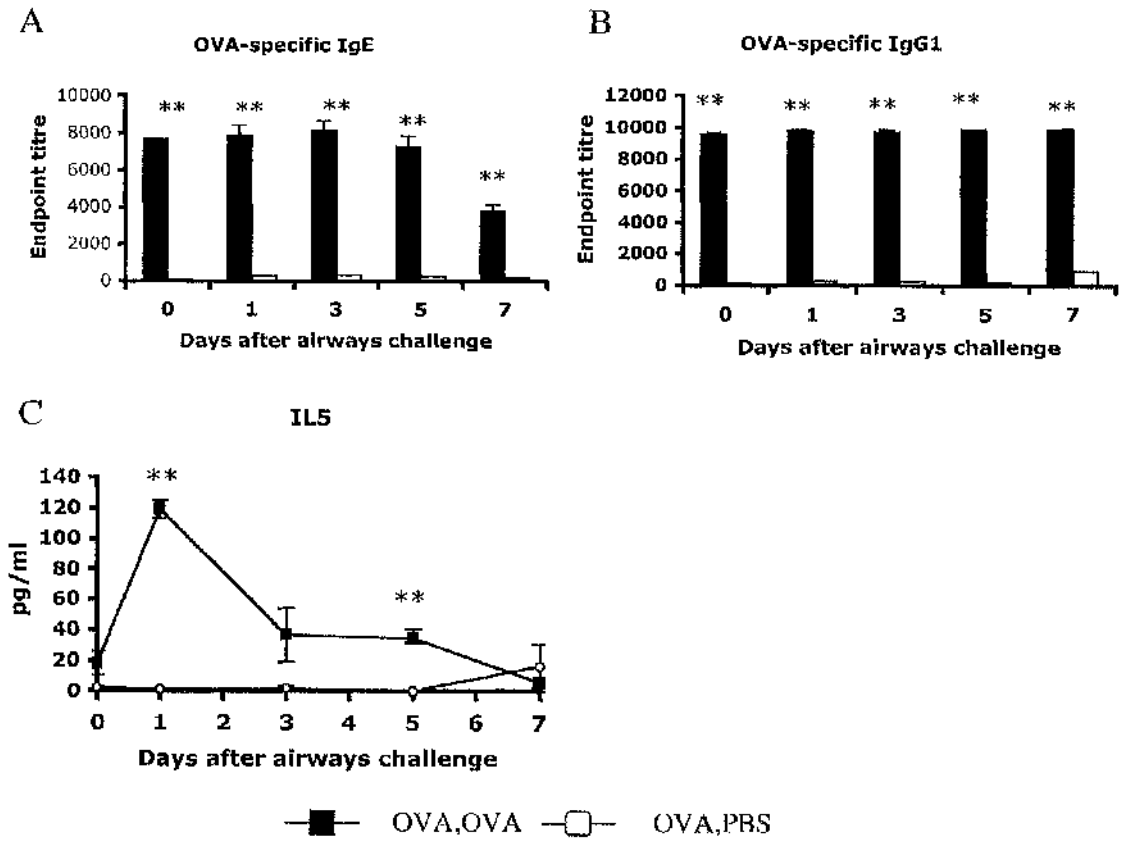

Figure 3.5: Antibody and cytokine production

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients by i.v. injection. Mice were then immunised with 100μg OVA/alum by i.p. injection 3 times, one week apart, followed by 1 intranasal challenge with 50μg OVA or PBS one week later. Mice were sacrificed at various timepoints thereafter. This resulted in a significant increase in antigen-specific IgE (A), IgG1 (B), and BAL IL-5 production (C). Values are represented as mean ± SEM, n= 3 per group, n= 2 repeats, (**$p<0.01$).

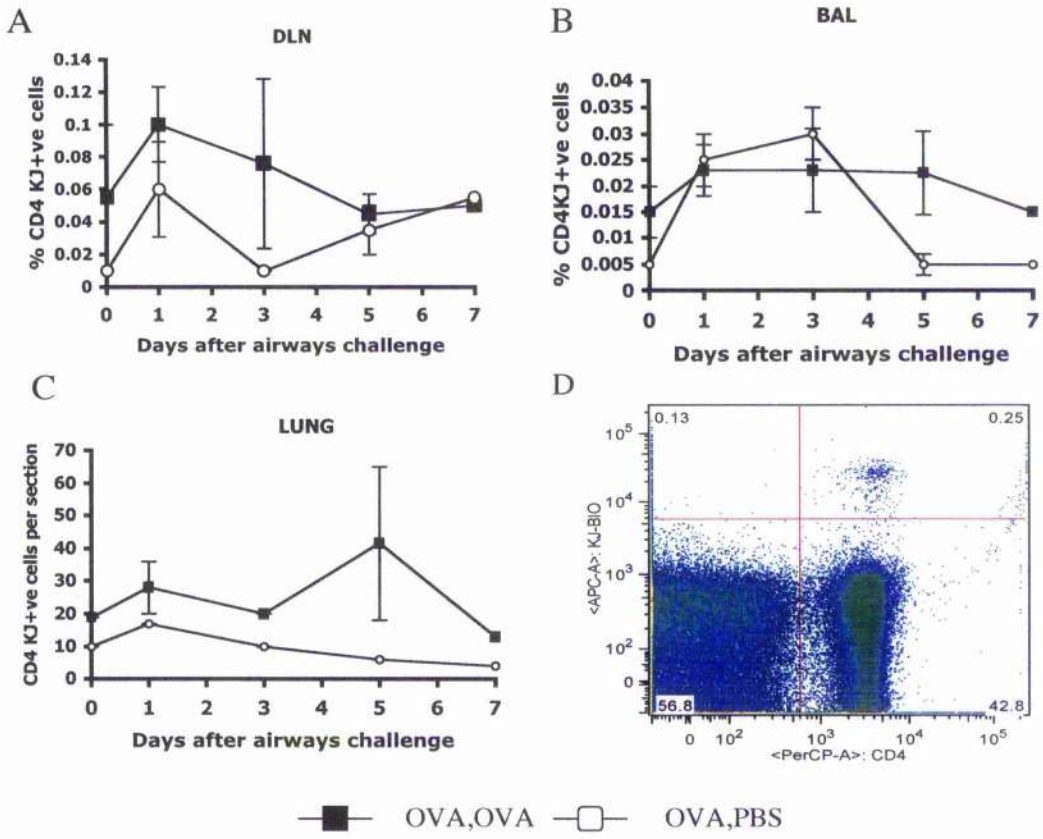

Figure 3.6: Detection of antigen-specific T cells

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients by i.v. injection. Mice were then immunised with 100μg OVA/alum by i.p. injection 3 times, one week apart, followed by 1 intranasal challenge with 50μg OVA or PBS one week later. Mice were sacrificed at various timepoints thereafter. Antigen-specific T cells were analysed by flow cytometry in the DLN (A) and BAL (B). LSC was used to analyse OVA-specific T cell in lung sections (C). Flow cytometry plot shows raw data for %KJ1.26 in DLN at day 3 post airways challenge (D). Values are represented as mean ± SEM, n= 3 per group, n= 2 repeats.

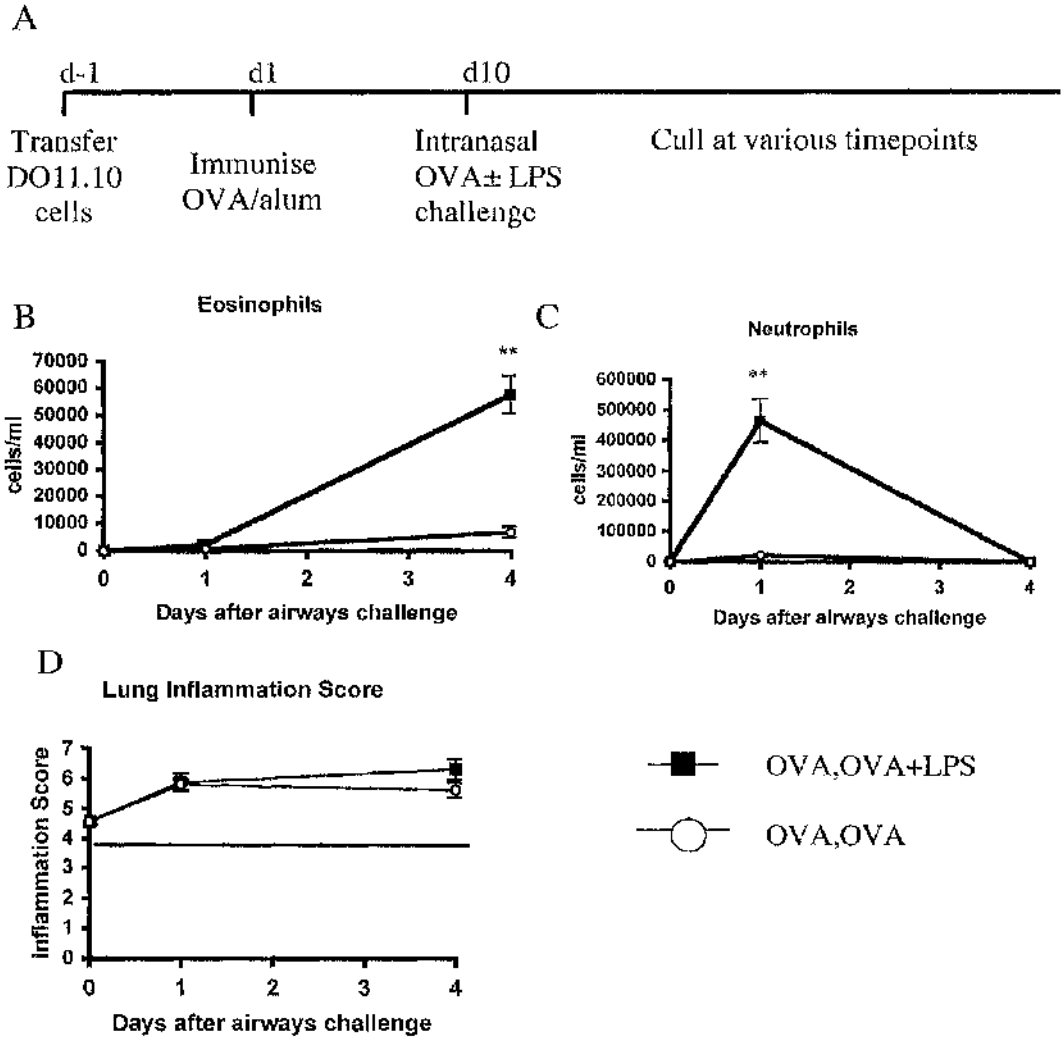

Figure 3.7: Addition of LPS to short airway inflammation model causes increased cosinophilia and neutrophilia

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with either 50μg OVA alone or 50μg OVA plus 2.5μg LPS (A). Mice were sacrificed at various timepoints. This

resulted in a significant increase in BAL eosinophilia (B) and neutrophilia (C). Lung sections were stained for H&E and scored for lung pathology (D). The solid black line represents the baseline pathology. Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (**p<0.01).

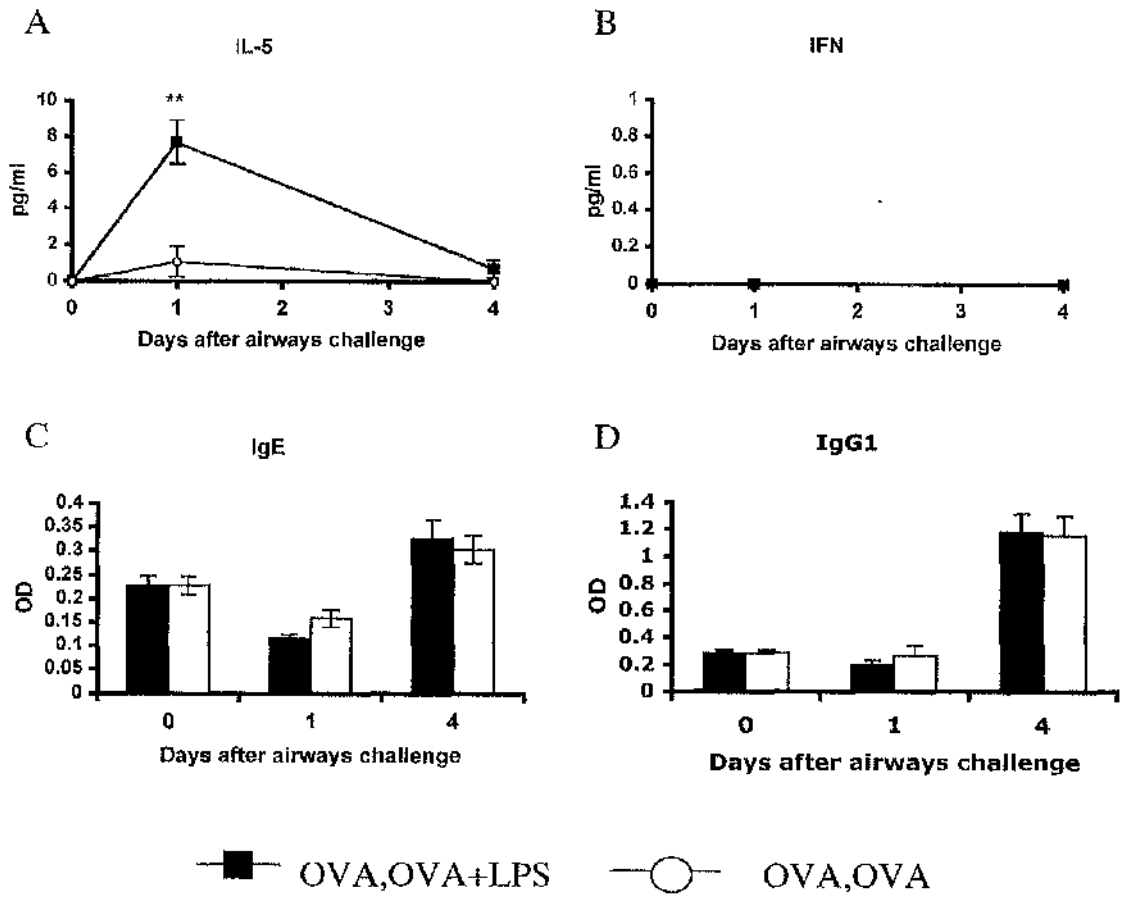

Figure 3.8: Cytokine and antibody production

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with either 50μg OVA alone or 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various timepoints. This resulted in a significant increase in BAL IL5 production (A). IFNγ was undetectable (B). Antigen-specific IgE (C) and IgG1 (D) serum levels were measured. Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (**p<0.01).

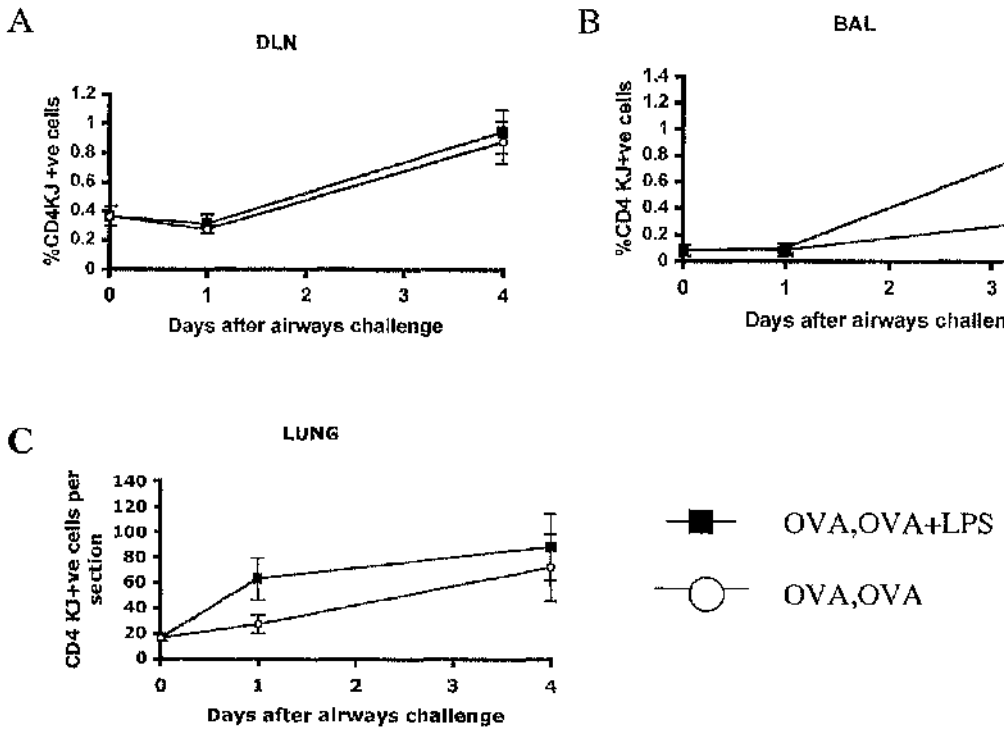

Figure 3.9: Antigen-specific T cells in DLN, BAL and lung tissue

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with either 50μg OVA alone or 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various timepoints. OVA-specific T cells were identified in DLN (A). A significant increase in antigen-specific T cells was seen in the BAL when challenged with OVA (B). Cells were identified by LSC analysis in lung sections (C). Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (**p<0.01).

Group / Gene	Long Lung OVA,PBS	Long LN OVA,PBS	Long Lung OVA,OVA	Long LN OVA,OVA	Short Lung OVA,OVA	Short LN OVA,OVA	Short Lung OVA,OVA+LPS	Short LN OVA,OVA+LPS
CD28	+	+++++	+	+	+	+	++	+
ICOS	+	+++++	+	++	+	+	+++++	+
OX40	+	+++++	+	++	+	+	+++++	+
CD40L	+	+++++	+	+++	+	+	+++	+
IL4	+	+++++	+	+	+	+	+	+
IL5	+	+	+	+++++	+	+	+	+
IL13	+	+	+	+	+	+	+++++	+
TNF α	+	+	+	+	+	+	+++++	+
IFNγ	+	+++	+	++++	++	++	+++++	+
CCR5	+	+	+	++++	+	+	+++++	+
CXCR4	+	+	+	+	+	+	+++++	+
Lymphotactin	+	+++++	+	+++	+	+	+++	+
RANTES	+	+	+	+	+	+	+++++	+
MIP1α	+	+	+	+	++++	+	++	+

Figure 3.10: Analysis of antigen-specific T cells from the long and short models.

Mice were treated as described in figures in figures 3.5 (long) and 3.6 (short) legends. 72 hours after airways challenge lung tissues and draining lymph nodes were removed and T cells were removed by positive selection for $CD4^+$ T cells and then negative selection for antigen specific T cells. RNA was then extracted form the samples and RT-PCR was used to quantify specific co-stimulatory molecules, cytokines and chemokines. The table summarises the results. (n=10 per group).

CHAPTER 4 - MICROBIAL PRODUCTS AND THEIR EFFECT ON ALLERGIC AIRWAYS INFLAMMATION

4.1 Introduction

The relationship between asthma and infection has been known for decades [11]. However, details of the interactions and mechanisms involved in this association remain unclear. Although the role of viral infections in childhood asthma have been studied quite extensively [307, 312, 389], the effect of bacterial infections is poorly understood [390]. Many epidemiological studies have shown an inverse relationship between living in rural areas and the incidence of atopy and asthma [391-393]. This is thought to be due to the increased level of bacterial endotoxin, suggesting that increased exposure to bacterial infection can be protective against the development of asthma [391, 394-396]. In contrast, several studies have shown that levels of endotoxin in house dust correlate with severity of asthma [397-400]. Experimental administration of LPS to asthmatic and healthy subjects results in AHR and respiratory symptoms [401, 402], with an exaggerated effect seen in asthmatics [403, 404].

Host defence against microbial pathogens is elicited through the innate immune system by means of TLRs. It is reasonable to assume that the effect of infectious agents on the development of allergy is closely linked to the activation of TLRs by PAMPs [405]. This is supported by studies using MyD88 deficient mice, showing that lack of TLR signalling resulted in Th2 responses and increased IgE production [406]. Most epidemiologic studies of exposure to TLR ligands and their effects on atopic phenotype have looked at LPS (endotoxin). Early studies showed that house dust mite endotoxin levels were lower in homes of allergen-sensitised verses non-sensitised infants, showing that indoor endotoxin exposure in early life may protect against allergen sensitisation [325]. This was further

supported by a study which identified livestock as a main source of endotoxin in homes and confirmed an inverse association between endotoxin exposure and atopy rates on farms [391].

The use of animal models to elucidate the mechanisms involved in these complex and contradictory studies has also proven inconclusive. The addition of LPS in murine models of allergic airways inflammation can result in either exacerbation or amelioration of pathology [332, 407, 408]. In animal studies it seems that the timing, route and dose of LPS administered are all able to affect the outcome of disease. However, several studies have consistently shown that LPS has a suppressive effect on asthma when administered at the time of antigen sensitisation [409, 410]. In contrast, no conclusive results have been reached when LPS is given after antigen sensitisation [407, 408]. It is clear from the studies outlined above that the underlying mechanisms of these effects are unclear and that more studies are needed to understand the complexities involved to be able to effectively treat airways inflammation.

To examine the effect of LPS administration on allergen-induced airways inflammation, I will use the adoptive transfer models described in chapter 3. The aim of this chapter is to examine and characterise the effects of exogenous inflammation, given locally or systemically, on airways inflammation. Our investigations show that LPS administered at the time of airways challenge can produce differing results depending on the route of administration. Given locally, LPS resulted in an increased antigen-driven eosinophilic inflammation in the airways, which was accompanied by increased antigen-specific T cell

numbers in the airways. When given systemically, LPS was shown to reduce airways inflammation, although no effect on antigen-specific T cells in the airways was found. There was also no affect on the memory response following subsequent antigen airways challenge, suggesting that LPS was affecting the inflammatory response and not the adaptive response. The results shown here were also mimicked using BLP, a TLR2 agonist, demonstrating the effects are not TLR4 specific. These results emphasize the many underlying factors involved in the immune response, and give an insight into the role exogenous factors play in mediating the immune response.

4.2 Results

4.2.1 Effect of addition of LPS to OVA airways challenge

In chapter 3 a model of allergic airways inflammation was established, in which adoptive transfer of Ag-specific T cells was followed by three immunisations, seven days apart and a single OVA airways challenge (Figure 3.4A). This model will be referred to as the "long model". This model will be modified to include the addition of LPS to the airways challenge to determine the effects of local administration of microbial products (Figure 4.1A). The LPS administered has been tested for impurities by SIGMA and was shown to contain less than five percent protein impurity. The airways challenge includes either OVA alone, LPS alone or a combination of OVA plus LPS. As expected OVA alone gave significant eosinophilia, with OVA plus LPS showing similar results. LPS alone was not sufficient to induce BAL eosinophilia (Figure 4.1B). When neutrophilia was assessed in the BAL, OVA alone did not induce neutrophilia. In contrast, the OVA plus LPS and the LPS alone treatments resulted in significantly higher levels of neutrophils compared with the OVA alone group (Figure 4.1C). These results suggests that the presence of antigen in the airway challenge is important in the induction of eosinophilia in this model, even though the mouse has been primed. When lung inflammation in histology sections was analysed, all three treatment groups induced inflammation higher than the baseline. OVA alone showed a constant level of inflammation throughout the timecourse. OVA+ LPS showed the highest level of inflammation, peaking at day three post airways challenge. LPS showed an early peak at day one post airway challenge, steadily declining over the timecourse (Figure 4.1D).

4.2.2 Cytokine and antibody production

IL-5 and IFNγ were measured to determine if the ability of LPS to exacerbate allergic airway inflammation was due to its capacity to affect cytokine production. BAL fluid was analysed by ELISA. OVA alone and OVA+LPS caused a significant increase in IL-5 production compared to LPS alone, however, the addition of LPS to Ova airways challenge decreased IL-5 compared to OVA alone(Figure 4.2 A). IFNγ could not be detected (Figure 4.2 B).

The production of OVA-specific antibodies was also assessed. Serum levels of Th2-associated OVA-specific antibodies IgE and IgG1 were not increased by the addition of LPS to OVA challenge (Figure 4.3 A and B respectively). However, the Th2 associated antibodies are not increased when LPS is added to OVA, suggesting that these mechanisms may not be involved in the increase in eosinophilia. It may be possible that antibody production is already maximal with OVA challenge alone and that the addition of LPS does not increase this.

4.2.3 OVA plus LPS enhances recruitment of antigen-specific T cells to the airway but not to the DLN

The recruitment of antigen-specific T cells to the DLN and BAL and was examined by flow cytometry. There was no difference in number of transgenic T cells in the DLN when all three treatment groups were compared (Figure 4.4 A). However, the numbers of transgenic T cells in the BAL fluid were found to be increased when OVA alone or OVA+LPS was given compared with LPS alone (Figure 4.4 B). Although the numbers are not significant, a similar trend was seen in further experiments. Furthermore, LSC analysis of lung sections

showed a significant increase in T cell numbers in OVA and OVA+LPS treated mice compared with the LPS alone treatment (Figure 4.4 C). This suggests that antigen-specific T cells may play a prominent role in the airways in the development of lung eosinophilia.

4.2.4 OVA plus LPS can induce airways eosinophilia in the short model

Initially, when the short model was carried out with one OVA/alum immunisation and a single intranasal OVA challenge (as described in chapter 3, Figure 3.5 A) this was insufficient to induce eosinophilia. As the addition of LPS increased airway eosinophilia in the long model, it was hypothesised that the addition of LPS to OVA airways challenge would be able to induce eosinophilia in the short model (Figure 4.5 A). As previously seen OVA alone did not induce eosinophilia in this short model. However, the addition of OVA+LPS led to a significant increase in BAL eosinophilia, seen at day four post airway challenge (Figure 4.5 B). This was accompanied by a significant increase in BAL neutrophilia seen at day one post airway challenge, not seen in the OVA alone group (Figure 4.5 C). Analysis of lung inflammation in histological sections showed a trend towards an increase in inflammation when OVA+LPS was given compared with OVA alone, although this was not significant (Figure 4.5 D).

4.2.5 Effect of LPS on cytokine and antibody production

The addition of LPS to OVA in the airway challenge resulted in a significant increase in BAL IL-5 production compared with OVA alone (Figure 4.6 A), as seen previously in the long model. The level of IFNγ was undetectable in both groups (Figure 4.6 B). The increase

in IL-5 production with the addition of LPS to OVA in the airways challenge may help to induce more eosinophilia in the short model.

The levels of OVA-specific IgE and IgG1 were measured in serum. No difference was seen between antibody levels when OVA and OVA+LPS were compared. (Figures 4.6 C and D). These results suggest that the levels of Th2-associated antibodies are not responsible for the levels of eosinophilia in the airways in this model.

4.2.6 LPS-induced BAL eosinophilia in the short model is associated with recruitment of antigen-specific T cells in the airway

Antigen-specific T cells were measured in the DLN and BAL fluid by flow cytometry. No difference was observed between groups in the DLN (Figure 4.7 A). When compared with the OVA alone treated mice, a significant increase in T cells was seen in the BAL fluid in mice treated with OVA+LPS (Figure 4.7 B). This was supported by a similar increase in transgenic T cells in lung sections analysed by LSC for OVA+LPS compared with OVA (Figure 4.7 C), although this data was not significant.

Overall, these results suggest that the addition of LPS to a model that previously showed no airway inflammation or associated pathology has been switched to a model with pathology. This is associated with an increase in IL-5 production and recruitment of antigen-specific T cells to the BAL. This recruitment of T cells also suggests a role for them in the effector phase of the response, after the initial sensitisation.

4.2.7 Pam3CSK4 (BLP) enhances eosinophilia airways inflammation when administered locally with antigen

To determine if the effects seen with LPS were limited to the TLR4 pathway, the experiments were repeated using BLP, a TLR2 ligand. When BLP was added to OVA in the airway challenge in the short model, a significant increase in BAL eosinophilia was seen (Figure 4.8 A) compared with OVA alone. This was accompanied by a significant BAL neutrophilia in the OVA plus BLP treated mice compared with OVA alone (Figure 4.8 B), as seen with the addition of LPS. As with LPS, BLP did not cause a change in recruitment of antigen-specific T cells to the DLN between the two groups (Figure 4.8 C). However, a significant increase in transgenic T cell was seen in the BAL when BLP was added to OVA (Figure 4.8 D). This is consistent with the results shown with the addition of LPS. These results show that it is possible to enhance airway inflammation by recruitment of T cells to the airways by a variety of PAMP-dependant pathways.

4.2.8 Effect of systemic LPS and BLP on airway eosinophilia

It has been shown in many studies that the route of administration of microbial products can affect the disease outcome in murine models [332, 407, 408, 410]. To determine whether the route of administration of LPS or BLP had any effect on the outcome of the adoptive transfer model of airway inflammation, they were introduced systemically into the long model (Figure 4.9 A), two hours after airways challenge. As shown by other groups [411] BAL eosinophilia was significantly reduced by both LPS and BLP (Figure 4.9 B).

4.2.9 Systemic effect of LPS and BLP is transient

These results suggest the effects of LPS and BLP may be due to a direct effect on the effector mechanisms of inflammation, rather than on the underlying immune response, due to the effects being seen after the airway challenge and also very rapid. If the effect is truly transient, then it should not affect immunological memory. However, it may be the case that LPS and BLP are acting as adjuvants to enhance the response to OVA exposure.

To elucidate how LPS and BLP are working, mice were exposed to the regime described earlier (Figure 4.9 A) with an initial OVA airway challenge followed by systemic LPS or BLP two hours later. The mice were then left to rest for two weeks before being given a second OVA airway challenge (Figure 4.10 A). The inflammatory response to the second challenge was then measured. Although LPS and BLP suppressed the BAL eosinophilia after the initial airway challenge (as previously seen), there was no difference between the groups after the second airway challenge (Figure 4.10 B). This shows that the suppressive effects of LPS and BLP are transient but also that they do not have any adjuvant effects. There was no difference in recruitment of antigen-specific T cells to the DLN or the BAL (Figure 4.10 C and D) or in antigen-specific IgE production (data not shown) between the different treatment groups, suggesting that there was no additional priming by the LPS or BLP injections. These results show that the effect of LPS and BLP is transient and may work directly on the local inflammatory response. The effects of LPS and BLP are also not due a change in T cell trafficking. However, it has not been shown that these agents lead to increased immunological sensitisation to airway antigens.

4.2.10 Cells are not sequestered to the peritoneal cavity after i.p. injection

Another possible explanation for the reduction in eosinophilia seen after i.p. injection of LPS or BLP, is that the injection causes an acute inflammatory reaction in the abdomen with consequent accumulation of inflammatory cells in that location, in turn reducing the cell number in the other location (i.e. airways). To examine this possibility, the cellular compartment of the peritoneal cavity was examined in the long model at various timepoints after the i.p. injection of LPS or BLP. Figure 4.11 A shows that there was no significant increase in total cell number in LPS or BLP treated mice compared to the control group. The eosinophil numbers were unaffected between the groups (Figure 4.11 B). The neutrophil number is increased in the LPS and BLP number (Figure 4.11 C), but decreased in the macrophages and lymphocytes (Figure 4.11 D and E). These results show that LPS and BLP cause an acute inflammatory response in the abdomen characterised by a neutrophil influx. However, this is not accompanied by enhanced recruitment of other inflammatory cells, in particular eosinophils. Thus the reduction in BAL eosinophils is not directly due to their sequestration to the peritoneal cavity.

4.3 Discussion

The first part of this chapter focused on the local administration of the TLR agonists LPS and BLP in conjunction with OVA airways challenge in previously sensitised mice. It was found that administration of the microbial products in addition to OVA in the airway exacerbated eosinophilic inflammation in the long model and induced it in the short model. This suggests that microbial products may play a role in regulating the severity of existing allergic airways inflammation, or that they could promote a switch to an eosinophilic response in previously non-allergic lung.

To try and elucidate the mechanisms involved in these responses, a series of experiments were carried out to determine if the microbial products were having a direct effect on the inflammatory pathway, or if they were altering the adaptive immune response. When LPS or BLP is added to the OVA airway challenge there is a significant increase in BAL eosinophilia. This is accompanied by a marked increase in BAL neutrophilia. These results are consistent with many human studies, who have shown an increase in neutrophil influx and AHR after inhalation of endotoxin in both normal [399, 402, 412] and asthmatic [413] subjects. The neutrophil influx was found not to be related to a change in lung function [402, 414]. This is consistent with the results in the long model, showing that although OVA airways challenge does not induce a neutrophil influx, lung inflammation is still observed, suggesting that neutrophils may not be necessary for lung pathology. However, many studies have shown that neutrophils are associated with clinical forms of asthma [415-418]. The increase in eosinophils seen with addition of LPS to OVA airways challenge is comparable to both human and murine studies. When atopic, but not normal, patients were

challenged with LPS an increase in eosinophils was observed in lavage fluid [419]. In mice previously sensitised to allergen, low doses of LPS resulted in exacerbation of eosinophilia [420].

As well as the increase in BAL cell content, other mediators were also measured in response to addition of LPS to the airways challenge. The production of cytokines in the BAL showed there was an increase in IL-5 production with addition of LPS to OVA, which was not present with LPS alone. IFNγ was not detectable in any group. Several mechanisms have been proposed to explain LPS induction of eosinophilia and proinflammatory cytokines. DCs are well recognized to play a central role in inflammatory reactions elicited by LPS [421]. When DCs are activated by LPS through TLR4, they become mature and acquire an increased ability to prime T cells [422]. A study by Eisenbarth *et al.*, reported that low level inhaled LPS signaling through TLR4 is necessary to induce Th2 responses to inhaled antigens in a mouse model of allergic sensitization. The mechanism by which LPS signaling results in Th2 sensitization was shown to involve the activation of antigen-containing dendritic cells [407]. However, a recent study proposed that LPS-induced MCs activation and modulation with increased production of Th2 cytokines, such as IL-5 and IL-13, appear to control the severity of eosinophilic airway inflammation [423]. It is known that IL-5 and IL-13 play a crucial role in the induction and the severity of eosinophilic infiltrate in airway inflammation in the lung [362]. IL-5 is a key factor for eosinophilia and could be responsible for some tissue damage in chronic asthma [424]. The data presented here suggests that the addition of LPS to an OVA airways challenge results in the exacerbation of eosinophilia..

It is possible that LPS and BLP may alter the underlying adaptive immune response, via different pathways including influence on B cells and IgE production, or by altering T cell number or function. An increase in IgE level could lead to an exaggerated response to antigen through enhanced cross-linking of IgE receptors on effector cells such as mast cells with consequent degranulation and release of inflammatory and bronchospasmogenic mediators. IgE has been shown to be important in humans [216, 217, 220-222, 425] and animal airways disease [426, 427], however, it may not be critical [224]. Serum levels of OVA-specific IgE were found to be no different between animals that received LPS or BLP with OVA and those that received OVA alone. The same was also true of IgG1 and IgG2a levels arguing against a shift towards a Th1-type phenotype.

The role of Ag-specific $CD4^+$ T helper cells in this model was explored using the DO11.10 transgenic OVA-specific T cell system. The commonly accepted idea of antigen presentation in airways inflammation is that antigen is presented to T cell in the draining lymph node, which then leads to increased production of Th2 cytokines and inflammation. But recent studies have shown that there is a population of memory T cells resident in the local tissue that are reactivated upon antigen re-stimulation [428-430], most likely by DCs [233]. However, antigen presentation in the airways has not been proven. The findings reported here concur with these studies. Although the proportion of Ag-specific T cells was increased in DLN after airway challenge, there was no difference in the increase between animals challenged with OVA plus LPS/BLP and those challenged with OVA alone. However, the number of Tg T cells was seen to increase in the BAL fluid when LPS/BLP

was included in the airway challenge, in both the long and short model. This strongly supports a role for T cells in the airways during the effector phase of the response.

These experiments have shown that LPS and BLP given in conjunction with OVA in an airways challenge, in previously sensitised mice, leads to an exaggerated airway response, predominantly eosinophilic. The underlying mechanism behind this response is still not fully understood, although it may be mediated by T cell number in the airways, but not the DLN.

The second part of the chapter focuses on the administration of TLR ligands by systemic injection at the time of airway challenge. Previous work has shown that the TLR2 agonist, BLP, has a suppressive effect on a model of allergic airways inflammation [411]. Work presented in this chapter shows that these effects can be mirrored in the long adoptive transfer model and is also reproducible using LPS. The work done using the model with BLP suggested that BLP may act by skewing the immune response towards a Th1 phenotype, causing a reduction in Th2 inflammation. However, Th1 responses do not always reduce or counteract allergic inflammatory responses [164, 167, 431].

In contrast to the results seen when LPS or BLP were administered locally at the time of challenge, systemic administration of LPS or BLP at the time of challenge resulted in amelioration of BAL eosinophilia compared with OVA alone. This data correlates to other studies which have also shown a decease in airways inflammation with systemic endotoxin treatment in murine models [408, 420, 432, 433]. When other parameters were analysed it

was found that systemic LPS or BLP did not affect the numbers of antigen-specific T cells in the DLN or in the BAL. There were also no differences between cytokine or antibody production when compared to the control groups, implying that there was not a shift from a Th2 phenotype to a Th1 phenotype. If the suppressive effect of the TLR agonists were acting through the adaptive response, it may be expected that the effect may also be seen in the memory response to antigen re-exposure. However, when mice were re-challenged with OVA fourteen days after the first airway challenge there was no change in the eosinophil response (speed or extent) in mice previously challenged systemically with LPS or BLP. Therefore, this data suggests that these agonists do not act via an effect on adaptive immunity.

Further experiments carried out were aimed at determining whether systemic injection was causing inflammatory cells to be sequestered into the peritoneum. Peritoneal lavage was carried out and cell contents compared to the BAL fluid. Both LPS and BAL caused an influx of neutrophils into the peritoneum, but there was no effect on eosinophil numbers and macrophage and lymphocyte numbers were reduced. Therefore, it seems improbable that inflammatory cells are being recruited into the peritoneum instead of the airways.

The experiments in this chapter have looked at the effect of TLR agonists, LPS and BLP, administered locally and systemically at the time of airways challenge. In these protocols the mice have already been sensitised to the allergen, which may represent a more clinical model of disease, as most patients will already have been sensitised when they seek treatment. This approach differs from many other studies that have looked at TLR

administration at the time of sensitisation [401, 407-409, 433-435]. The results presented in this chapter suggest that exposure to microbial products lead to the worsening of airways inflammation in a murine model of allergic airways inflammation. This is supportive of studies done in humans [436-440], which showed that inhalation of LPS by allergic asthmatics induced airways inflammation, including increased eosinophilia in the airways. The experiments in this chapter imply that this may be due to an accumulation of antigen-specific T cells in the airways, which may affect the underlying adaptive immune response. This may also lead to increased sensitisation to other allergens, as Th2 cells have been shown to facilitate priming of naïve T cells to unrelated antigen through IL-4 secretion [441]. This has been seen in atopic patients that accumulate allergens over time [442, 443].

Systemic treatment at the time of allergen challenge was shown to reduce subsequent inflammation, which means that there is a possibility that there may be therapeutic benefits of these agents. The ability to reduce inflammation is important but a problem with these agents is that LPS may act as an adjuvant on antigen re-exposure. However, my results imply that this is not the case, although the effects of LPS and BLP in reducing inflammation after a subsequent antigen challenge were diminished. The transient effects of systemic LPS and BLP therefore may not be of great therapeutic benefit, although understanding the underlying mechanisms may allow the development of ways to prolong their effects.

When compared to other studies, when LPS is given locally during antigen challenge, my results are in concurrence, showing exacerbation of airways inflammation [332, 407, 433]. However, there are also conflicting studies showing amelioration of the inflammatory

response [410, 433, 444, 445]. The use of systemic LPS to suppress the allergic response agrees with the data presented in this thesis [432, 444]. When LPS has been administered during sensitisation either locally or systemically most studies have shown a reduction in airways inflammation [423, 432, 446, 447]. In spite of this, caution must be taken when comparing these studies as different routes of administration, doses, timing and species have been used throughout the publications.

Overall, these results have shown that LPS administered at the time of antigen challenge can produce differing results depending on the route of administration. When LPS was given locally it resulted in an increased antigen-driven eosinophilic inflammation in the airways, accompanied by an increase in antigen-specific T cell numbers in the airways. This implies a role for T cells in the effector phase of the immune response, showing that LPS can modulate the adaptive immune response. When given systemically, LPS reduced airways inflammation, although there was no change in T cell numbers in the DLN or the airways. There was also no affect on the memory response following subsequent antigen challenge, suggesting that the effect is on the inflammatory response. The results have also been shown using BLP, a TLR2 agonist, demonstrating that the effects are not TLR4 specific. These results highlight the many underlying factors involved in the immune response and help our understanding of how exogenous inflammatory mediators can affect airways inflammation.

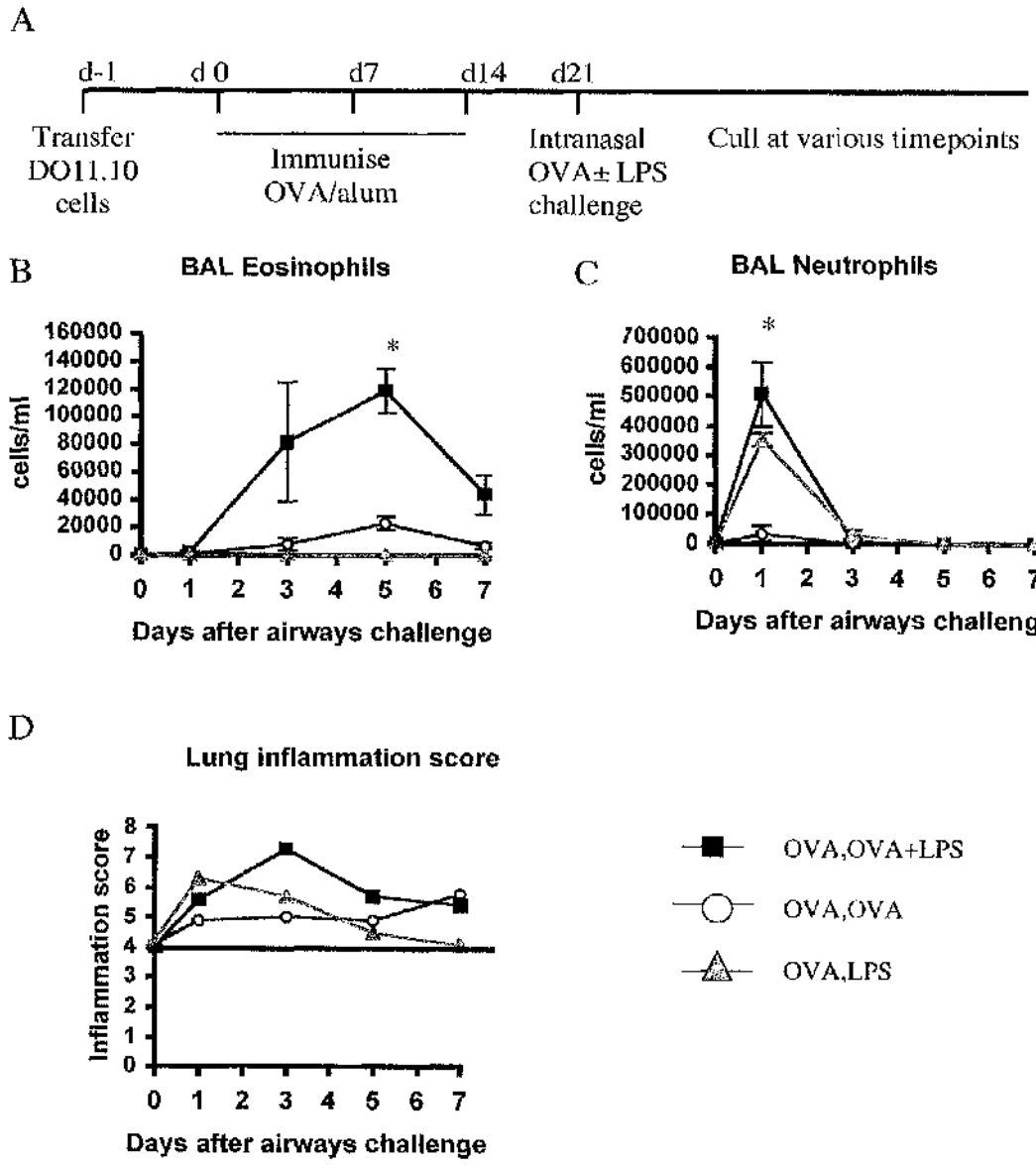

Figure 4.1: Adoptive transfer model of allergic airways inflammation with addition of LPS.

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days

later with either 50μg OVA alone, 50μg OVA plus 2.5μg LPS, or 2.5μg LPS alone (A). Mice were sacrificed at various timepoints. BAL preparations were sampled and analysed for eosinophil (B) and neutrophil (C) content from cytospins. Lung sections were stained for H&E and analysed for lung pathology (D). Baseline pathology represented by solid black line. Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (*$p<0.05$).

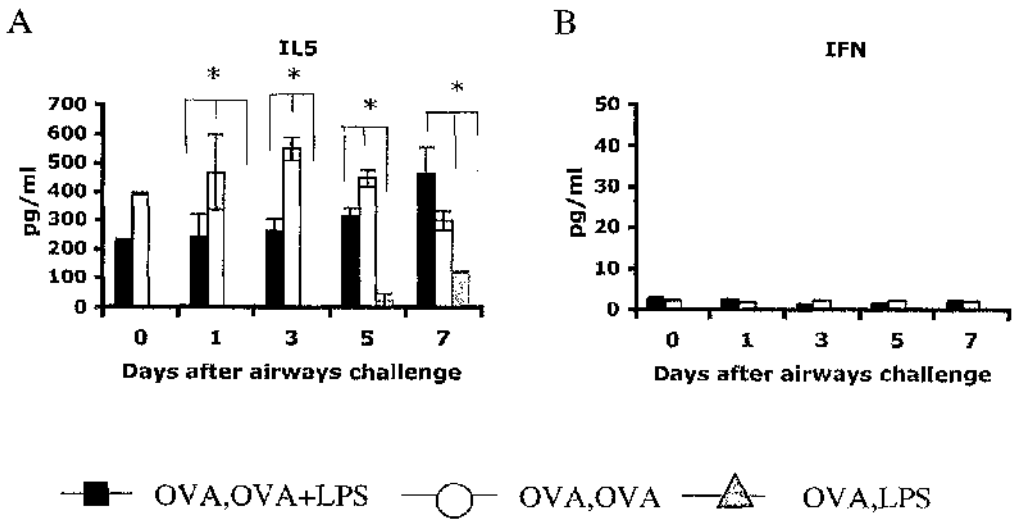

Figure 4.2: Cytokine production

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days later with either 50μg OVA alone,50μg OVA plus 2.5μg LPS, or 2.5μg LPS alone. Mice were sacrificed at various timepoints. BAL supernatants were analysed for IL5 (A) and IFNγ (B). Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (*$p<0.05$).

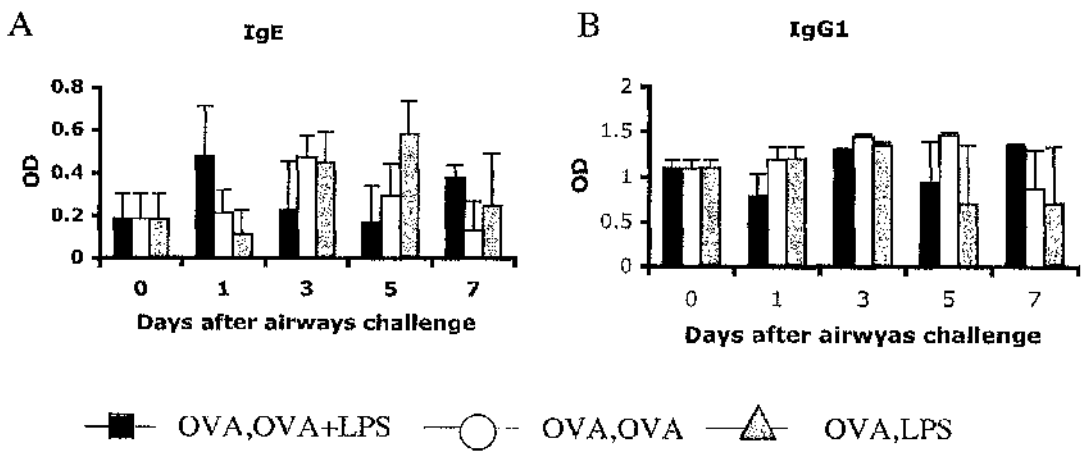

Figure 4.3: Antibody production

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days later with either 50μg OVA alone,50μg OVA plus 2.5μg LPS, or 2.5μg LPS alone. Mice were sacrificed at various timepoints. Serum was analysed for IgE (A) and IgG1 (B). Values are represented as mean ± SEM, n=5 per group, n= 2 repeats.

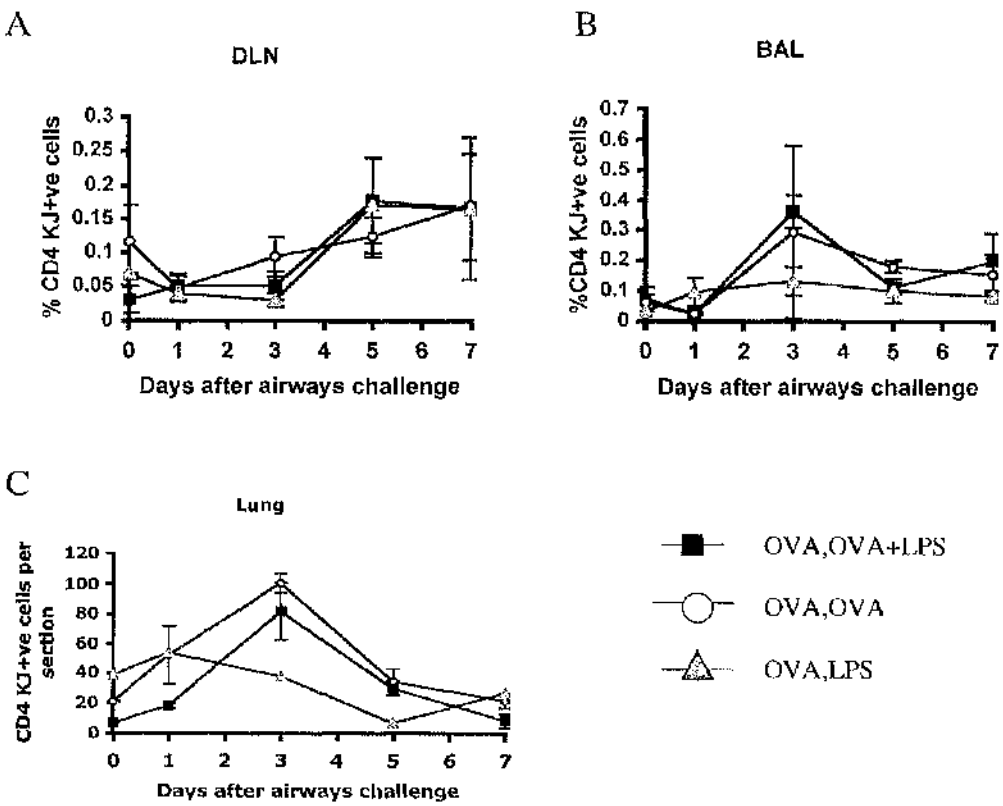

Figure 4.4: Identification of antigen-specific T cells in DLN, BAL and lung tissue

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection,three times, 7 days apart, followed by one intranasal challenge 7 days later with either 50μg OVA alone,50μg OVA plus 2.5μg LPS, or 2.5μg LPS alone. Mice were sacrificed at various timepoints. Antigen-specific T cells were identified in the DLN (A) and BAL fluid (B) by flow cytometry. Lung sections were stained for antigen-specific T cells and analysed by LSC (C). Values are represented as mean ± SEM, n=5 per group, n= 2 repeats.

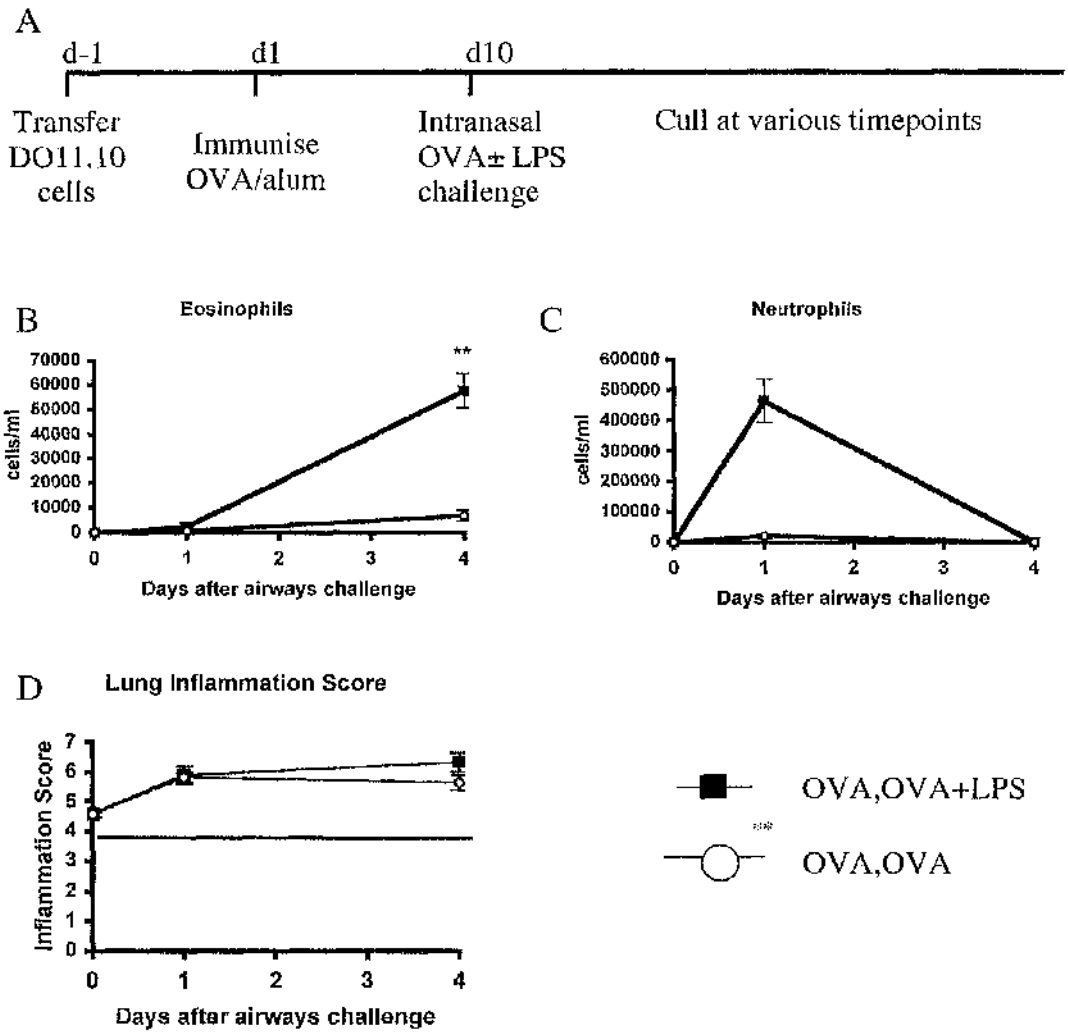

Figure 4.5: Addition of LPS to short airway inflammation model causes increased eosinophilia and neutrophilia

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with either 50μg OVA

alone or 50μg OVA plus 2.5μg LPS (A). Mice were sacrificed at various timepoints. This resulted in a significant increase in BAL eosinophilia (B) and neutrophilia (C). Lung sections were stained for H&E and scored for lung pathology (D). The solid black line represents the baseline pathology. Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (**$p<0.01$).

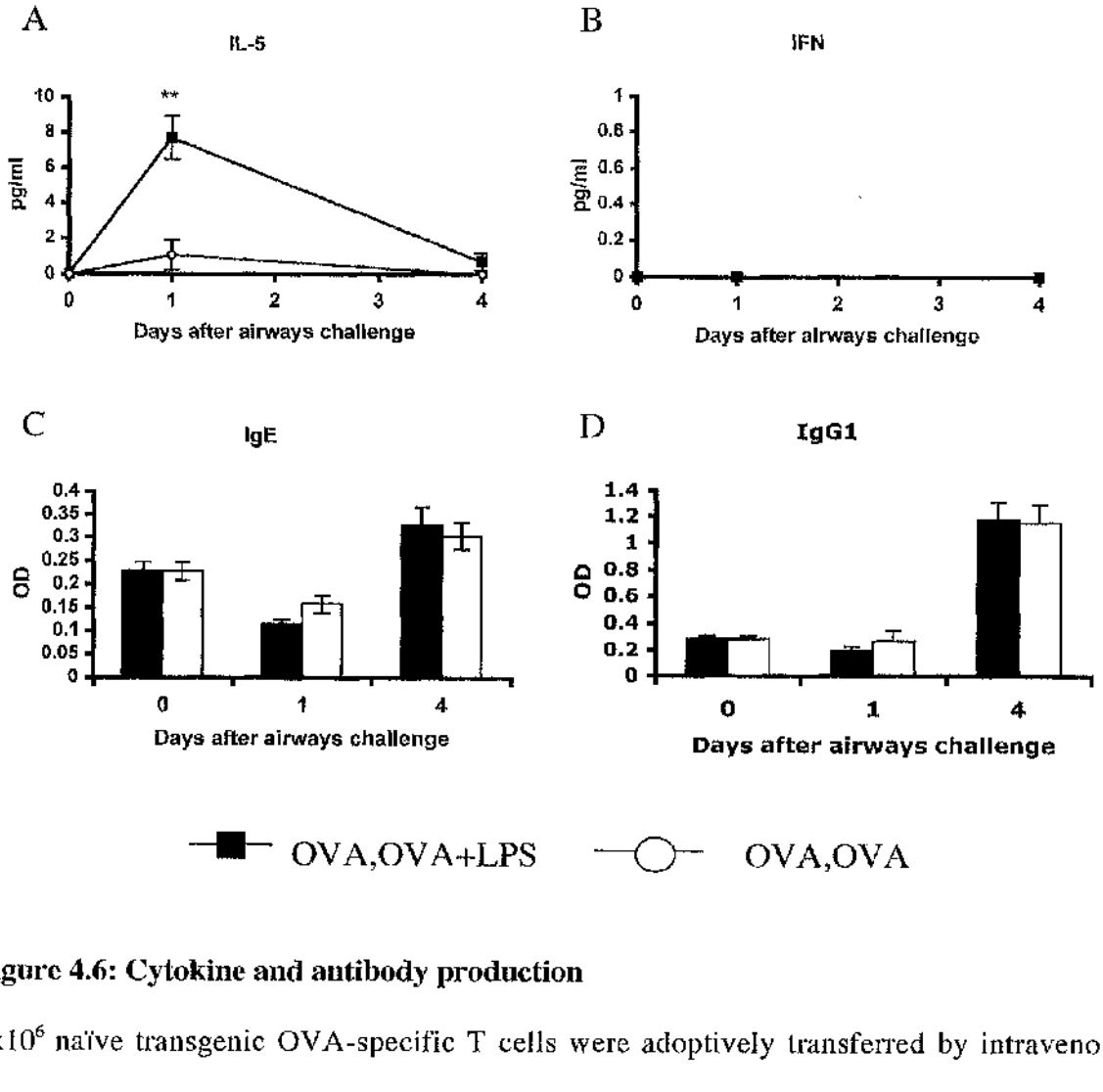

Figure 4.6: Cytokine and antibody production

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with either 50μg OVA alone or 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various timepoints. This resulted in a significant increase in BAL IL5 production (A). IFNγ was undetectable (B). Antigen-specific IgE (C) and IgG1 (D) serum levels were measured. Values are represented as mean ± SEM, n=5 per group (**p<0.01).

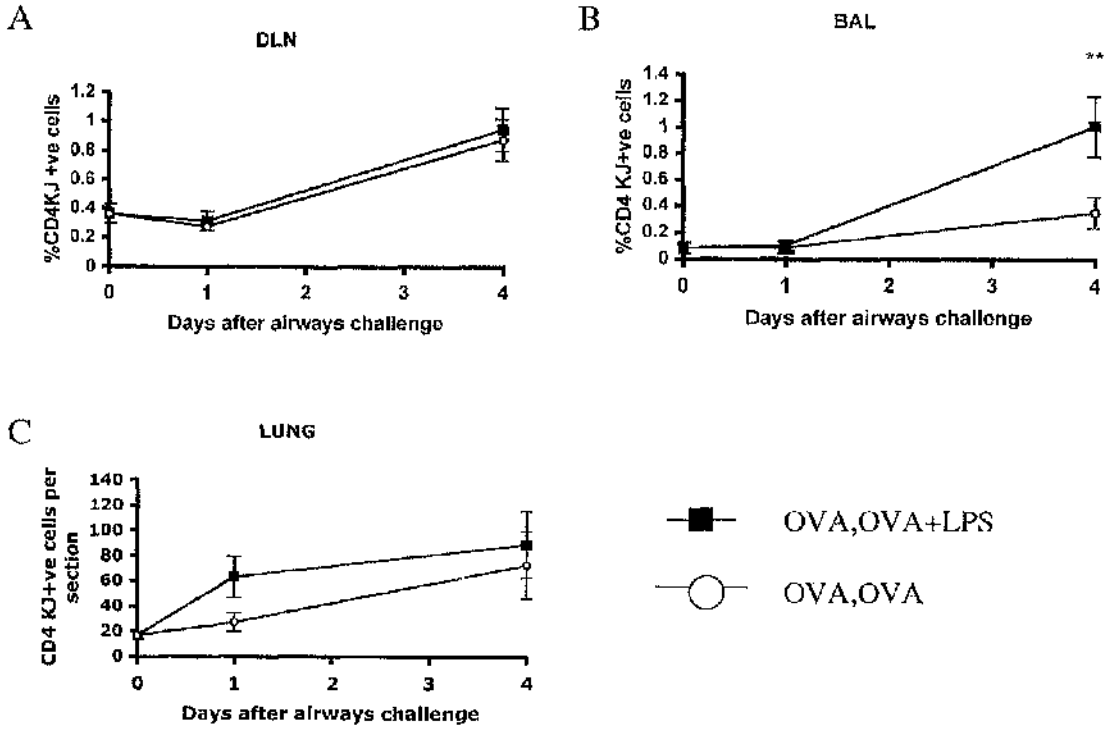

Figure 4.7: Antigen-specific T cells in DLN, BAL and lung tissue

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with either 50μg OVA alone or 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various timepoints. OVA-specific T cells were identified in DLN (A). A significant increase in antigen-specific T cells was seen in the BAL when challenged with OVA (B). Cells were identified by LSC analysis in lung sections (C). Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (**p<0.01).

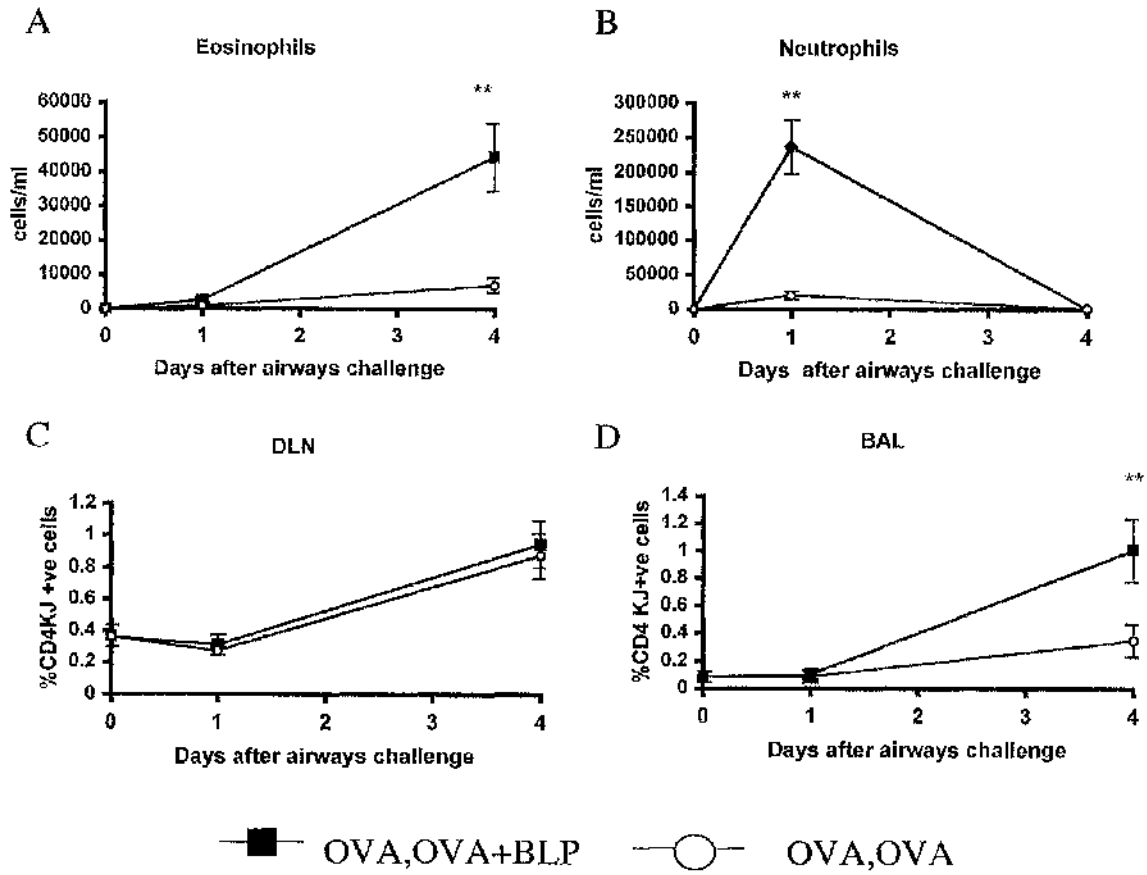

Figure 4.8: Short adoptive transfer model with addition of BLP causes BAL eosinophilia and neutrophilia

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with either 50μg OVA alone or 50μg OVA plus 2.5μg BLP. Mice were sacrificed at various timepoints. This resulted in a significant increase in BAL eosinophilia (A) and neutrophilia (B). Antigen specific T cells were identified in DLN (C) and BAL (D) by flow cytometry. Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (**$p<0.01$).

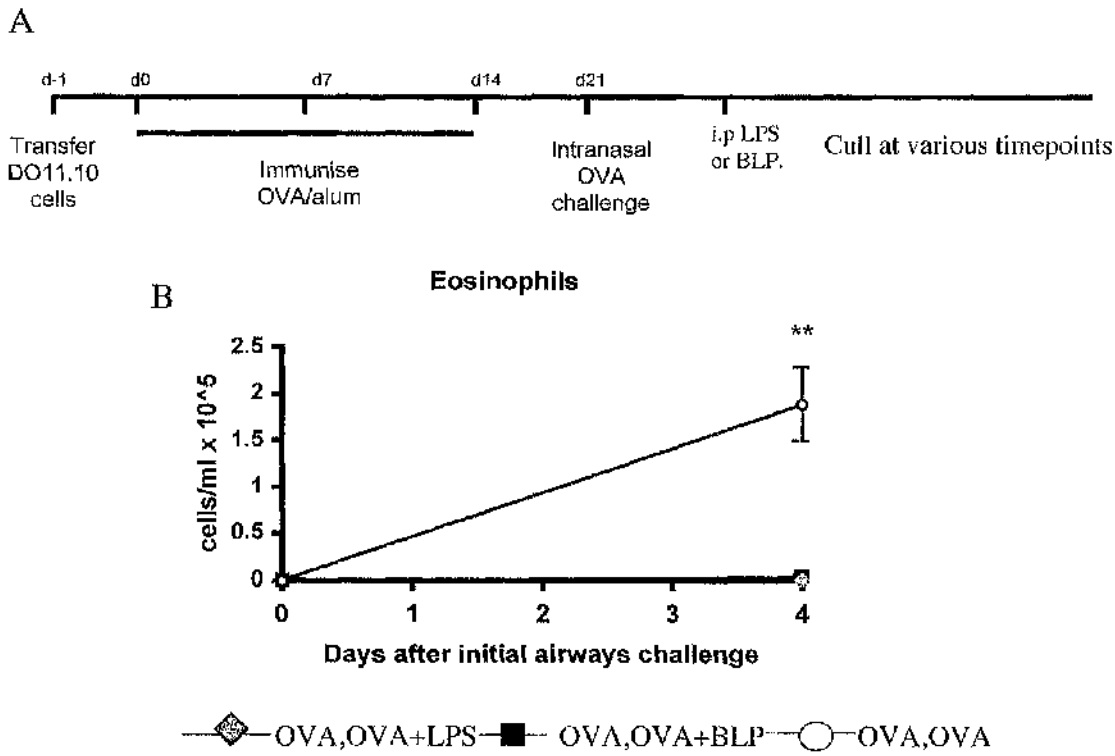

Figure 4.9: Short adoptive transfer model with systemic LPS or BLP re-challenge BAL suppresses BAL eosinophilia

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with 50μg OVA. Mice were then challenged with LPS or BLP by i.p. injection two hours later (A). Mice were sacrificed at various timepoints. Re-challenge with LPS or BLP resulted in significant suppression of BAL eosinophilia (B). Values are represented as mean ± SEM, n=5 per group, n= 2 repeats (**$p<0.01$).

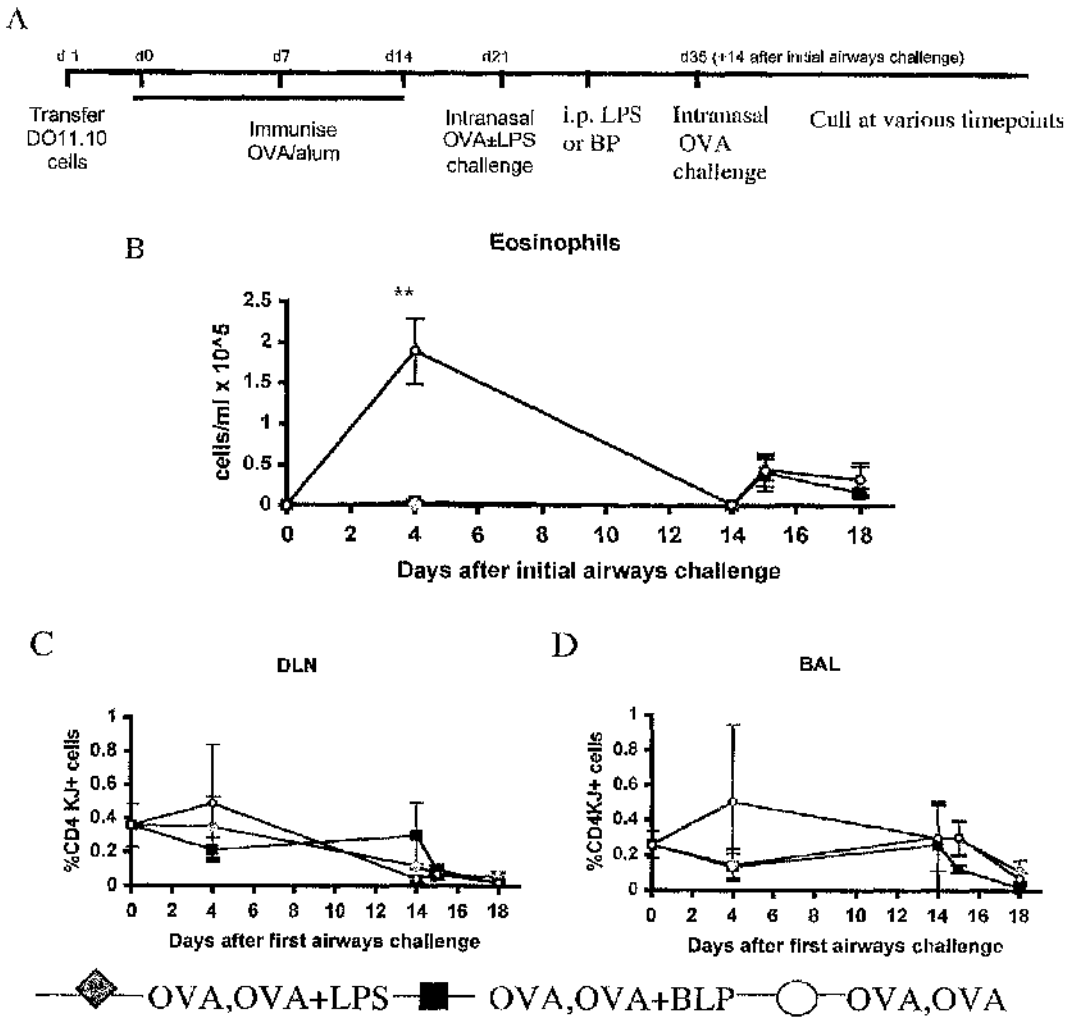

Figure 4.10: Effect of systemic LPS or BLP on antigen-rechallenge

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge seven days later with 50μg OVA. Mice were then challenged with LPS or BLP by i.p. injection two hours later. Fourteen days later mice received an intranasal challenge with 50μg OVA (A). Mice were sacrificed at various timepoints. BAL samples were analysed for

eosinophilia (B). Antigen-specific T cells wee identified in DLN (C) and BAL fluid (D). Values are represented as mean ± SEM, n=5 per group, n= 1 repeat (**p<0.01).

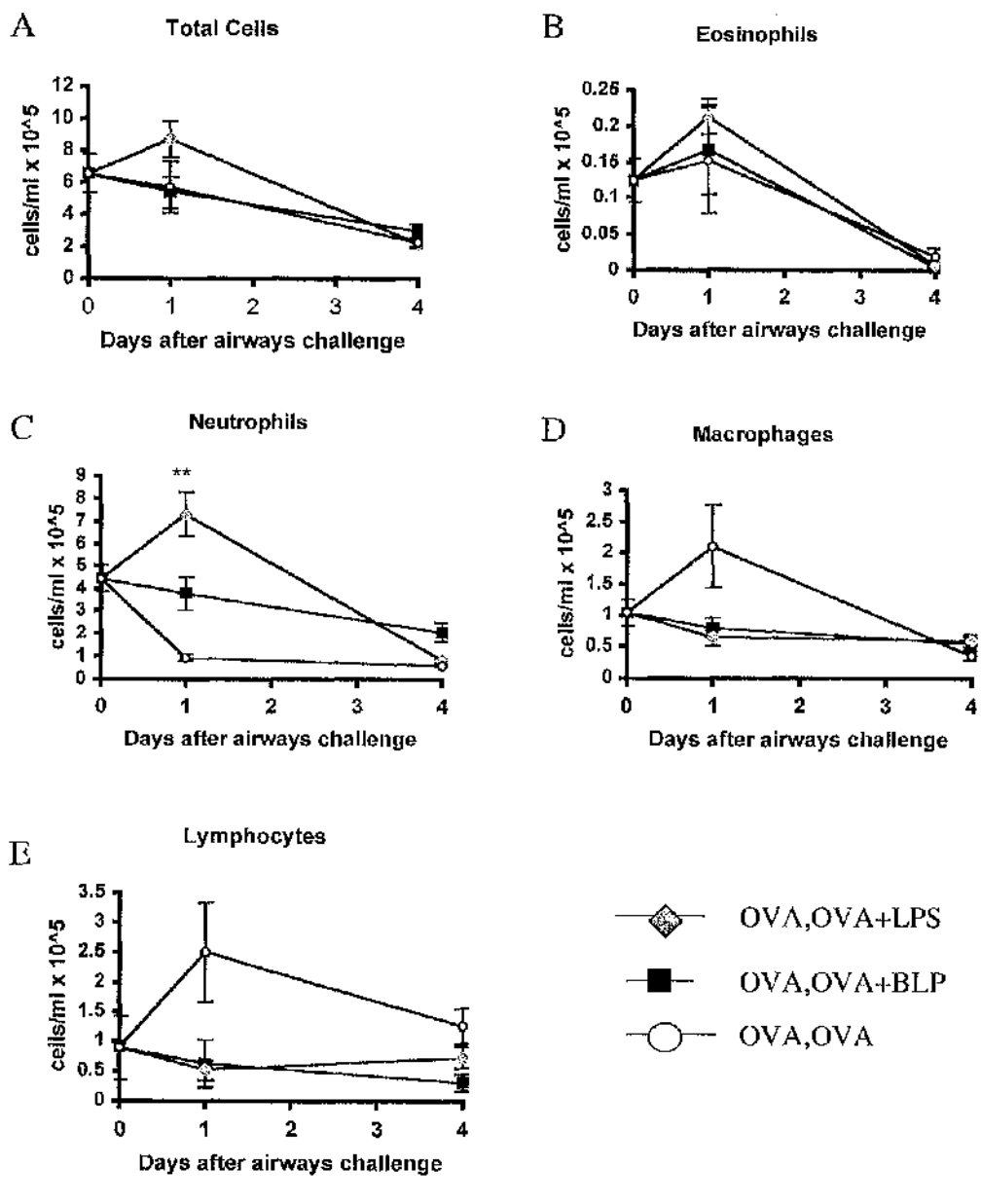

Figure 4.11: Effect of systemic LPS or BLP on peritoneal washes

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge seven

days later with 50μg OVA. Mice were then challenged with LPS or BLP by i.p. injection two hours later. Fourteen days later mice received an intranasal challenge with 50μg OVA (A). After sacrifice, peritoneal lavage was performed on each mouse and the cell content analysed by cytospin preparation. Graphs show: total cells (A), eosinophils (B), neutrophils (C), macrophages (D) and lymphocytes (E). Values are represented as mean ± SEM, n=5 per group, n= 1 repeat (*$p<0.05$).

CHAPTER 5 EFFECT OF TNFα BLOCKADE ON AIRWAYS INFLAMMATION

5.1 Introduction

Anti-TNFα therapy was the first of a new class of drugs called biologic therapies, mostly monoclonal antibodies and soluble receptors, which were designed to target specifically critical points in pathways, with the aim of producing potent but precise effects on disease pathophysiology. TNFα blockade was initially directed against the synovial inflammation seen in rheumatoid arthritis [448] but has found a role in several other rheumatic diseases as well as inflammatory conditions such as inflammatory bowel disease and psoriasis [449, 450]. Despite much research into the immunological mechanisms underlying asthma, relatively few biologic therapies have been investigated in asthma: notably, anti-IL-5 and anti-CD4 agents have not shown great benefit in clinical trials [141, 142, 156, 157] and anti-IgE mAb is only now beginning to enter clinical practice as an adjunct to standard treatment in patients with atopic asthma and evidence of sensitisation to perennial allergens [220, 221, 451].

TNFα is a potent pro-inflammatory cytokine with immunoregulatory activities, and is produced by many cell types including monocytes, macrophages, lymphocytes, neutrophils, eosinophils, and mast cells [283, 290, 452]. In the lung, TNFα is synthesised and stored mainly in mast cells and alveolar macrophages [300]. It functions as a chemoattractant for neutrophils and monocytes, increases microvascular permeability, and activates T cells, eosinophils, and mast cells. Furthermore, there is increased expression of TNFα in the airway of asthmatic patients compared to normal subjects [300, 301]. Mast cells are unique in being the only tissue-resident cells with granules containing preformed TNFα and consequently during the early stages of inflammation or infection they may be the sole

source of this cytokine [302]. TNFα is able to stimulate the production of IL-8, regulated on activation normal T expressed and secreted (RANTES) and granulocyte macrophage colony stimulating factor (GM-CSF) by airway epithelial cells, which increases the expression of vascular adhesion molecules such as intercellular cell adhesion molecule 1(ICAM-1) and vascular adhesion molecule 1 (VCAM-1), which are involved in the recruitment of inflammatory cells to the airways [303]. TNFα is also thought to be involved in airway remodelling through its effects on fibroblasts and by inducing eosinophils to release matrix metalloproteinase 9 [304]. Furthermore, experimental administration of TNF to the airways can induce AHR [453, 454].

Data such as these have been sufficient to initiate a number of small trials of anti-TNF therapy in human asthmatics. Of these, one has shown no benefit [455], whereas two more recent trials, both looking at severe asthmatics, show that there is some improvement in lung function with treatment [277]. However, these have been small studies and large-scale double-blind randomised control trials are awaited.

However, despite the trials of these therapies, the mechanisms of action of anti-TNFα therapies remain unclear. A novel mode of action is suggested by McLachlan and colleagues which emphasises the link between mast cells, TNFα and the regulation of inflammation and immunity [305, 456]. They demonstrated in a murine model, that injection of *E. coli* into the footpad led to hypertrophy of the popliteal (draining) lymph node, attributable to an influx of T and B lymphocytes and associated with mast cell degranulation in the footpad. Mast cell deficient mice (W/W^v) mice showed significantly

reduced lymph node swelling which was reversed by reconstitution of the mice with wild-type mast cells, but not with TNFα-deficient mast cells. As lymph nodes are the central inductive site of the adaptive immune response, facilitating the interaction of T cells and APCs, such effects could be significant in a wide range of conditions including infection, autoimmune conditions and allergies where there is immune activation.

To examine the effects of TNFα blockade on airways inflammation, I will use the adoptive transfer model described in chapter 3. The aim of this chapter is to examine the effects of anti-TNFα given systemically on airways inflammation and to elucidate the mechanism underlying the response. Our investigations show that TNFα blockade during either the induction or effector phases caused no reduction in pathology associated with the disease. However, blockade during the induction phase prevented lymph node hypertrophy. When TNFα blockade was administered throughout the induction and effector phases of disease, lymph node hypertrophy and lung pathology were reduced, although the reduction in BAL eosinophilia was not significant. This was associated with a reduction in BAL IL-5 production, showing that anti-TNFα treatment is needed throughout the course of the disease to reduce inflammation. When anti-TNFα treatment was administered in a more acute model of airways inflammation, the result was more pronounced, showing a reduction in BAL eosinophilia, pathology, lymph node hypertrophy, BAL IL-5 production and IgE production. When L-selectin expression was assessed on antigen-specific T cells it was found that the draining lymph nodes and the lung tissue had increased expression in both anti-TNFα and control mice. These data suggest that although anti-TNFα treatment affects

the accumulation of T cells in draining lymph nodes, it does not mediate its effects via the expression of L-selectin. Thus anti-TNFα may be useful in severe and acute asthma.

5.2 Results

In chapter 3 a model of allergic airways inflammation was established, in which adoptive transfer of Ag-specific T cells was followed by three immunisations, seven days apart and a single OVA airways challenge (Figure 3.4 A). This model will be referred to as the "long model". In chapter 4 a similar model was adapted, in which adoptive transfer of Ag-specific T cells was followed by a single immunisation, followed by a single OVA plus LPS airways challenge ten days later (Figure 4.5 A). This model will be referred to as the "short model". These models will be used to investigate the effects of anti-TNFα treatment on airways inflammation.

It has been shown that TNFα may regulate hypertrophy of lymph nodes following infection via an effect on T cells [305]. If TNFα is important in the lymph node response, then blocking its action during the induction phase would suppress the initial primary immune response. However, a later effect of TNFα on T cell effector function cannot be ruled out, therefore it is important to assess the effects of TNFα blockade during the effector phase of the disease. To determine if TNFα blockade had an effect on the induction or effector phase of the long model, anti-TNFα treatment was given at a dose of 50mg/kg (previously optimised dose: personal communication by Simon Cruwys, Astra Zeneca) by i.p. injection the day before and two days after OVA/ALUM immunisation (Figure 5.1 A).

5.2.1 Effect on TNFα blockade during induction or effector phase of airway inflammation.

To determine if anti-TNFα treatment affected lymph node hypertrophy when given during the induction or effector phase of the long model, the draining and non-draining lymph nodes were removed and weighed. When TNFα was blocked during the induction phase, the draining (mediastinal) lymph node did not increase in weight as was seen in the control group (Figure 5.1 B). However, this difference was only seen day seven post airways challenge. This difference was not seen when TNFα was blocked during the effector phase (Figure 5.1 C). No difference was seen in the non-draining (inguinal) lymph nodes when treated with anti-TNFα during the induction or the effector phase (Figure 5.1 D and E). When the cellularity of the lymph nodes was determined, no significant differences were seen between the two groups after any of the treatment regimes (Figure 5.2). The increase in weight of the LN was not accompanied by any changes in cellularity. This may be explained by oedema, which would increase the weight of the lymph node but not the cellularity.

When cell infiltrates were analysed in the BAL fluid it was found that there was no significant difference in BAL eosinophils or neutrophils when TNFα was blocked during the induction or the effector phase of the model (Figure 5.3 A to D). Consistent with this result, there were no significant differences in lung pathology with either treatment regime (Figure 5.3 E and F).

The use of OVA-specific TcR Tg T cells in our airway inflammation model allows the kinetic analysis of antigen-specific T cells as the disease progresses. Lymph nodes and BAL fluid were analysed by flow cytometry for KJ1.26$^+$ T cells. There were no significant

differences in numbers of antigen-specific T cells recruited to the draining or non-draining lymph nodes, or to the BAL fluid when TNFα was blocked during either the induction or effector response (data not shown).

These results suggest that TNFα blockade during the induction phase of OVA-induced airway inflammation is able to stop lymph node hypertrophy in the draining lymph node but has no other significant effects on inflammation in the induction or effector phase.

5.2.2 Treatment with anti-TNFα causes a significant reduction lung pathology and reduction in BAL eosinophilia

As the previous results did not show any effect on the pathology of airway inflammation, the experiment was repeated with TNFα being blocked throughout both the induction and effector phase of the long model (Figure 5.4 A).

TNFα blockade throughout the long model appeared to cause an overall reduction in BAL eosinophilia compared to control mice (Figure 5.4 B). However, this figure was not statistically significant. Histological analysis of inflammation in lung sections showed that anti-TNFα treatment produced a significant reduction in lung pathology compared to the control group, consistent with the possible decrease in eosinophilia seen in the BAL fluid (Figure 5.4 C). Decreased cell infiltrate and inflammation in anti-TNFα treated mice can be seen in H&E stained sections, compared to the control group (Figure 5.4 D-G). This suggests that TNFα blockade can have an effect on inflammation when blocked throughout the course of the disease, possibly not solely through an effect on eosinophilia.

5.2.3 Anti-TNFα treatment prevents hypertrophy of the draining lymph nodes of the lung.

The draining lymph nodes are important in adaptive immune responses and are known to accumulate large numbers of circulating lymphocytes following infection [457]. This increase in lymph node size is important for the interaction of T cells and APCs and further induction of immune responses [73]. To determine if TNFα blockade has any effect on lymph node hypertrophy the mice were treated as described above and draining and non-draining lymph nodes were weighed and cellularity determined after airway challenge. After anti-TNFα treatment the draining lymph node did not increase in weight, whereas those from the control group did show an increase in weight. (Figure 5.5 A). Non-draining lymph nodes from both the etanercept and the control group showed an equal increase in weight (Figure 5.5 B). The cellularity of the draining lymph nodes and the non-draining lymph nodes was determined. In the draining lymph nodes, the increase in cellularity observed in control mice was decreased with anti-TNFα treatment (Figure 5.5 C). There was no difference in cellularity between the two treatment groups in the non- draining lymph nodes (Figure 5.5 D). This suggests that blockade of TNFα may have an effect on cell movement to the draining lymph node and this decrease in cell number may account for the draining lymph nodes not increasing in weight as seen in the control group. However, analysis of antigen-specific T cells by flow cytometry showed no difference in cell number between groups (Figure 5.5 E and F).

5.2.4 BAL cytokine and serum antibody responses after airways challenge.

BAL fluid was collected and analysed for the presence of IL-5 and IFNγ. IL-5 was significantly reduced in anti-TNFα treated mice compared to the control group, which correlates with the reduction in eosinophilia observed in the BAL fluid (Figure 5.6 A). IFNγ production could not be detected in either of the groups (data not shown). Serum was assessed for OVA-specific antibody production. There was no significant difference between OVA-specific IgE in the anti-TNFα group and the control group (Figure 5.6 B).

5.2.5 Effect of etanercept in the short model.

Anti-TNFα drugs are now being considered as a possible therapy for asthma and recent clinical trials have shown beneficial effects in asthmatic patients [458, 459]. These studies looked at severe asthmatics, characterised by chronic airways inflammation, and high neutrophils numbers in the airways [415, 416, 460]. The short model characterised in chapter 4, where one immunisation and one airways challenge with OVA plus LPS may be more representative of chronic airways inflammation as the eosinophilia is accompanied by a marked neutrophil influx. Furthermore, when OVA-specific T cells were analysed by RT-PCR (as described in chapter 3) it was found that they expressed a high level of TNFα compared to any of the other groups in the short or long model. For these reasons, it was decided to determine if anti-TNFα would have a more pronounced effect in this model.

5.2.6 Etanercept treatment in the short model reduced BAL eosinophilia, DLN weight and cellularity.

When anti-TNFα treatment was administered throughout the short model (Figure 5.7 A), it resulted in a significant decrease in eosinophilia compared to the control group (Figure 5.7 B). There was no difference observed in neutrophil number between the two groups (Figure 5.7 C). When DLN were removed and weighed it was found that anti-TNFα treated mice did not show an increase in weight as seen in the control group (Figure 5.8 A). There was no difference in weight between the two groups in the non-DLN (Figure 5.8 B). The same trend was seen when cellularity was determined, showing that there were significantly more cells in the control group of the DLN (Figure 5.8 C) and no difference between the two groups in the non-DLN (Figure 5.8 D). However, when antigen-specific T cells were analysed by flow cytometry, no difference was seen between the groups in the DLN, non-DLN or the BAL (Figure 5.9). These results show that etanercept treatment throughout the short model is able to reduce eosinophilia, which was not seen in the long model, and DLN weight and cellularity, in a T cell-independent manner.

5.2.7 Effect of etanercept on cytokine and antibody production in the short model.

BAL fluid was collected and analysed for the presence of IL-5 and IFNγ. IL-5 was significantly reduced in the etanercept treated group compared with the control group (Figure 5.10 A). IFNγ could not be detected in either group. Serum was analysed for OVA-specific antibody production. There was a significant reduction in IgE production at day

four post airways challenge in the etanercept treated group compared to the control group (Figure 5.10 B).

Overall, anti-TNFα treatment in the short model caused a significant reduction in BAL eosinophilia, IL-5 production, DLN weight and cellularity and OVA-specific IgE production compared with the control group, independent of T cell number. The reduction in eosinophilia and IgE production was not seen in the long model. These results are consistent with clinical trials in which anti-TNFα had been shown to be more effective in patients with severe asthma (short model), than mild to moderate asthma (long model)suggesting that anti-TNFα therapy is beneficial in airways inflammation.

Despite these results, it is still unclear as to how anti-TNFα therapies actually work. These results suggest that etanercept may be affecting the migration of cells to the DLN, causing a reduction in weight and cellularity compared with the control group. Adhesion molecules are known to play vital roles in numerous cell processes, including cell transmigration and response. T lymphocytes migrate from circulation to the lung to initiate and propagate airways inflammation. The adhesion molecules mediating lymphocyte influx into the airways during an allergic response have been poorly defined. Thus L-selectin expression, which has been shown to be important in access to lymph nodes [461], was measured on antigen-specific T cells in the short model after anti-TNFα treatment.

5.2.8 L-selectin expression on antigen specific T cells.

The short model was run as described previously (Figure 5.7 A), with etanercept treatment throughout. Antigen-specific T cells in DLN, non-DLN and lung tissue were assessed by flow cytometry for the expression of L-selectin low (effector) and L-selectin high (naïve) cells. There was significantly increased KJ1.26 $^{+}$, L-selectin^{+} low cells at day four post challenge in both etanercept and control mice in the DLN (Figure 5.11 A). There was no difference in L-selectin expression between the anti-TNFα treated mice and the control mice in non-DLN (Figure 5.11 B). In the lung tissue there were increases in the number of L-selectin low expressing T cells at day one post challenge in anti-TNFα and control groups and as seen in the DLN, there was significantly increased KJ1.26 $^{+}$, L-selectin^{+} low cells at four post challenge in mice treated with etanercept and the control mice (Figure 5.11 C).

5.3 Discussion

TNFα is a major therapeutic target in a range of chronic inflammatory diseases e.g. rheumatoid arthritis, ankylosing spondylitis, and Crohn's disease [462-464]. These diseases are mostly Th1-type diseases associated with increased TNFα production. Genetic studies have also shown an association between TNFα, AHR and asthma [465] and murine studies have shown that inhalation of TNFα leads to AHR [466]. Many cells are able to produce TNFα, but those most relevant to asthma are T cells, monocytes, macrophages, mast cells, eosinophils and epithelial cells. These cells are involved in tissue remodelling, epithelial cell barrier permeability, recruitment of inflammatory infiltrates to the site of inflammation and upregulation of adhesion molecules [467]. Anti-TNFα drugs are now being considered as a possible therapy for asthma and recent clinical trials have shown beneficial effects in asthmatic patients [458, 459] .

Even though asthma is a Th2 disease characterised by eosinophils, recent interest has been shown in TNFα as a therapy for asthma. Studies that have looked at TNFα inhibition in animal models have shown a suppressive effect on eosinophils [468-471], however one study using TNFα receptor knockout mice did not [471]. The differences in results observed might be due to the TNFα inhibition employed or the models used. Most of these studies have used TNFα receptor knockout mice or anti-TNF antibodies, unlike etanercept, which binds to and functionally inhibits soluble receptors [468-471]. Clinical studies using differing strategies of TNFα blockade have shown differential effects in diseases, where anti-TNF antibody but not soluble receptor is beneficial in Crohn's disease [450, 472].

The experiments in this chapter have investigated a role for TNFα in the treatment of allergic airways inflammation. The airways model can be considered in two phases: the sensitisation phase, where the mouse is immunised with the OVA antigen, developing a systemic immunological response, and the effector or challenge phase, where rechallenge with the Ag via the airways leads to eosinophilic inflammation. The effect of etanercept treatment may depend on the phase in which it is administered. If TNFα were important in the lymph node response to antigen then it would seem plausible that blocking its action during the sensitisation phase would suppress the primary immune response to OVA. To examine this, etanercept was given either in the sensitisation or effector phases.

When anti-TNFα was administered during the induction phase of the model it prevented lymph node swelling. However, this was not accompanied by any reduction in eosinophilia or pathology. Similarly, in experiments where anti-TNFα was given during the effector phase only, no significant effect on lung inflammation, eosinophilia or lymph node size was observed. This suggests that although anti-TNFα treatment during induction was able to reduce lymph node swelling, the effect may be independent of the mechanisms leading to eosinophilic inflammation. Anti-TNFα treatment throughout the long model of airways inflammation (possibly representative of mild to moderate asthma) prevented hypertrophy of the draining lymph nodes of the lung. This was accompanied by a decrease in BAL eosinophilia (although not statistically significant) and IL-5 production, and a reduction in lung pathology. This data is representative of several murine and human studies that show administration of anti-TNFα leads to the amelioration of BAL eosinophilia and associated Th2 cytokines [277, 455, 466, 470].

However, recent clinical trials have shown beneficial effects of TNF blockade in patients with severe asthma, a clinical phenotype which is characterised by chronic airways inflammation, relative corticosteroid-resistance and a tendency towards high numbers of neutrophils in the airways [415, 416, 460]. It seems likely that the acute model used here, in which inhaled allergen results in a predominantly eosinophilic infiltrate, does not represent the pathophysiological changes ongoing in this subgroup of patients. Indeed, in a clinical trial of mild-moderate asthmatics, whose disease the murine model is more likely to resemble, no improvement in airway eosinophils or AHR was found [455].

In chapter 3 it was demonstrated that the short model, which has an airways challenge of OVA plus LPS significantly worsened airways inflammation, not only in terms of eosinophils but also neutrophils. Although this does not resemble all aspects of severe asthma (the model provides an acute, as opposed to chronic inflammation and the neutrophilia is transient) it may provide the basis for further studies to investigate how therapeutic agents such as etanercept may work. Furthermore, when OVA-specific T cells were analysed by RT-PCR (as described in chapter 3) it was found that they expressed a high level of TNFα compared to any of the other groups in the short or long model. Therefore it was hypothesised that anti-TNFα treatment may have a more profound effect in this model.

As hypothesised, when anti-TNFα was administered throughout the short model it caused a significant reduction in BAL eosinophilia, IL-5 and antigen-specific IgE production. This was accompanied by a decrease in DLN weight and cellularity. This data correlates with the

recent studies suggesting that anti-TNFα therapies are more beneficial in severe asthmatics compared to patients that exhibit mild or moderate symptoms [415, 416, 460].

One mode of action by which TNFα may be exerting its effects is by blocking lymph node hypertrophy. It is known that naïve T cells continually enter and exit lymphoid organs in a recirculation process that is essential for immune surveillance. During an immune response the egress process can be shut down transiently [474]. When this occurs locally it increases lymphocyte numbers in the responding lymphoid organ. Several mediators of the innate immune system are known to cause shutdown, including TNF α, although the mechanisms are still unclear [475-477]. TNFα has also been shown to be capable of altering the number and specificity of lymphocytes recirculating through stimulated lymph nodes by selectively altering the entry of lymphocytes into the efferent lymphatics of inflamed lymph nodes *in vivo* [478-480]. This would correlate with the decrease in cellularity and weight observed in the draining lymph nodes of anti-TNFα treated mice, suggesting that blocking TNFα allows cells to leave the lymph node instead of being trapped and initiating a subsequent immune response.

Lymphocyte trafficking is not a random event as although naïve T cells appear to circulate without preference for any one group of lymph nodes, memory T cells preferentially return to the tissues associated with the lymph node groups in which they became sensitised: as process known as lymphocyte homing [481]. This process is controlled by adhesion receptors on the lymphocyte binding to vascular addressins selectively expressed on the endothelium [482, 483]. L-selectin is expressed on most lymphocytes and is the key

receptor that initiates lymphocyte capture events in high endothelial venules in secondary lymphoid organs and at peripheral sites of injury and inflammation [461]. L-selectin has been shown to mediate arteriolar and venular rolling on inflamed pulmonary endothelium [484-486], and has been shown to be important in lymphocyte migration during an allergic inflammatory response [487]. Most studies demonstrate the absence of L-selectin results in reduced inflammation [488-491]. However, some studies have shown that L-selectin is required for the development of airway hyperresponsiveness but not airway inflammation in murine models of asthma [492].

To determine if anti-TNFα treatment was affecting the expression of L-selectin and therefore lymphocyte migration, its expression was measured on antigen-specific T cells from DLN, non-DLN and lung tissue. When L-selectin expression was measured in the DLN and the lung it was found that both the etanercept treated mice and the control mice showed increased expression after airways challenge. When L-selectin levels were measured in the non-DLN there was no difference between the groups and expression was not changed after administration of airways antigen. This data suggests that anti-TNFα may be affecting the migration of cells to the DLN in an L-selectin independent manner. It has also been observed that 50,000 L-selectin molecules per cell are required for efficient rolling in lymph node HEVs [493], therefore the presence of L-selectin on lymphocytes may not predict their potential to home to lymph nodes [494].

Overall, these results have shown that when anti-TNFα treatment was administered during the induction phase of the long model it prevented lymph node swelling. However, this was not accompanied by any reduction in eosinophilia or pathology. Similarly, in experiments where anti-TNFα was given during the effector phase only, no significant effect on lung inflammation, eosinophilia or lymph node size was observed. This suggests that although anti-TNFα treatment during induction was able to reduce lymph node swelling, the effect may be independent of the mechanisms leading to eosinophilic inflammation. Blocking TNFα throughout both the induction and effector phases of the long model showed effects of anti-TNFα in reducing overall lung inflammation and preventing lymph node hypertrophy in an antigen-induced model of airway inflammation, with a less pronounced effect on lung eosinophilia. In the short model, TNFα blockade throughout caused a reduction in BAL eosinophilia and associated pathology, and DLN weight and cellularity did not increase compared to control mice.

The data presented in this chapter suggests a novel mechanism of action of anti-TNFα therapy in immune diseases, by inhibiting lymph node responses in an L-selectin independent manner. A more pronounced reduction in lung inflammation and eosinophilia was seen in the short model, which may be more representative of a sever asthmatic response correlates with recent studies stating that anti-TNFα therapies are more effective in this group of asthmatic patients.

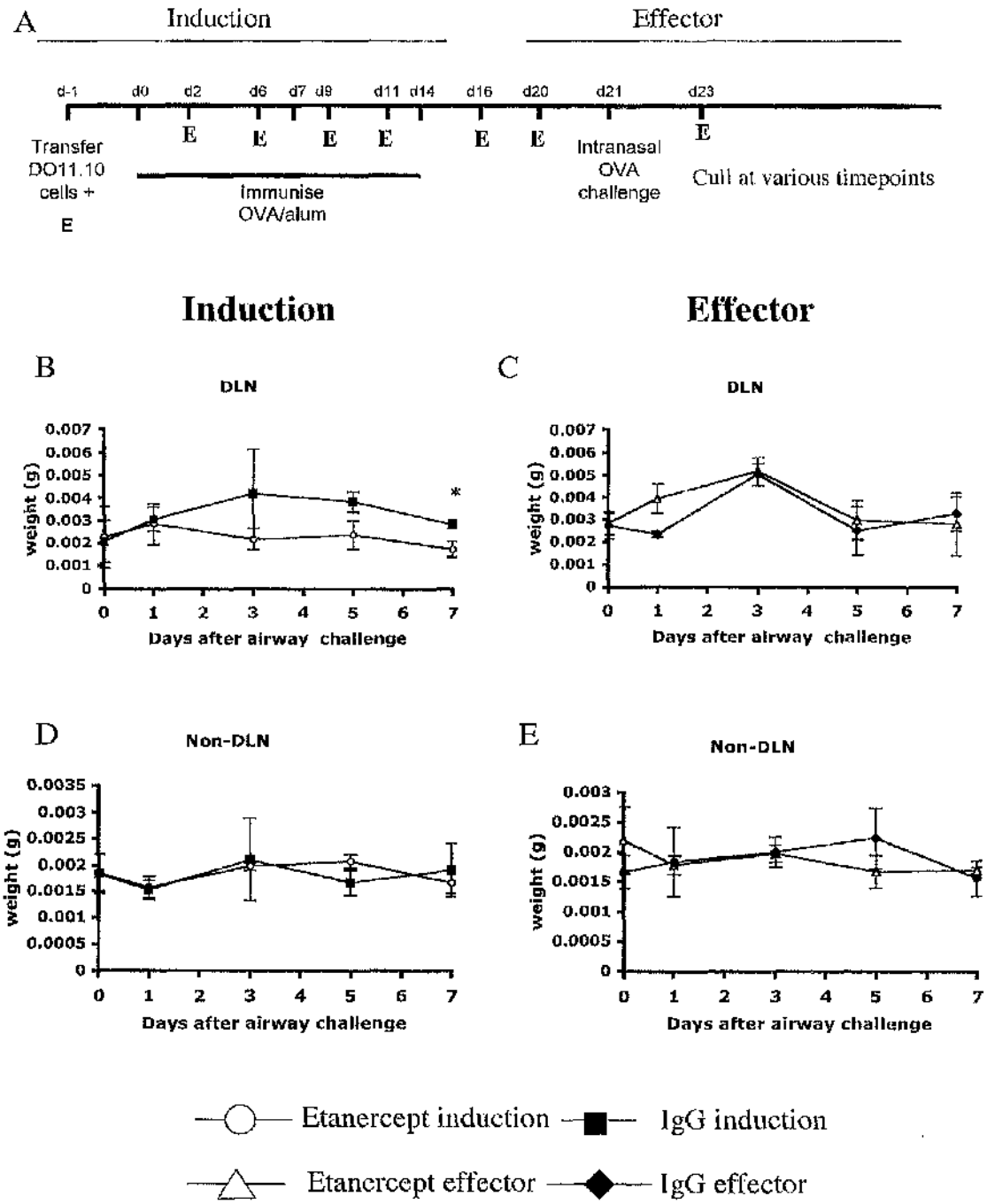

Figure 5.1: Effect of etanercept on lymph node weights

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100μg OVA and 1% alum by i.p. injection on three occasions, each a week apart. One week after the final immunisation, mice were challenged by intranasal administration of 50μg OVA. Mice were treated during the induction phase with 50mg/kg etanercept (E on timeline) by i.p. injection one day before and two days after each immunisation. In other experiments etanercept were treated with etanercept during the effector phase (A). Control mice were given 50mg/kg human IgG instead of etanercept during the induction or effector phase. Draining lymph nodes (B and C) and non-draining lymph nodes (D and E) were removed and weighed. Results are expressed as mean ± SEM (* $P<0.05$), n=3-4 per group, n= 1 repeat.

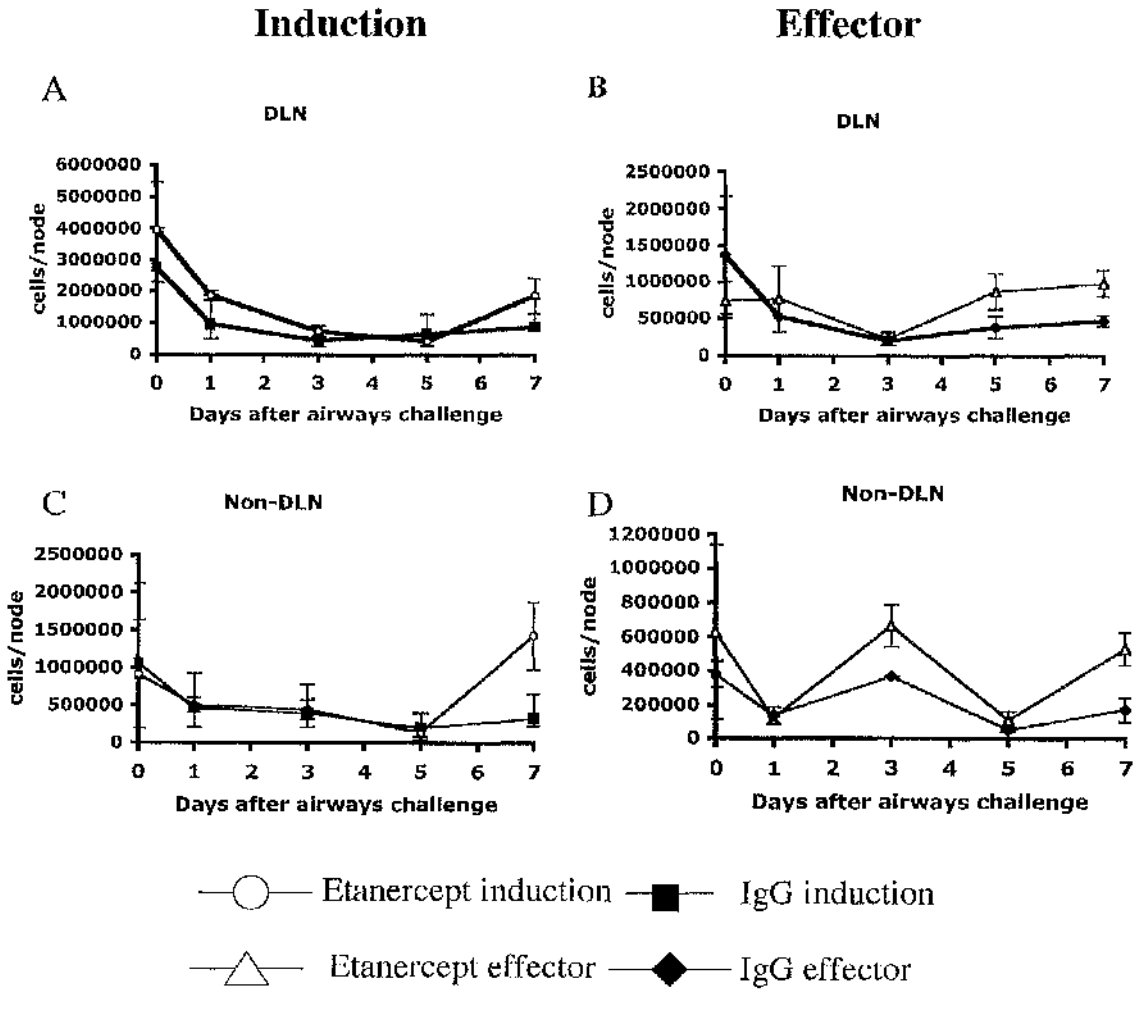

Figure 5.2: Effect of etanercept on lymph node cellularity

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100μg OVA and 1% alum by i.p. injection on three occasions, each a week apart. One week after the final immunisation, mice were challenged by intranasal administration of 50μg OVA. Mice were treated during the induction phase with 50mg/kg etanercept by i.p. injection one day before and two days after each immunisation. In other experiments etanercept were treated with etanercept during the effector phase. Control mice were given 50mg/kg human IgG instead of etanercept during the induction or

effector phase. Draining lymph nodes (A and B) and non-draining lymph nodes (C and D) were removed and cellularity was determined. Results are expressed as mean ± SEM, n=3-4 per group, n= 1 repeat.

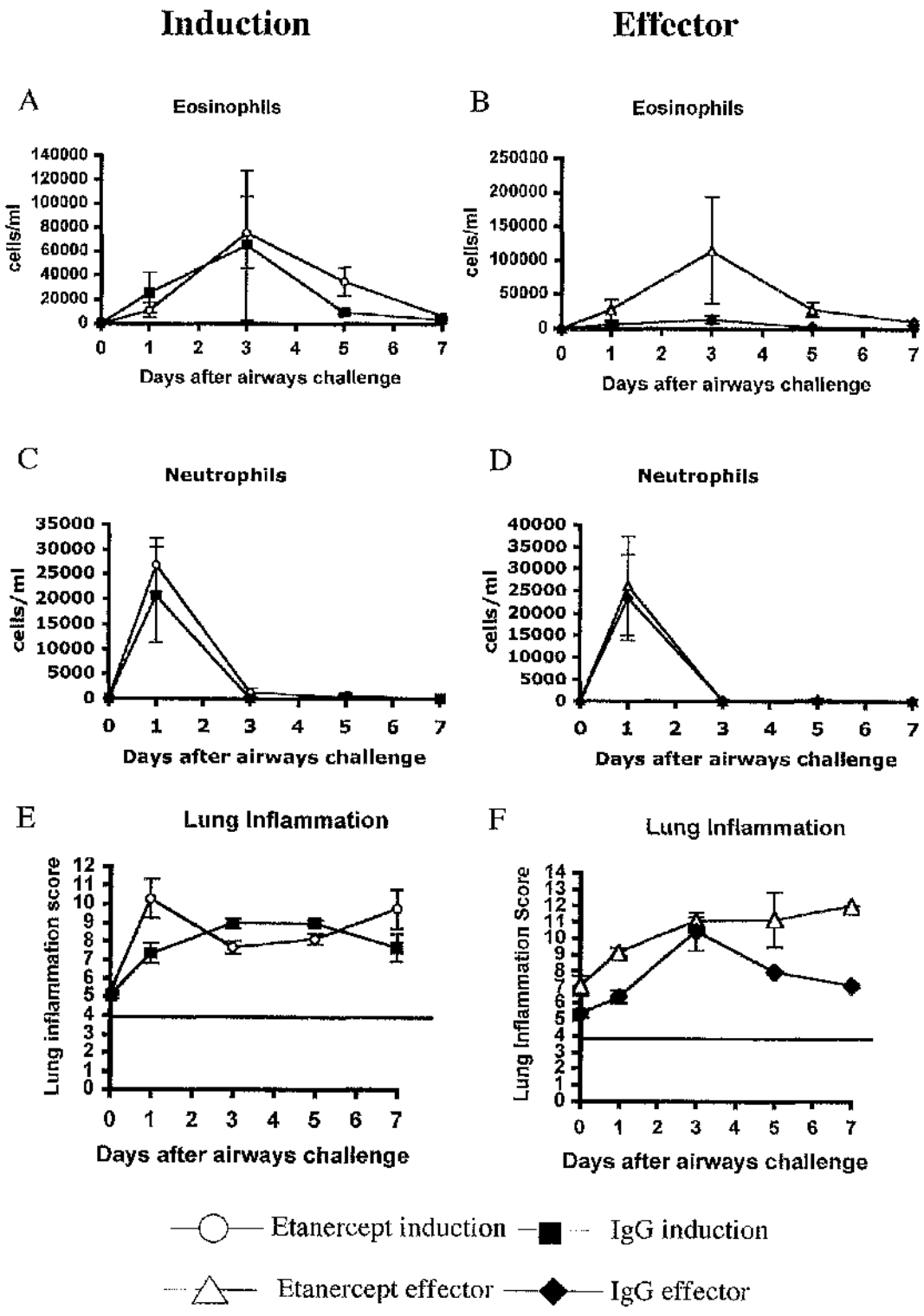
Induction
Effector
A
Eosinophils
cells/ml
140000
120000
100000
80000
60000
40000
20000
0
0 1 2 3 4 5 6 7
Days after airways challenge
B
Eosinophils
cells/ml
250000
200000
150000
100000
50000
0
0 1 2 3 4 5 6 7
Days after airways challenge
C
Neutrophils
cells/ml
35000
30000
25000
20000
15000
10000
5000
0
0 1 2 3 4 5 6 7
Days after airways challenge
D
Neutrophils
cells/ml
40000
35000
30000
25000
20000
15000
10000
5000
0
0 1 2 3 4 5 6 7
Days after airways challenge
E
Lung Inflammation
Lung inflammation score
12
11
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7
Days after airways challenge
F
Lung Inflammation
Lung Inflammation Score
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7
Days after airways challenge
Etanercept induction
IgG induction
Etanercept effector
IgG effector

Figure 5.3: Etanercept treatment does not affect lung inflammation

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100μg OVA and 1% alum by i.p. injection on three occasions, each a week apart. One week after the final immunisation, mice were challenged by intranasal administration of 50μg OVA. Mice were treated during the induction phase with 50mg/kg etanercept by i.p. injection one day before and two days after each immunisation. In other experiments etanercept were treated with etanercept during the effector phase. Control mice were given 50mg/kg human IgG instead of etanercept during the induction or effector phase. BAL fluid was sampled and eosinophils (A and B), and neutrophils (C and D) were analysed by cytospin preparation. Lung sections were harvested and analysed for pathology (E and F). Baseline inflammation represented by solid black line. Results are expressed as mean ± SEM, n=3-4 per group, n= 1 repeat.

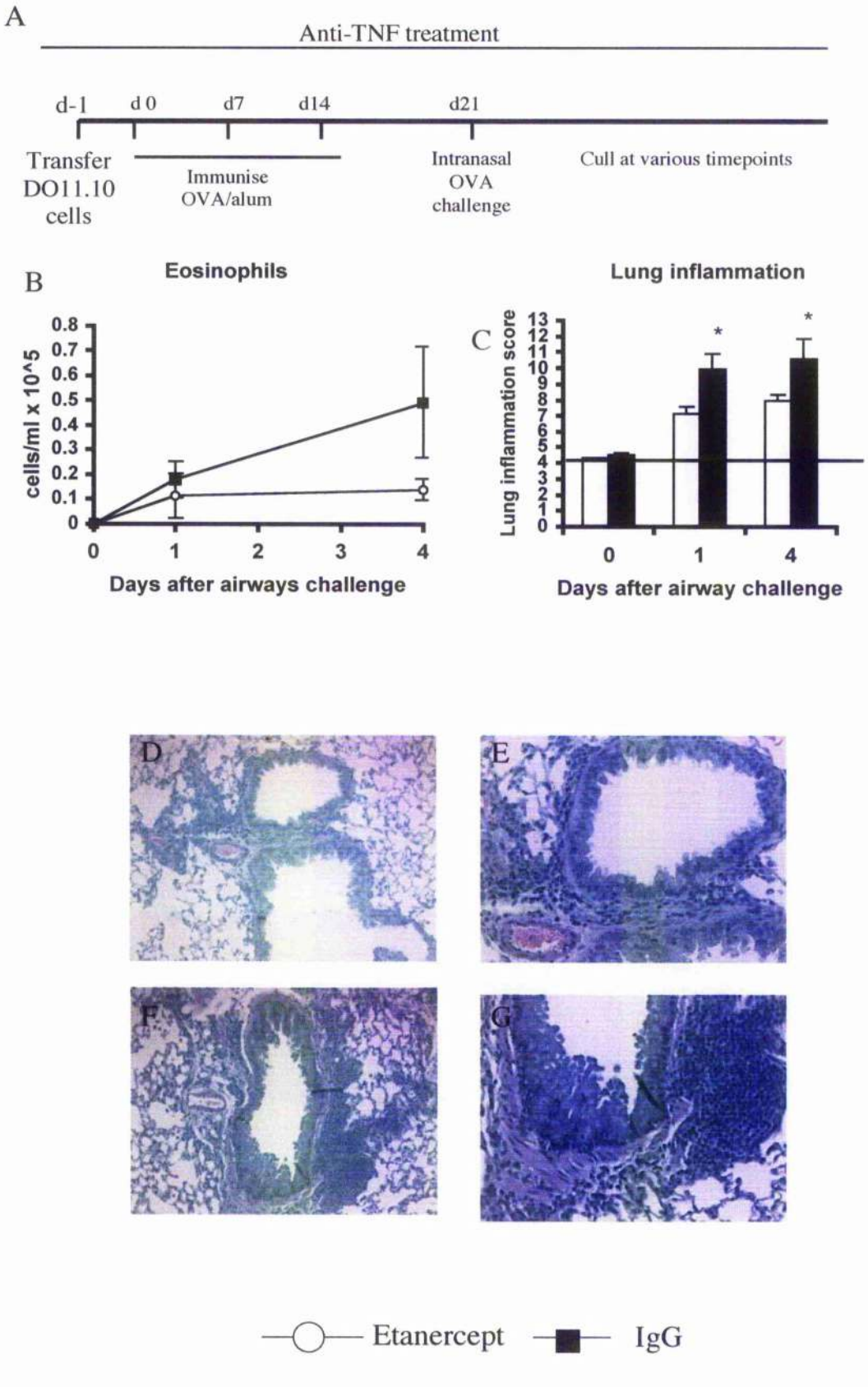
A
Anti-TNF treatment
d-1
d 0
d7
d14
d21
Transfer DO11.10 cells
Immunise OVA/alum
Intranasal OVA challenge
Cull at various timepoints
B
Eosinophils
cells/ml x 10^5
Days after airways challenge
C
Lung inflammation
Lung inflammation score
Days after airway challenge
D
E
F
G
Etanercept
IgG

Figure 5.4: Etanercept treatment throughout reduces lung pathology

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100μg OVA and 1% alum by i.p. injection on three occasions, each a week apart. One week after the final immunisation, mice were challenged by intranasal administration of 50μg OVA. Mice were treated throughout with 50mg/kg etanercept by s.c. injection every two days. Control mice were given 50mg/kg human IgG instead of etanercept (A). BAL fluid was sampled and eosinophil content was analysed by cytospin preparation (B). Lung sections were scored for inflammation (C). Lung sections were stained with H&E. Sections show anti-TNFα (D,E) and IgG (F,G) treated mice at day 4 post airways challenge at 10x and 20x magnification. Results are represented as mean ± SEM (* $p< 0.05$), n=3-6 per group, n= 2 repeats.

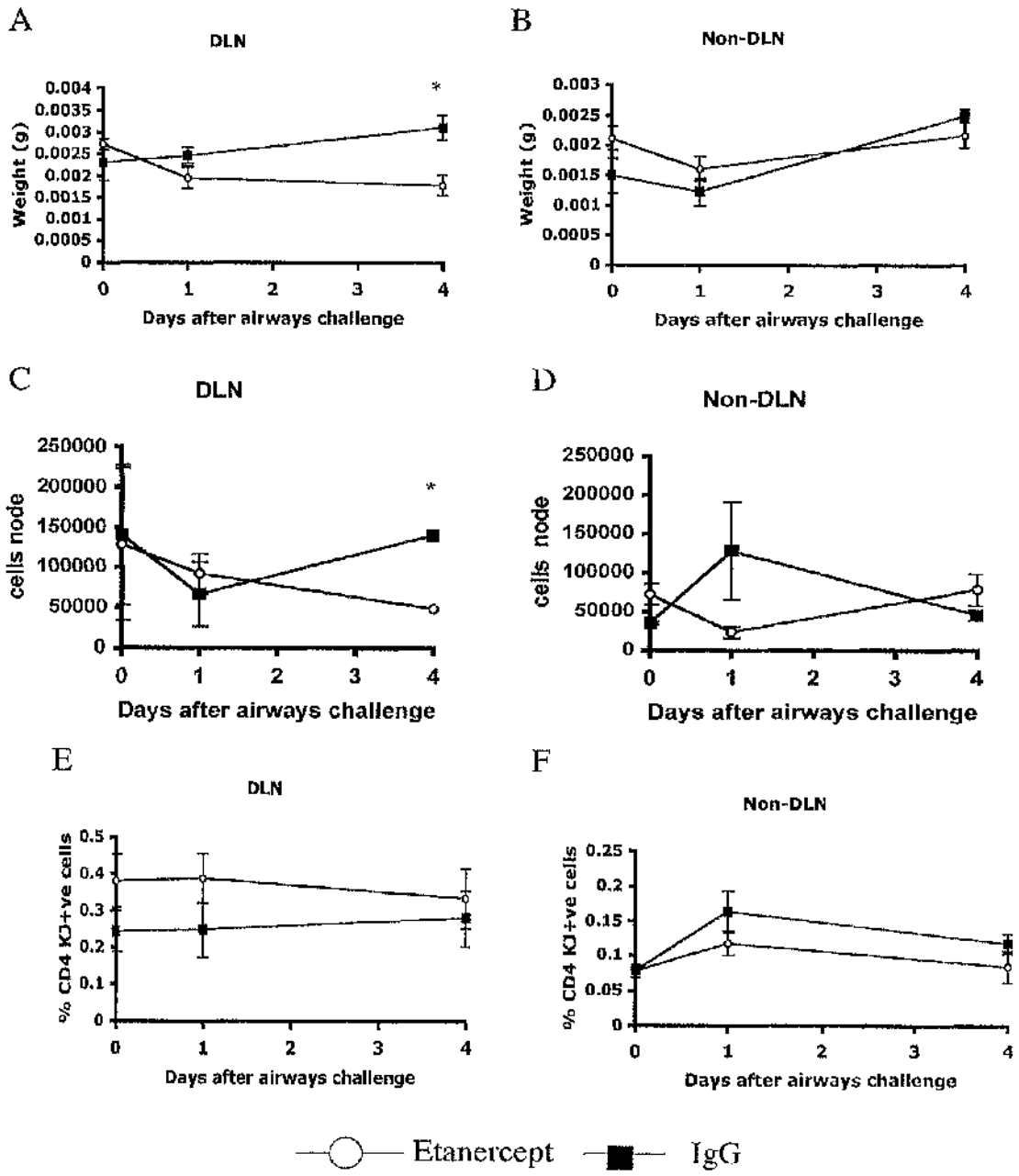

Figure 5.5: Etanercept treatment throughout reduces DLN weight and cellularity.

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100μg OVA and 1% alum by i.p. injection on three occasions, each a week apart. One week after the final immunisation, mice were challenged by

intranasal administration of 50µg OVA. Mice were treated throughout with 50mg/kg etanercept by s.c. injection every two days. Control mice were given 50mg/kg human IgG instead of etanercept. Draining and non-draining lymph nodes were removed and weighed (A and B) and cellularity was determined (C and D). OVA-specific T cell numbers were determined by flow cytometry (E and F). Results are represented as mean ± SEM (* $p < 0.05$), n=6 per group, n= 2 repeats.

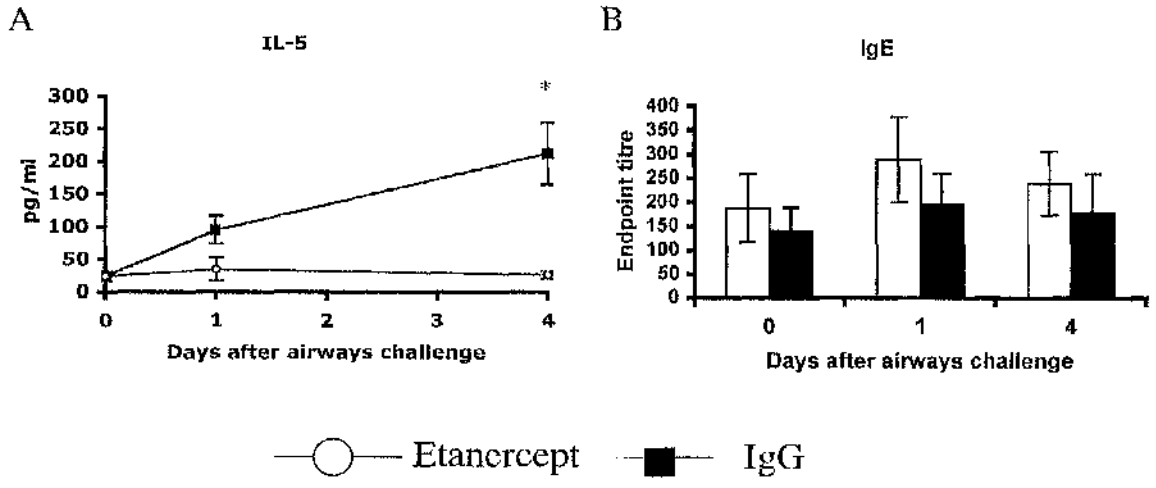

Figure 5.6: Etanercept reduces IL-5 production

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100μg OVA and 1% alum by i.p. injection on three occasions, each a week apart. One week after the final immunisation, mice were challenged by intranasal administration of 50μg OVA. Mice were treated throughout with 50mg/kg etanercept by s.c. injection every two days. Control mice were given 50mg/kg human IgG instead of etanercept. BAL fluid was sampled and analysed for IL-5 (A). Serum from the same mice was removed and analysed by ELISA for Ag-specific IgE (B). Results are represented as mean ± SEM (* $p< 0.001$), n=3-6 per group, n= 2 repeats.

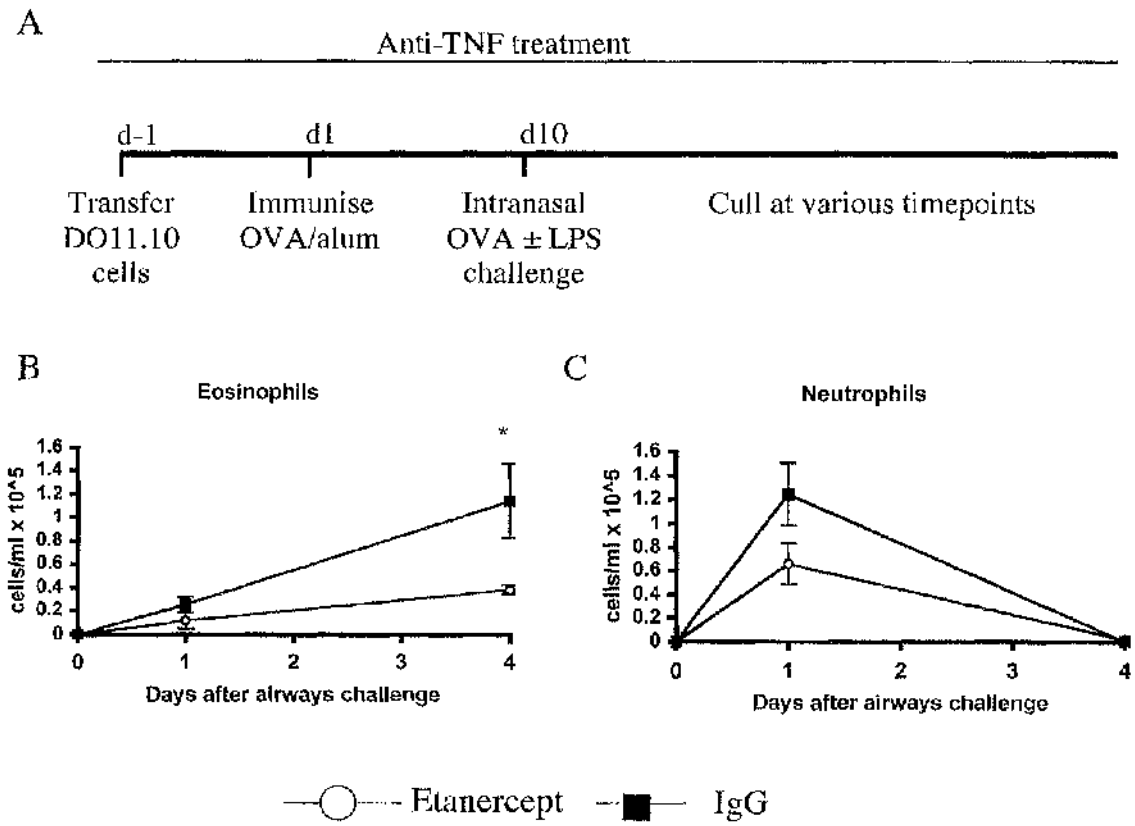

Figure 5.7: Etanercept treatment throughout the short model reduces BAL eosinophilia.

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100μg OVA and 1% alum by i.p. injection. Ten days later mice were challenged by intranasal administration of 50μg OVA. Mice were treated throughout with 50mg/kg etanercept by s.c. injection every two days (A). Control mice were given 50mg/kg human IgG instead of etanercept. BAL fluid was sampled and eosinophil content was analysed by cytospin preparation for eosinophils (B) and neutrophils (C). Results are represented as mean ± SEM (* $p< 0.05$), n=5 per group, n= 2 repeats.

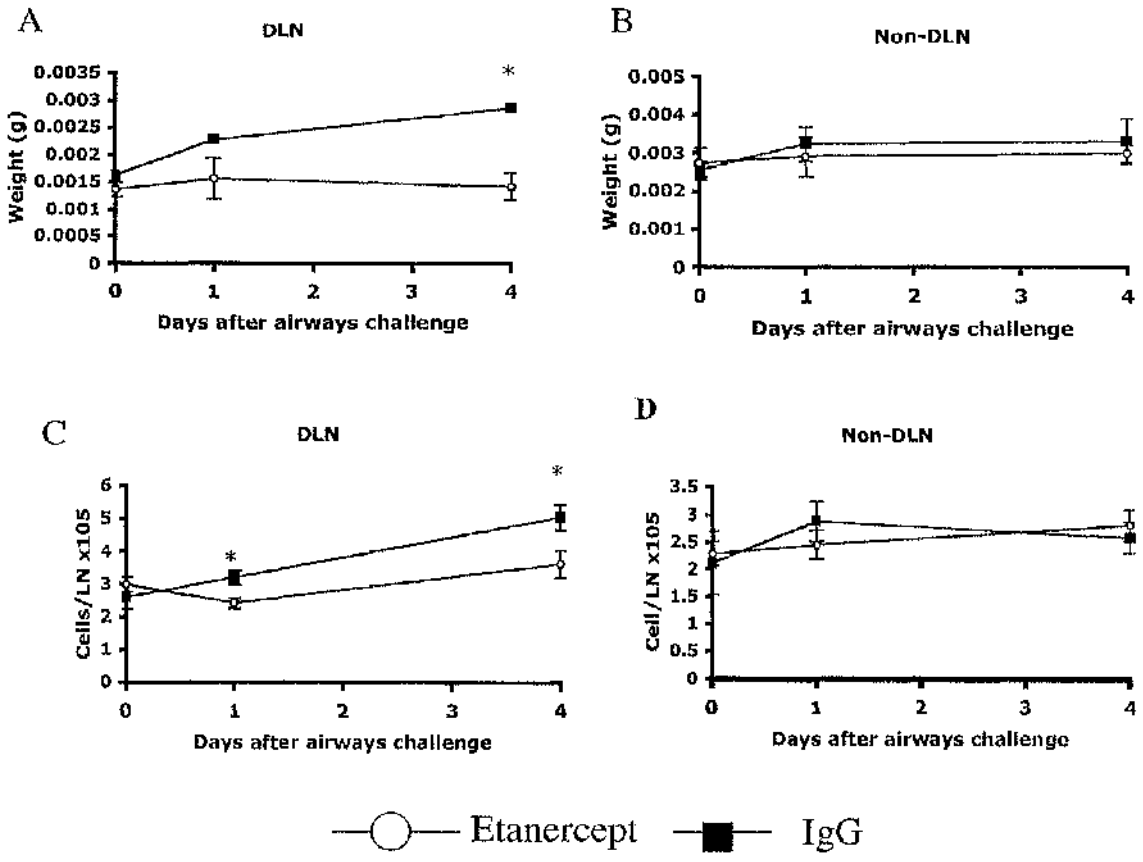

Figure 5.8: Etanercept treatment throughout the short model reduces DLN weight and cellularity.

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100μg OVA and 1% alum by i.p. injection. Ten days later mice were challenged by intranasal administration of 50μg OVA. Mice were treated throughout with 50mg/kg etanercept by s.c. injection every two days. Control mice were given 50mg/kg human IgG instead of etanercept. DLN and non-DLN were removed and weighed (A and B) and cellularity calculated (C and D). Results are represented as mean ± SEM (* $p< 0.05$), n=5 per group, n= 2 repeats.

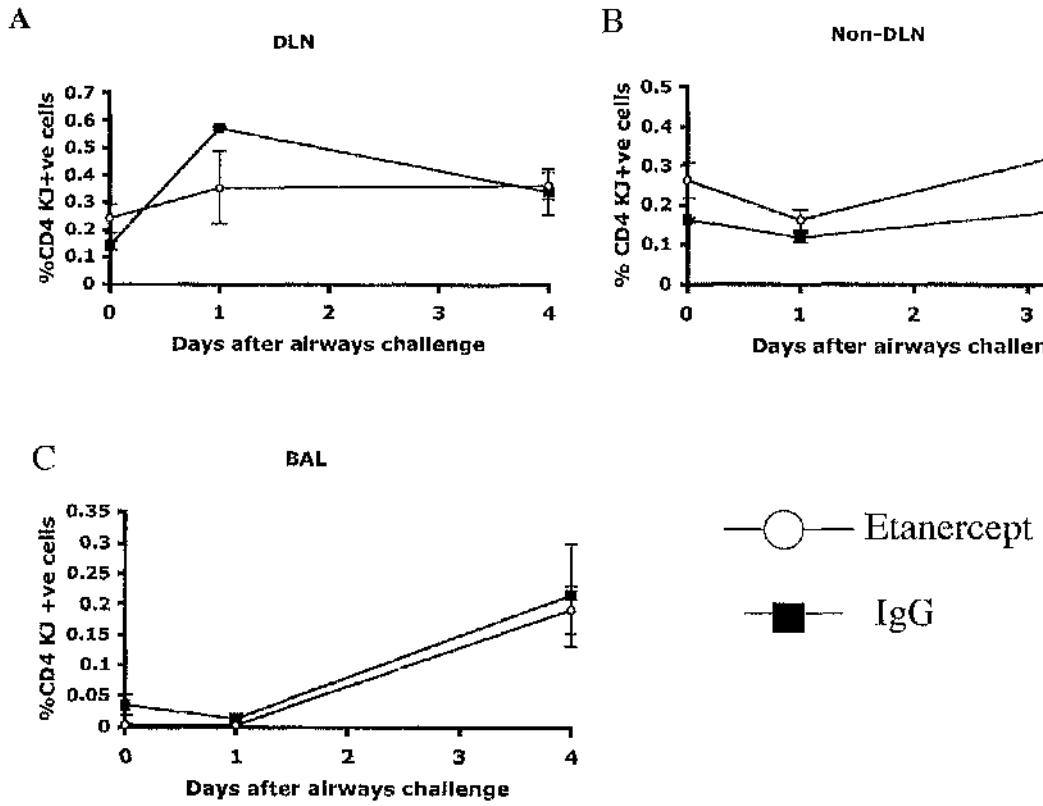

Figure 5.9: Effect of etanercept on antigen-specific T cells.

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100µg OVA and 1% alum by i.p. injection. Ten days later mice were challenged by intranasal administration of 50µg OVA. Mice were treated throughout with 50mg/kg etanercept by s.c. injection every two days. Control mice were given 50mg/kg human IgG instead of etanercept. Antigen-specific T cells were analysed by flow cytometry in DLN (A) non-DLN (B) and BAL fluid (C). Results are represented as mean ± SEM, n=5 per group, n= 2 repeats.

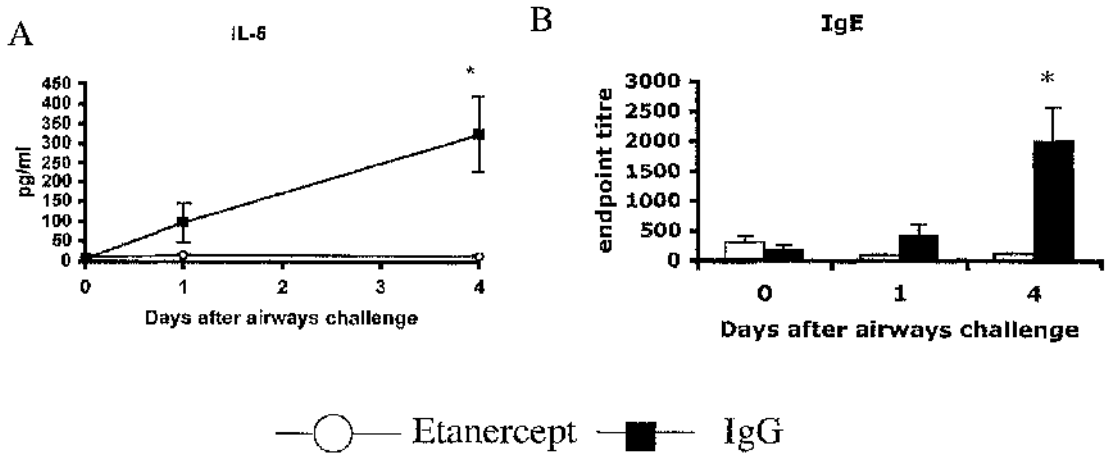

Figure 5.10: Etanercept reduces IL-5 and IgE in the short model.

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100µg OVA and 1% alum by i.p. injection. Ten days later mice were challenged by intranasal administration of 50µg OVA. Mice were treated throughout with 50mg/kg etanercept by s.c. injection every two days. Control mice were given 50mg/kg human IgG instead of etanercept. BAL fluid was sampled and analysed for IL-5 (A). Serum form the same mice was analysed for antigen-specific IgE (B). Results are represented as mean ± SEM, (*$p<0.05$), n=5 per group, n= 2 repeats.

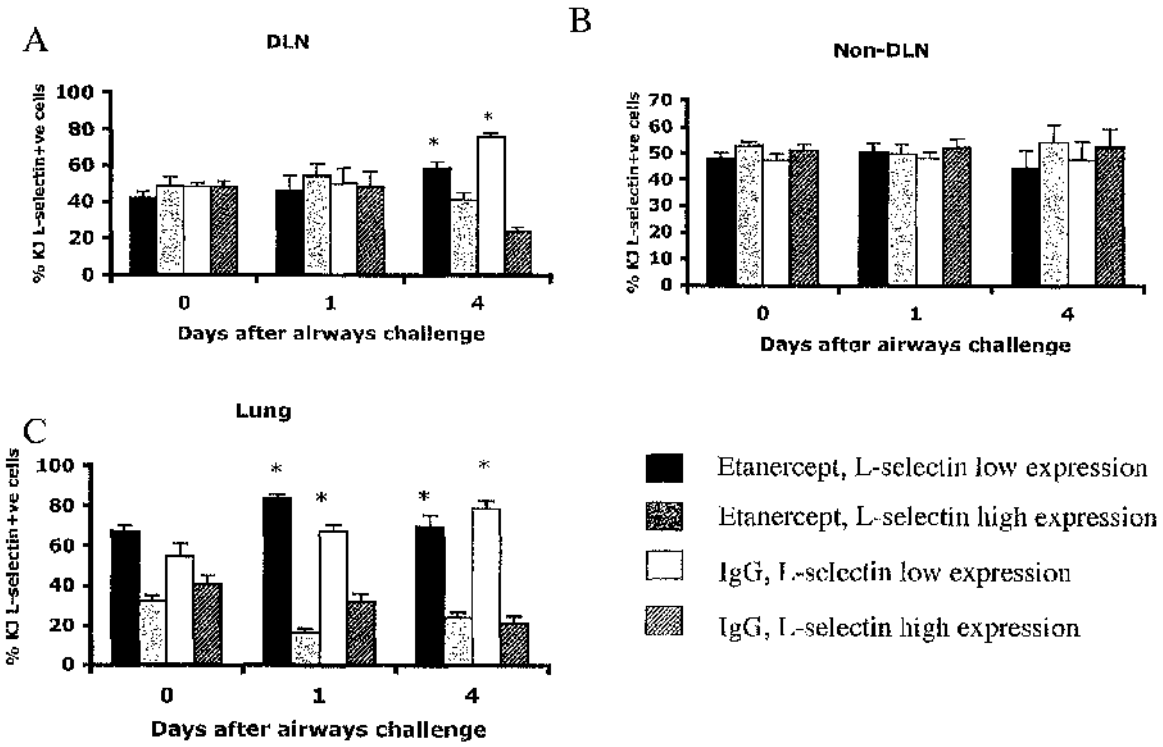

Figure 5.11: L-selectin expression on antigen-specific T cells.

$3x10^6$ OVA-specific T cells were adoptively transferred into naïve Balb/c recipients, which were then immunised with 100µg OVA and 1% alum by i.p. injection. Ten days later mice were challenged by intranasal administration of 50µg OVA. Mice were treated throughout with 50mg/kg etanercept by s.c. injection every two days. Control mice were given 50mg/kg human IgG instead of etanercept. Antigen-specific T cells were analysed by flow cytometry in DLN (A) non-DLN (B) and BAL fluid (C) for L-selectin expression. Results are represented as mean ± SEM, (*$p<0.05$), n=5 per group, n= 2 repeats.

CHAPTER 6 WHEN AND WHERE DOES T CELL DIVISION AND ACTIVATION TAKE PLACE IN ALLERGIC AIRWAYS INFLAMMATION?

6.1 Introduction

The recirculation of T cells through secondary lymphoid organs and peripheral non-lymphoid tissues is fundamental to the maintenance of immune surveillance. It is believed that naïve T cells recirculate randomly through secondary lymphoid sites until they are stimulated by antigen conveyed there from tissues by APCs [495, 496]. During T cell activation, naïve T cells are imprinted with a template of the site in which they were initially stimulated [497]. These cells then differentiate to acquire a tissue specific homing phenotype via expression of different receptor and signalling molecules, which facilitate return to that site [498]. Allergic asthma is characterised by chronic eosinophilic airway inflammation, believed to be orchestrated by Th2 lymphocytes specific for inhaled allergen [499] and pulmonary immune responses can be generated by the introduction of antigen into the airways [500, 501]. The development of specific cell-mediated pulmonary immunity requires an afferent phase, during which inhaled antigens are captured, processed and presented to T cells in the context of MHCII on APCs [502] and an efferent phase in which T cells traffic through lymph nodes to sites of inflammation. Thus T cells are potentially involved in two phases of airway inflammation.

Drugs that interfere with the activation of T cells or more selectively Th2-specific signalling molecules, and drugs that prevent the selective migration into lung tissue are promising novel strategies for the treatment of allergic asthma. Previous studies have shown that depletion of $CD4^+$ T cells by administration of antibody clearly inhibited allergen-induced airway eosinophilia and AHR [124, 503]. It has also been shown that $CD4^+$ T lymphocytes are critical for the development of airway epithelial changes and subepithelial fibrosis as

well as AHR in a murine model of chronic asthma [504]. A study in IL-4 knockout mice, using mAb against CD4 in a murine model of allergic airways showed that the drug inhibited allergen-induced increases in airways responses, the number of eosinophils in BAL fluid, serum antigen-specific IgE levels and IL-13 in BAL fluid [505], suggesting that Th2 responses play a critical role for the development of allergen induced airway remodelling and that inhibition of Th2 responses may be a viable therapeutic approach.

Thus, although T cells play an important role in asthma pathogenesis, it is still unclear as to when and where T cell activation and division takes place in allergic airways inflammation. An extensive network of dendritic cells line the conducting airways and interstitium of the lung and are capable of presenting inhaled antigen to naïve and memory T cells *ex vivo* [506-509]. Maturation and migration of airway dendritic cells to the T cell areas of the lymph nodes are poorly understood but are thought to be essential for mature DCs to interact with naïve recirculating T cells [502, 510]. Thus understanding the timing and location of T cell divisions and activation in airways inflammation should provide insights into the pathogenesis and treatment of infectious and allergic diseases of the lung.

The activation and division of naïve T cells *in vivo* is difficult to detect because of the low frequency of high affinity antigen-specific T cells. Although unmanipulated TCR transgenic animals make normal high affinity T cell responses to immunogenic and tolerogenic antigens, adoptive transfer of CFSE labelled transgenic T cells into a normal recipient offers a number of advantages. The main advantage is that the T cell division profile can be

correlated to *in vivo* distribution and recirculation of antigen-specific T cells to lymph nodes and non-draining lymphoid sites.

In this chapter antigen-specific T cells will be used in conjunction with CFSE labelling and assessment of proliferating cell nuclear antigen (PCNA) expression to allow identification of T cell division after antigen airways challenge. To model the early events during T cell activation and division in response to inhaled antigen, adoptive transfer of CFSE labelled antigen-specific T cells in an acute lung injury model will be used. This will allow naïve T cells to be studied in different compartments involved in the response and the kinetics of the response assessed. The adoptive transfer model described in chapter 3 will be employed to examine PCNA expression by specific-antigen experienced cells as an indication of their recent division in the DLN, PLN, BAL and lung in the model of airways inflammation.

Our investigations show that cell division of antigen-specific T cells after an airways challenge of OVA plus LPS to naïve BALB/c mice could be seen by day three post airways challenge in the DLN, BAL and lung tissue. There is no cell division seen in the PLN throughout the timecourse. Interestingly, when the number of cell divisions was analysed it was found that although the three compartments show cell division by day three, T cells in the BAL and lung have divided more times than those in the DLN. This suggests that the response in these tissues is more vigorous than in the DLN or that more differentiated cells are remaining in these compartments. When antigen-specific T cell division was quantified by LSC in tissue sections of DLN, PLN and lung it was found in the long adoptive transfer model that there was an early wave of T cell division at day one post airways challenge in

the lung, followed by similar in the DLN. This leads to another wave of recently divided T cells in the lung, which corresponds to peak BAL eosinophilia. It is possible that the second wave of T cell division may be needed for the chronicity of asthma. In the short model of airways inflammation it was found that by day four post airways challenge both the DLN and lung showed significant T cell division compared with control groups. As seen in the long model there was no T cell division in the PLN. Although the kinetics are not as clear in this experiment it still suggests that T cell division in the lung and DLN is important to get airways pathology. This data gives an insight into the timing and location of antigen-specific T cell division in airways inflammation and will be important for rational design and application of therapeutics targeted against leukocyte migration.

6.2 Results

T cells play a major role in regulating inflammatory responses and have been shown to be important in asthma pathogenesis. However, it is still unclear when and where T cells divide or are activated in asthma. This chapter will use OVA-specific T cells and the adoptive transfer model of airways inflammation characterised in Chapter Three to try and elucidate where T cells are dividing after antigen challenge in the DLN, PLN BAL and lung tissue.

6.2.1 T cells divide in the DLN, BAL and lung after airways challenge.

An initial experiment was set up to investigate if we were able to detect antigen-specific T cell division. CFSE labelled OVA-specific T cells were adoptively transferred into naïve BALB/c mice, which then received an airways challenge of OVA plus LPS. T cell division was then tracked in the DLN, PLN, BAL and lung over a timecourse (Figure 6.1).

When T cell division was analysed in the different compartments after airways challenge it was found that there was no division in any compartment at day 0 post challenge. By day 3 after airways challenge the same percentage of T cells in the DLN, BAL and lung have all undergone division. No T cell division is seen in the PLN over the timecourse measured. Both the DLN and BAL T cells continue to divide at day five and seven. After the peak of T cell division in the lung at day three, the percentage of division drops at day five and seven (Figure 6.2 A). This may be due to the T cells dying or migrating to another compartment.

Although this data shows when and where antigen-specific T cells are dividing, it does not give any information about the extent of division. To determine if there was any difference in the number of cell divisions between the different compartments, the data was analysed to show T cells that had divided 1-3 times and T cells that divided 4-6 times.

6.2.2 BAL and lung T cells divide more vigorously than DLN T cells.

When T cells that divided 1-3 times were analysed over the timecourse, at day 3 post challenge when previously it looked as though the DLN, BAL and lung T cells were dividing at the same rate, the DLN have a greater percentage of T cells that have divided in this group. BAL and Lung have the same percentage of T cells dividing 1-3 times at this timepoint (Figure 6.2 B). As seen for the total percentage of T cell division, the DLN and BAL T cells continue to divide at day five and seven post airways challenge, with the lung T cell division declining (Figure 6.2 B).

Interestingly, when T cells that had divided 4-6 times were analysed it was found that BAL and lung T cells had a higher percentage of cells dividing than the DLN at day 3 post airways challenge (Figure 6.2 C). On days five and seven after antigen challenge DLN T cells were dividing more times that the BAL and lung. There was no difference in the percentage of T cells dividing 4-6 times in the BAL or lung at days 5 and 7 post challenge. This shows that although the overall percentage of T cell division is not different between the DLN, BAL and Lung at day 3 post airways challenge, the number of cell divisions is

greater in the BAL and lung at earlier time points, with the DLN dividing more than the BAL and lung T cells at days five and seven post airways challenge.

It is important to remember when analysing this data that the T cells involved were naïve and are making a primary response, which may differ from T cells that have previously been exposed to antigen. To address this issue the adoptive transfer model of airways inflammation was used to track division in T cells already sensitised to OVA. However, due to the length of the protocol, CFSE could not be used as the immunisation regime means that all the labelled T cells will have divided by the time antigen challenge is administered, meaning newly divided cells could not be identified in the compartments. Several studies have shown proliferating cell nuclear antigen (PCNA) as a reliable marker of division. Its temporal expression and relatively short half-life mean that cells expressing the PCNA are likely to be recently divided in the location they are observed in.

6.2.3 Long adoptive transfer model.

To enable the quantification of antigen specific T cell expression of PCNA, tissue samples from the long adoptive transfer model, previously described in chapter three, were analysed by LSC. The long model consists of the transfer of OVA-specific T cells into naïve BALB/c mice, followed by three OVA/alum immunisations seven days apart. Mice then receive an intranasal challenge seven days later of OVA, OVA plus LPS or LPS alone (Figure 6.3 A). As described previously this model results in significant BAL eosinophilia in the OVA plus LPS and the OVA alone groups, but not in the LPS alone group (Figure 6.3 B). When BAL was analysed for cell infiltrates it was found that OVA plus LPS and LPS alone resulted in a transient influx of neutrophils at day 1 post airways challenge (Figure 6.3 C). As shown in

clinical and other murine models of airways inflammation, eosinophilia is associated with an increase in BAL IL-5 production and increased antigen-specific serum IgE levels (Figure 6.3 D and E).

6.2.4 PCNA expression.

Lymph node and lung tissue sections from frozen sections stored from the experiment described above were stained with fluorescent antibodies against OVA-specific TcR transgenic T cells (KJ1.26) and PCNA and lymph nodes were also stained for the B cell marker B220 to allow visualisation of the lymph node architecture. LSC was used to allow identification of double positive cells *in situ* as described in chapter 2. Figures 6.4 and 6.5 show antibody staining in lymph nodes and lung tissue respectively. Green staining in the lymph node represents B cell follicles and autofluorescent structure in lung tissue, PCNA expressing cells are blue and KJ1.26 transgenic T cells are red. Antigen-specific T cells expressing PCNA, i.e. recently divided cells, are coloured pink.

When cells were quantified in DLN sections it was apparent that there were no $KJ1.26^+$ $PCNA^+$ cells at day one post airways challenge. However, by day three after challenge antigen specific T cells in the OVA plus LPS and OVA alone groups had undergone division. The LPS alone challenged animals showed little evidence of cell division (Figure 6.6 A). The percentage of double positive ($KJ1.26^+$ $PCNA^+$) cells declined after the peak at day three post airways challenge through days five and seven. In lung sections an initial burst of T cell division was seen at day one post airways challenge in the OVA plus LPS and OVA alone groups, again very little division was seen in the LPS alone group (Figure 6.6 B). The T cell division increases in the OVA plus LPS and OVA groups through day three and

peaks at day five post airways challenge, dropping by day seven. There was no difference between any of the treatment groups in the PLN (Figure 6.6 C), and the division that was seen was very low, as might be expected, suggesting that the low levels of division seen in the LPS groups in the OVA plus LPS and OVA alone groups in the DLN and the lungs may be non-specific staining.

When the data from the DLN and the lung sections are analysed in parallel (Figure 6.7) it can be seen that there is an initial wave of T cell division, presumably driven by local antigen presentation after airways challenge seen in the lung followed by similar in the DLN, probably driven by migrating dendritic cells. This leads to another wave of recently divided T cells accumulating in the lung. When compared with the data shown in figure 6.3 A and B it can be seen that the initial division of T cells in the lung corresponds to the transient neutrophil influx seen in the BAL. It is also of note that the peak T cell division in the lung corresponds to the peak of BAL eosinophilia (Figure 6.8).

6.2.5 Short adoptive transfer model.

As well as the long adoptive transfer model described above, PCNA expression was also assessed in the short model, previously described in chapter three, to determine if there was a difference in timing or location of T cell division between the models. The short model consists of the adoptive transfer of naïve OVA-specific T cells into naïve BALB/c mice followed by one immunisation with OVA/alum. Ten days later mice received an intranasal challenge with OVA plus LPS or OVA alone (Figure 6.9 A). As described in chapter three, airways challenge with OVA plus LPS in this model results in significant BAL eosinophilia

at day four post challenge compared with the OVA alone group (Figure 6.9 B). The OVA plus LPS group induces an influx of neutrophils at day one post challenge as previously seen. This was not seen in the OVA alone group (Figure 6.9 C). This protocol also induces significant BAL IL-5 production in the OVA plus LPS group compared with the OVA along group (Figure 6.9 D) and an increase in serum IgE levels at day 4 post airways challenge in both OVA and OVA plus LPS treated mice (Figure 6.9 E).

6.2.6 Cell division in the short model.

As in the long model, lymph node and lung sections were stained for antigen-specific T cells and PCNA expression and analysed by LSC. In the DLN there is no T cell division at day one post airways challenge, but by day four post challenge there is significantly more division in the OVA plus LPS group compared with the OVA alone group (Figure 6.10 A). The lung mirrors the division seen in the DLN, with no division at day one post challenge and significant division in the OVA plus LPS group compared with the OVA alone group by day four after airways challenge (Figure 6.10 B). As seen in the long model there was no difference in the groups in the PLN (Figure 6.10 C), and the level of division was very low again suggesting that it might be non-specific staining.

6.3 Discussion

The $CD4^+$ Th2 cell is a critical mediator of the asthma phenotype. Adoptive transfer of Th2 cells into naïve mice followed by aerosol challenge has clearly demonstrated that Th2 cells are recruited into the lung and are critical for inducing the characteristic hallmarks of asthma, including AHR, eosinophilia and mucus production [353]. Although it is know that T cells are present in the airways and the draining lymph nodes [511] it has been difficult to determine where, how and when they interact with the rest of immune system. Many studies have looked at the migration of lymphocytes between lymph nodes and airways [242, 512-515] and the subsequent interaction between T cells and DCs [351, 516-518]. However, it is not known if T cells are activated and divide in the lung itself or the lymph nodes.

The experiments in this chapter have investigated the timing and location of antigen-specific T cell division after antigen airways challenge. Naïve T cells have been studied after transfer of CFSE labelled cells into BALB/c mice followed by intranasal challenge. When the division of naïve T cells was analysed in the DLN, PLN, BAL and lung tissue it was found that there was division by day three post airways challenge in the DLN, BAL and lung. There was no division seen in the PLN throughout the timecourse. Although the three locations showed division at the same timepoint, the magnitude of the response was different. In the BAL and lung tissue it was shown that the T cells had divided more in these compartments than the T cells in the DLN. This data correlates with data showing that naïve T cells are recruited to the airways after antigen challenge [516, 519], and similar studies using fluorescent beads as a model of inhaled antigen have been detected in lung and BAL after intranasal administration [351].

The majority of studies looking at T cell activation and division in airways inflammation have looked in murine models where dendritic cells primed for a specific antigen are transferred into naïve mice, which receive a subsequent airways challenge, and the response analysed [513, 516, 517, 520]. The adoptive transfer model used in this chapter allows the identification of naïve T cells transferred into mice and given an airways challenge to induce airways pathology. The use of laser scanning cytometry allows the quantification of recently divided antigen-specific T cells *in situ* providing a novel model to study these parameters. When T cell division was quantified by LSC in the long model it was found that there was an early wave of T cell division seen in the lung at day one post airways challenge, followed by similar in the DLN at day three post challenge. At day five post challenge there was another wave of antigen-specific T cell division seen in the lung tissue. The early T cell division may have been caused by uptake and presentation of antigen by resident dendritic cells, which have been shown to be present in the lung [518]. The location of these dendritic cells allows them to have direct contact with incoming antigens. It is known that there is a dendritic cell network situated immediately above and beneath the basement membrane of the upper and lower airways enabling these cells to sample the epithelium for inhaled antigens [225, 509, 521]. It has also been shown in experimental models of respiratory virus infection that a population of memory T cells are established in the lung parenchyma and the lung airways [522, 523]. Airway resident memory cells contribute significantly to recall responses by providing immediate effector activity at the site of antigen entry [524]. A population of resident memory T cells may account for the early T cell division seen day one post airways challenge in the lung.

Previous studies depleting $CD4^+$ T cells in a murine model of airways inflammation before antigen challenge have shown reduction in AHR response to acetylcholine challenge and reduced eosinophilia, IgE and IL-13 production [505]. A study using A/J mice in an OVA induced model of airways inflammation with administration of anti-CD4 mAb before airways challenge caused reduction in AHR and lung eosinophilia [124] and lung eosinophilia was also prevented in a murine model of airways inflammation in CD4 deficient mice [363]. These studies suggest an important role for T cells in the induction and maintenance of immune responses to antigen. Gaining and insight into the location and timing of T cell division and activation will enable more accurate therapeutic intervention in airways inflammation.

To determine the site of T cell division in airway inflammation, antigen-specific T cells were tracked in different compartments throughout the course of the disease and proliferating cell nuclear antigen (PCNA) expression was measured on antigen-experienced T cells as an indication of their recent division. When T cell division was quantified by LSC in the long model it was found that there was an early wave of T cell division seen in the lung at day one post airways challenge, followed by similar in the DLN at day three post challenge. At day five post challenge there was another wave of antigen-specific T cell division seen in the lung tissue. The early T cell division may have been caused by uptake and presentation of antigen by resident dendritic cells, which have been shown to be present in the lung [518]. The location of these dendritic cells allows them to have direct contact with incoming antigens. It is known that there is a dendritic cell network situated immediately above and beneath the basement membrane of the upper and lower airways enabling these cells to sample the epithelium for inhaled antigens [225, 509, 521]. It has also been shown in

experimental models of respiratory infection that a population of memory T cells are established in the lung parenchyma and the lung airways [522, 523]. Airway resident memory cells contribute significantly to recall responses by providing immediate effector activity at the site of antigen entry [524], showing that there is local antigen presentation in the lung [525].

Upon antigen encounter, respiratory tract DCs migrate to the draining mediastinal lymph nodes where they control the activation and differentiation of antigen-specific T cells [526]. Activated effector T cells will migrate back to the site of inflammation and will be (re)stimulated locally by activated DCs [525]. It has been shown that different subsets of DCs are responsible for antigen transportation in response to different types of antigen [527]. A study by De heer *et al.*, showed both myeloid and plasmacytoid DCs take up antigen within the draining lymph node in response to OVA inhalation, but only the myeloid subset was shown to present antigen to naïve $CD4^+$ T cells [231]. As well as DCs, macrophages are able to uptake and process antigen to T cells and may be the first cell to encounter antigen in the airways (REF). Alveolar macrophages play a key role in the maintenance of immunological homeostasis in the respiratory tract and represent the most abundant cells in the conducting airways (REF). Macrophage numbers are increased in the lung and alveolar space of patients with asthma and COPD and are localised to sites of alveolar destruction (REF). This movement and activation of T cells corresponds to the peak of antigen-specific T cell division seen in the DLN at day three post airways challenge. The subsequent wave of T cell expansion seen in the lung may be due to T cells returning to the site of inflammation where they initially encountered antigen after being imprinted in the DLN. It

has been hypothesised that during activation naïve T cells are imprinted with a template of the site in which they were initially stimulated [497]. These cells then differentiate to acquire a tissue-specific homing phenotype which facilitates there return to that site [481, 498]. The idea that antigen-specific T cells can home back to the site of initial antigen encounter parallels the data shown in this study in which there is a second wave of T cell division seen in the lung tissue at day five post airways challenge. The two stages of T cell division in the lung mimics the data shown by Catron *et al.*, who showed that as well as an initial T cell response to antigen, a population of "late-arriving" T cells are present after antigen challenge that may be central memory cells and were shown to proliferate more extensively than the resident T cells [513]. These cells may be critical for the chronic pathology that is seen in asthma patients. This data gives an insight into the timing and location of Ag-specific T cell division in airways inflammation and will be important for rational design and application of therapeutics.

When all the data form the experiment was analysed in parallel it was seen that the initial T cell division seen in the lung corresponded to the influx of neutrophils in the BAL, and the second wave of T cell division in the lung coincided with the peak of BAL eosinophilia. Allergic asthma is associated with Th2 responses and the presence of T cells have been shown to be crucial in the development of asthma pathogenesis [2]. The presence of neutrophils have been shown to be associated with severe asthmatics [416, 528] and are known to be chemotactic for T cells [529], suggesting that there presence in the BAL early in the response may cause the recruitment of activated T cells to the site of inflammation and ensuing pathology. T cells are crucial in the development of eosinophilia in airways

inflammation [2, 190, 235, 363, 364, 530] and the peak of T cell division in this model is consistent with the peak of BAL eosinophilia.

When T cell division was measured in the short model, no division was seen at day one post challenge in the DLN or the lung. This is in contrast to the data seen in the long model where T cell division was seen in the lung at this timepoint. This may be due to the T cells in this model only being primed once as apposed to three times. By day four after airways challenge, there was division seen in both the DLN and the lung. No division was observed in the PLN over the timecourse. The peak of T cell division in both the DLN and the lung corresponds to the peak BAL eosinophilia seen in this model. Although the data from this timecourse is not as detailed as the long adoptive transfer model, it still implies that T cell division in the DLN and lung is associated with BAL eosinophilia and lung pathology.

The migration of T cells between lymph nodes and the site of inflammation is crucial to the resulting immune response. Inhibiting T cell trafficking has been shown to be an effective strategy for treating a host of inflammatory diseases such as multiple sclerosis and Crohns' disease [461], thus the increasing knowledge being gathered on the involvement of T cells in diseases such as asthma will enable therapies to be targeted more effectively. Recently there has been increased interest in the use of FTY720 as a therapeutic drug in models of transplantation and autoimmunity and has recently been shown to be important in kidney transplantation [531-534]. FTY720 selectively and reversibly sequesters lymphocytes, via the sphingosine 1-phosphate receptor, but not monocytes or granulocytes from blood and spleen in to secondary lymphoid organs, preventing their migration towards sites of

inflammation [535-537]. More recently a study using FTY720 in a murine model of allergic airways inflammation showed administration of the drug before antigen airways challenge abrogated airways inflammation by inhibition of the migration of dendritic cells to draining lymph nodes, which in turn inhibited the formation of allergen-specific Th2 cells in lymph nodes [538]. This study again highlights the importance of using systems such as the adoptive transfer system, where tracking of cells of known specificity allows identification of both T cells and dendritic cells and their possible interactions in diseases.

Overall these results have shown that antigen administration to naïve antigen-specific T cells results in cell division in the DLN, lung and BAL all at day three post challenge. However, it was found that the T cells in the BAL and lung divided more in these compartments. This suggests that the response seen in the BAL and lung was more vigorous than in the DLN. When memory T cells divisions were quantified in the long adoptive transfer model by LSC it was found that an early wave of T cell division, presumably driven by local Ag presentation after airways challenge, was seen in the lung followed by similar in the DLN, probably driven by migrating dendritic cells. This leads to another wave of recently-divided T cells accumulating in the lung. Peak T cell division in the lung corresponded to BAL eosinophilia, which is mimicked in the short adoptive transfer model where peak T cell division at day four post airways challenge mirrors peak BAL eosinophilia. This data gives an insight into the timing and location of Ag-specific T cell division in airways inflammation and will be important for rational design and application of therapeutics.

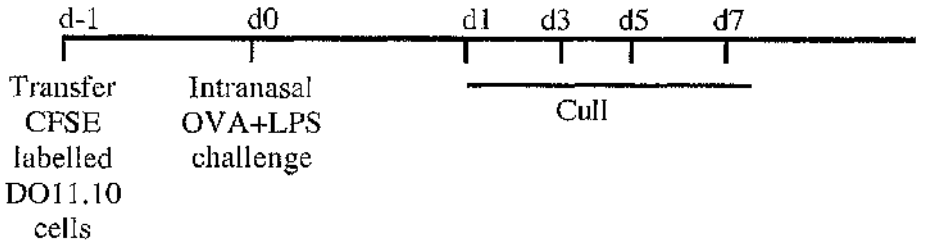

Figure 6.1: CFSE Timeline

$3x10^6$ naïve CFSE labelled transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients which were given an intranasal challenge one day later with 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various time points.

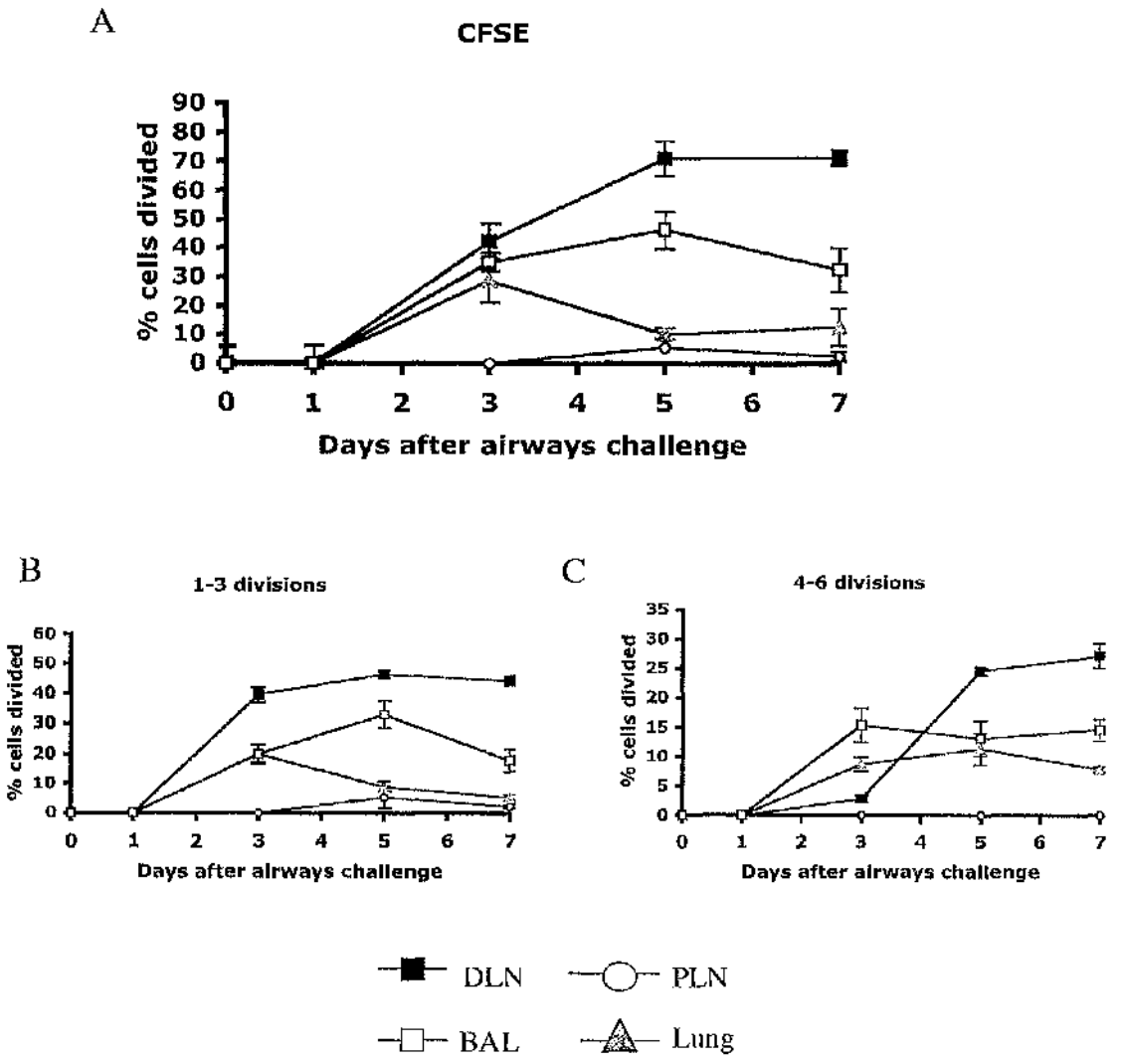

Figure 6.2: CFSE division in DLN, PLN, BAL and lung

$3x10^6$ naïve CFSE labelled transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients which were given an intranasal challenge one day later with 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various time points. OVA-specific CFSE labelled T cells were analysed for number of cell divisions in DLN, PLN, BAL and lung tissue (A). Data was analysed by the number of cell divisions: graph B

shows 1-3 division, graph C shows 4-6 divisions. Values are represented as mean ± SEM, n=3 per group, n= 1 repeat.

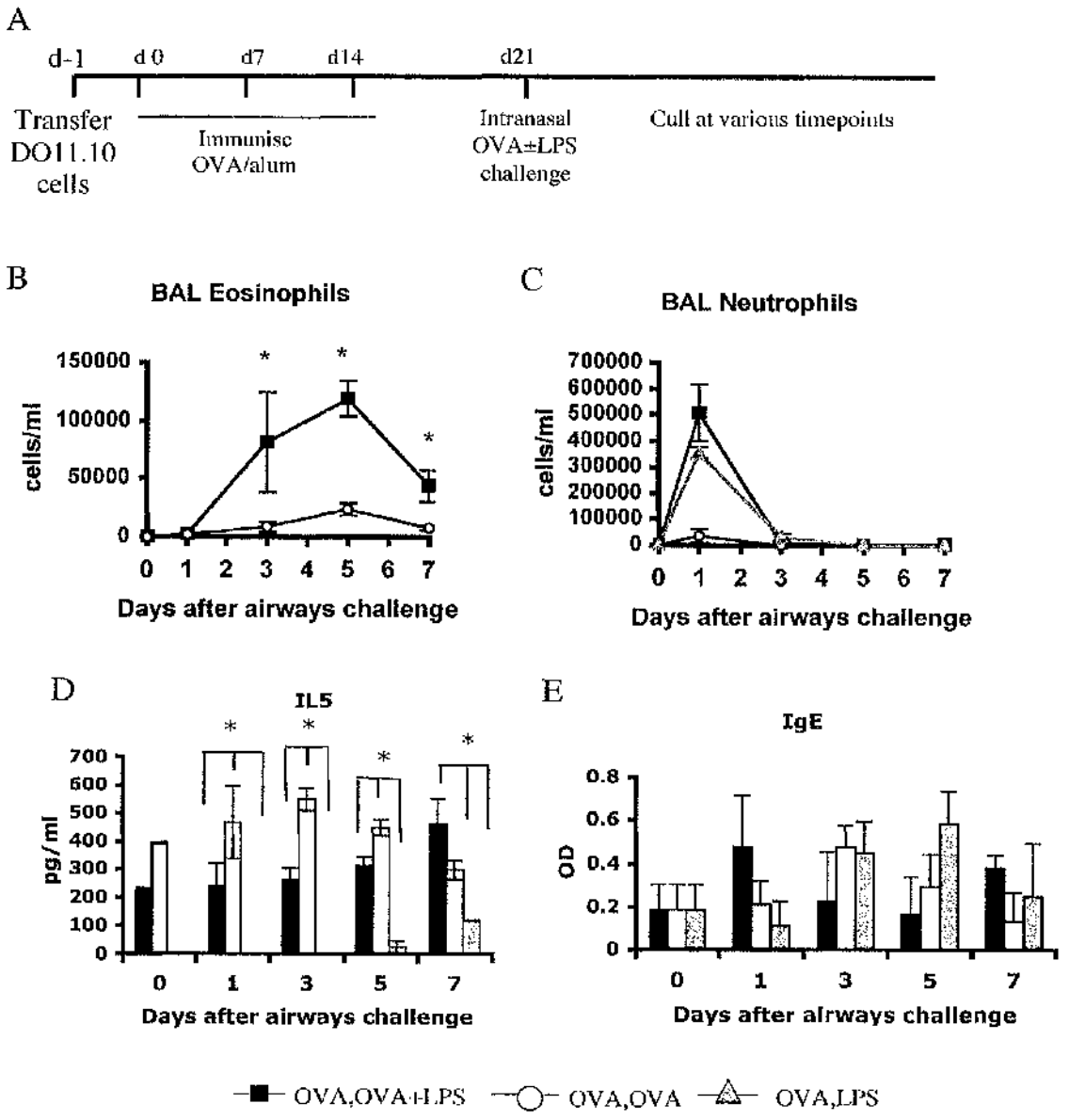

Figure 6.3:Long adoptive transfer model (Discussed in chapter 3)

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days

later with 50μg OVA, 50μg OVA plus 2.5μg LPS or 2.5μg LPS alone (A). Mice were sacrificed at various timepoints. BAL fluid was sampled and cytospins preparations were analysed for the presence of eosinophils (B) and neutrophils (C). BAL IL-5 production (D) and serum IgE levels (E) were measured in the same mice. Values are represented as mean ± SEM, n=3 per group , n= 2 repeats (*$p<0.05$).

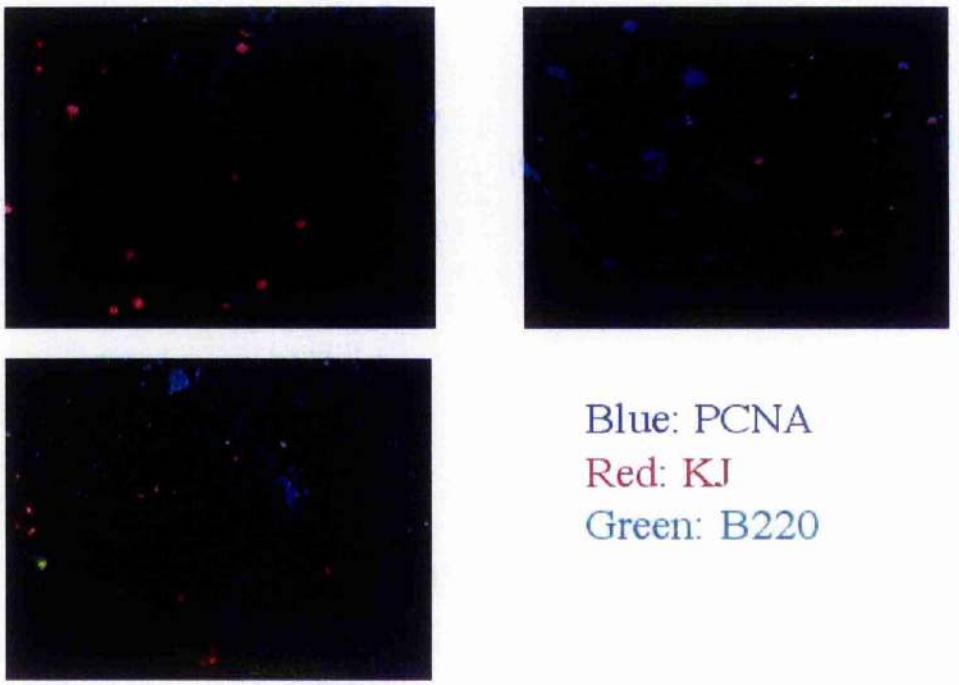

Figure 6.4: KJ and PCNA staining in lymph nodes

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days later with 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various timepoints. LN sections from day five post airways challenge were stained with fluorescent antibodies for OVA-specific T cells (KJ), PCNA and B220 and analysed by laser scanning cytometry.

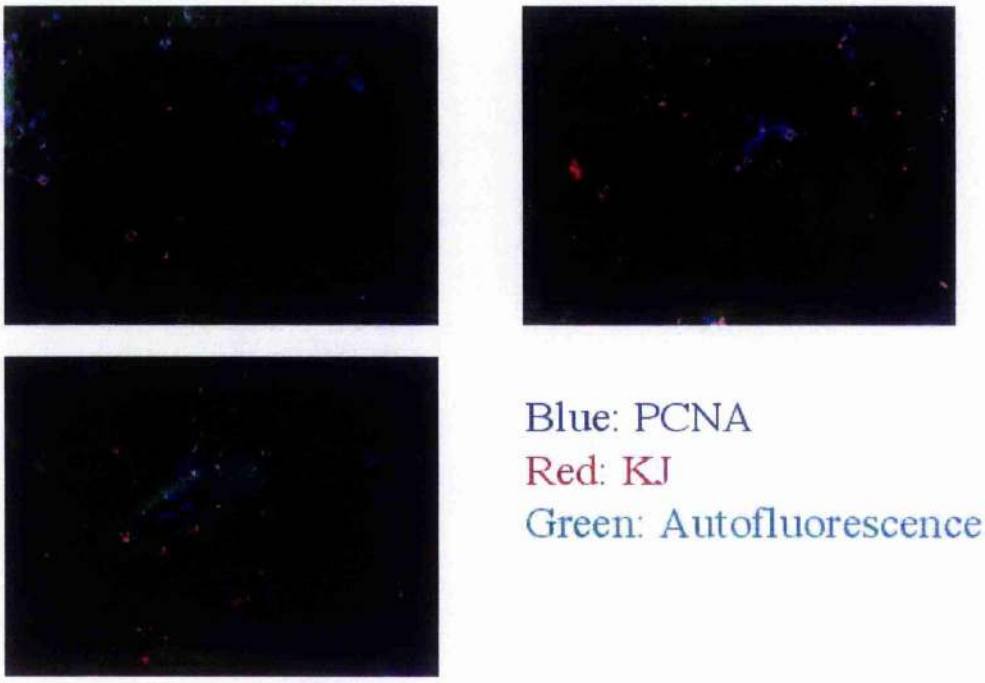

Figure 6.5: KJ and PCNA staining in lung tissue

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days later with 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various timepoints. Lung sections from day five post airways challenge were stained with fluorescent antibodies for OVA-specific T cells (KJ) and PCNA and analysed by laser scanning cytometry.

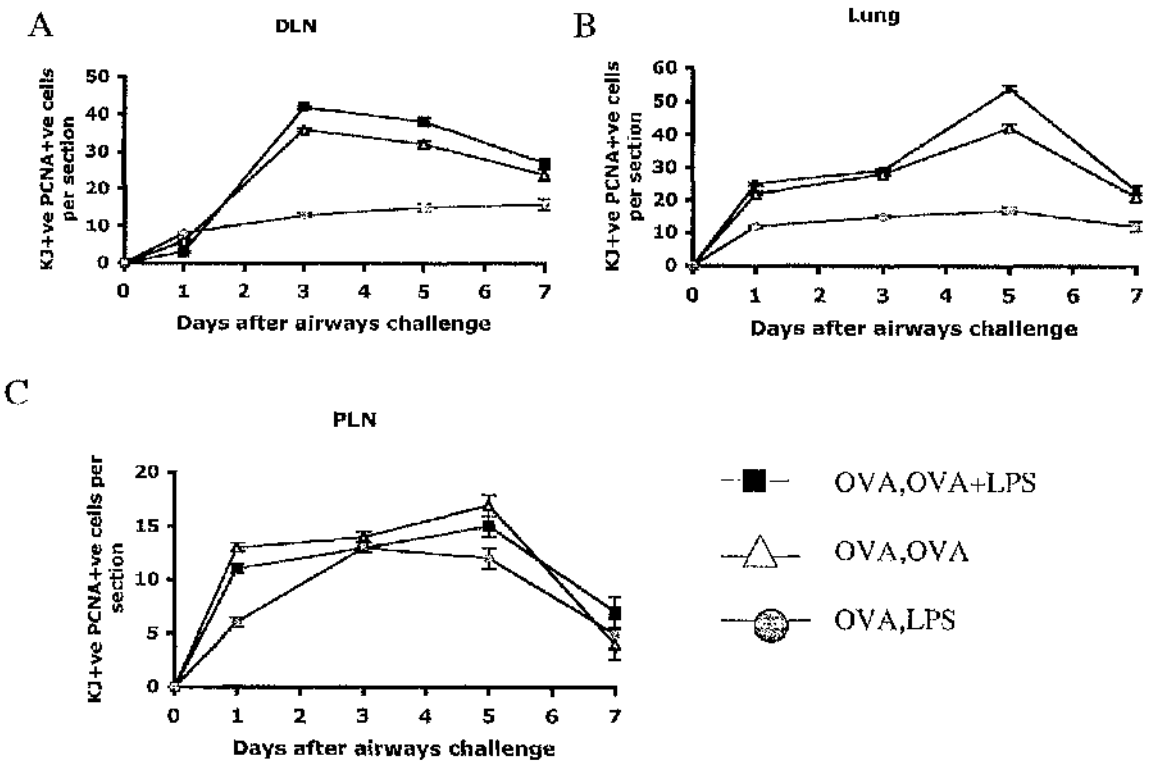

Figure 6.6: Quantification of KJ+ve/PCNA+ve cells by LSC

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days later with 50μg OVA, 50μg OVA plus 2.5μg LPS or 2.5μg LPS alone. Mice were sacrificed at various timepoints. LN and lung sections were stained for OVA-specific T cells and PCNA. Double positive cells were quantified by LSC. Values are represented as mean ± SEM, n=3 per group, n= 2 repeats.

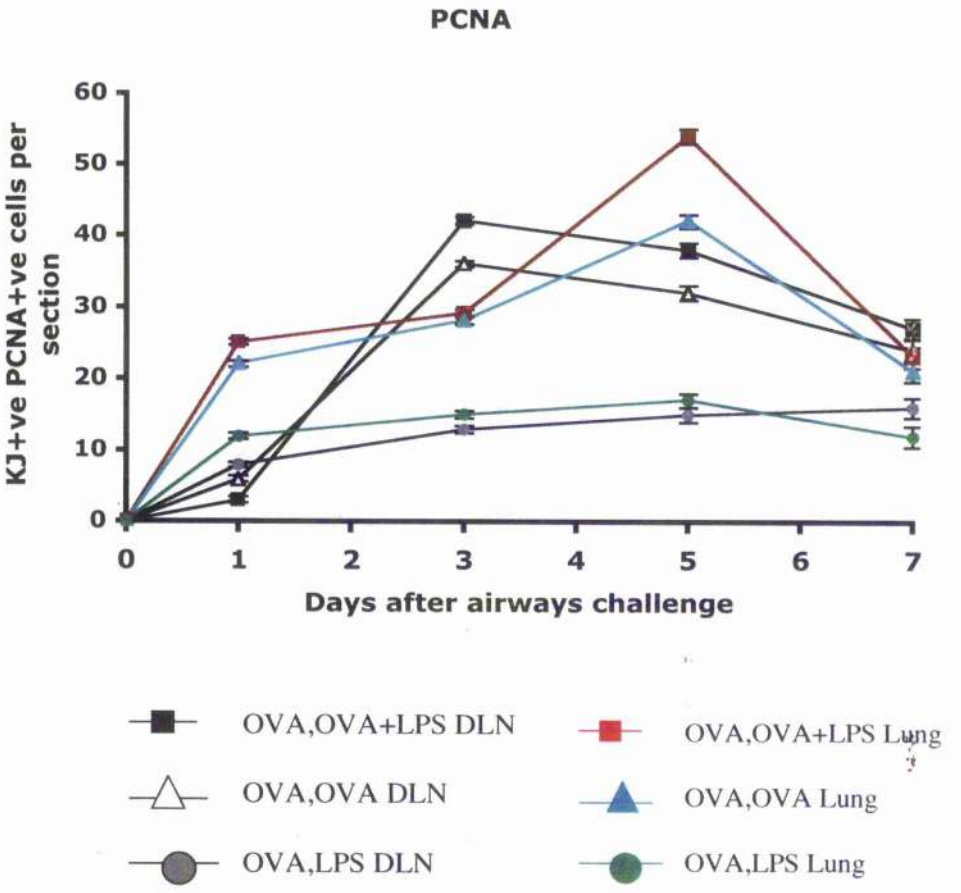

Figure 6.7: DLN and Lung overlay

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days later with 50μg OVA, 50μg OVA plus 2.5μg LPS or 2.5μg LPS alone. Mice were sacrificed at various timepoints. LN and lung sections were stained for OVA-specific T cells and PCNA. Double positive cells were quantified by LSC. Values are represented as mean ± SEM, n=3 per group, n= 2 repeats.

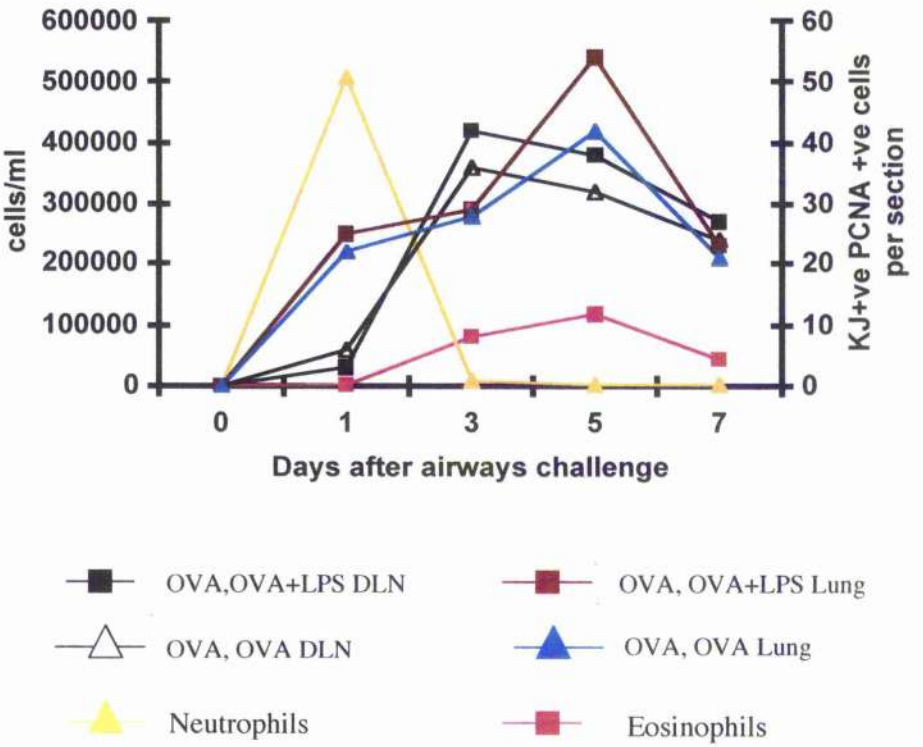

Figure 6.8: DLN and Lung and BAL overlay

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, three times, seven days apart, followed by one intranasal challenge 7 days later with 50μg OVA, 50μg OVA plus 2.5μg LPS or 2.5μg LPS alone. Mice were sacrificed at various timepoints. LN and lung sections were stained for OVA-specific T cells and PCNA. Double positive cells were quantified by LSC. BAL fluid was sampled and cytospins analysed for neutrophil and eosinophil content. Values are represented as mean ± SEM, n=3 per group, n= 2 repeats.

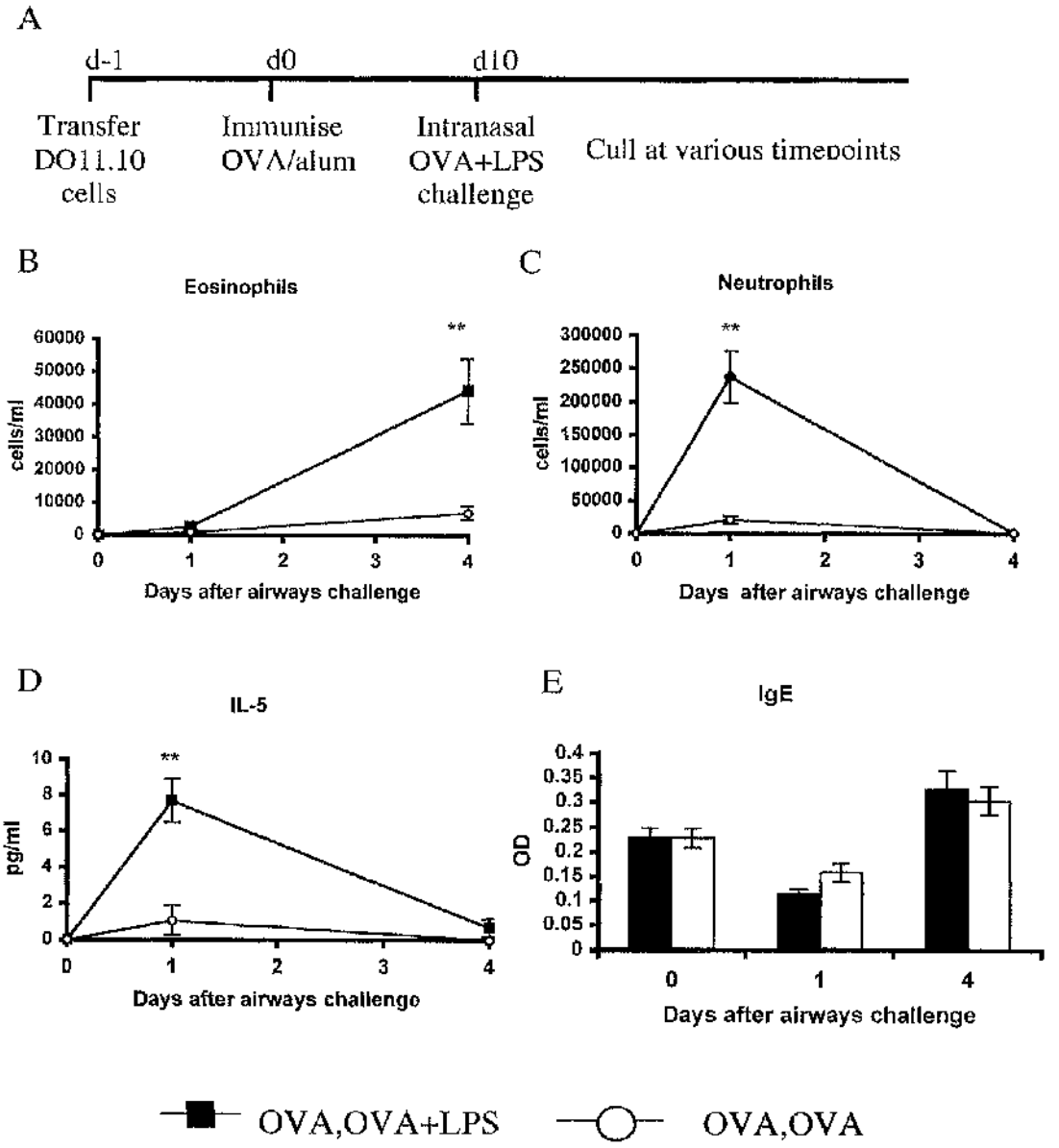

Figure 6.9: Short adoptive transfer model (Discussed in chapter 3)

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with 50μg OVA or 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various timepoints. BAL fluid was sampled

and cytospins preparations were analysed for the presence of eosinophils (B) and neutrophils (C). BAL IL-5 production (D) and serum IgE levels (E) were measured in the same mice. Values are represented as mean ± SEM, n=5 per group, (**p<0.01) , n= 2 repeats.

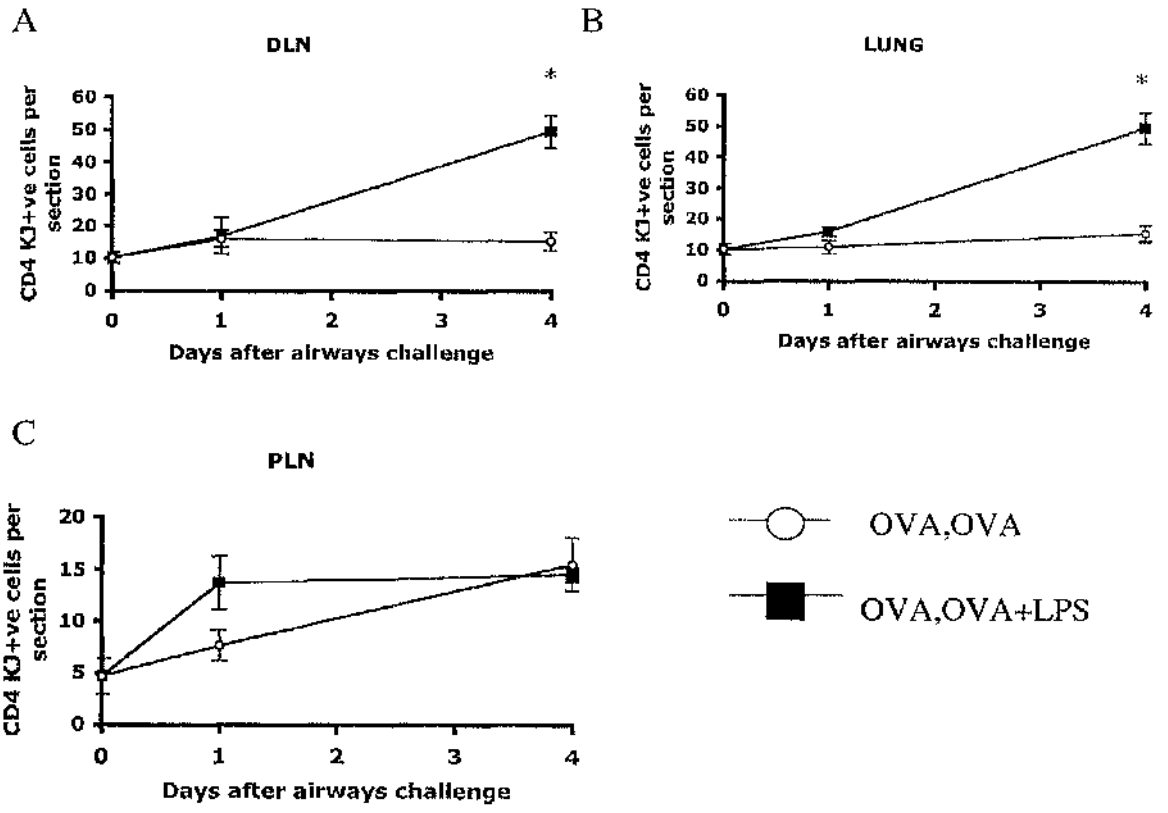

Figure 6.10: Quantification of KJ+ve/PCNA+ve cells

$3x10^6$ naïve transgenic OVA-specific T cells were adoptively transferred by intravenous injection into BALB/c recipients, which were then immunised with 100μg OVA/alum 1%, by i.p. injection, followed by one intranasal challenge 10 days later with 50μg OVA or 50μg OVA plus 2.5μg LPS. Mice were sacrificed at various timepoints. LN and lung sections were stained for OVA-specific T cells and PCNA. Double positive cells were quantified by LSC. Values are represented as mean ± SEM, n=5 per group (*$p<0.05$), n= 2 repeats.

CHAPTER 7 - GENERAL DISCUSSION

7.1 Introduction

Asthma is a complex chronic inflammatory disease of the lung characterised by airflow obstruction, airway hyperresponsiveness, and airway inflammation [539]. The inflammatory response is characterised by infiltration of the airway wall by mast cells, lymphocytes and eosinophils, together with the elevation of antigen-specific IgE. Several studies have shown a critical role for Th2 cytokines such as IL-4, IL-5 and IL-13 in the disease process [136, 154, 385]. Furthermore, abundant clinical and experimental evidence suggests that T helper cells initiate and maintain inflammation in allergic asthma [105, 124, 353]. Through the cytokines they secrete, Th2 lymphocytes drive the production of allergen-specific IgE and orchestrate the recruitment and activation of the primary effector cells, such as mast cells, and eosinophils. Despite recent advances in understanding of the pathology of asthma, there are still gaps in our knowledge regarding the mechanisms initiating, maintaining and regulating airway inflammation resulting in the disease.

Though antigen-specific T cells are widely accepted to be critical in allergic airways inflammation it has proved difficult to study where, when and how they interact with the rest of the immune system due to their low precursor frequency [355]. While the presence of isotype switched antibodies (e.g. IgE) indicate that antigen-specific T cells play a crucial role in allergic airways inflammation these cells could potentially make contributions in a variety of ways [540, 541]. For example, they may have indirect effects on other cells (e.g. eosinophils) via their production of cytokines (IL-5) and/or direct effects via production on cytokines and/or surface molecules [344, 542, 543]. As such in this thesis I developed model systems to allow me to track antigen-specific T cells in the context of allergic airways

inflammation. Using these models I have been able to describe where and when T cell activation and division likely occurs in allergic airways inflammation, the impact of non-specific inflammatory events (e.g. equivalent to infection) on this condition and how therapeutic intervention may be working.

7.2 Development of novel models of airways inflammation

The DO11.10 adoptive transfer system is well-established in studies of T cell interactions [339, 348] and has also been used in models of experimental asthma although in most cases the transgenic cells were used principally to investigate the different effects of transferring Th1 or Th2 cells rather than to examine compartment-specific contributions by initially naïve T cells [194, 242, 349, 350]. Where Ag-specific T cells have been tracked into different tissues following transfer, some studies have identified, but not quantified, transgenic T cells [121]. The remainder of studies have almost exclusively used T cells which have been polarised and activated *in vitro* to either a Th1 or Th2 phenotype before transfer [168, 195, 251, 351-354]. The model established here differs in that the development of eosinophilic airways inflammation is combined with the transfer of naïve antigen (OVA)-specific T cells. This enables the developing T cell responses to be analysed *in vivo* via the ability both to identify and quantify Ag-specific T cells in different anatomical locations (lymph nodes, lungs and BAL fluid) through every stage from initiation of activation, through differentiation to effector function.

The adaptation of the well established OVA induced airways inflammation model [341] used by many groups to study airways inflammation resulted in pathology as previously shown.

This model, consisting of three OVA immunisation followed by an OVA airways challenge (long model), produces BAL eosinophilia, lung inflammation, increased OVA-specific IgE and IgG1, and increased IL-5 production. The response to antigen challenge in this model is similar to that seen in humans, which consists of an IgE-mediated inflammatory cascade in the airways, characterised by the influx of Th2 lymphocytes and eosinophils and chronic pulmonary inflammation [361]. Several studies have shown the importance of T cells in the effector phase of asthma, from their ability to migrate to the lung in response to antigen challenge, and their role in the development of eosinophils and IL-5 production [362-368]. Previous work carried out in this lab has shown that the depletion of $CD4^+$ T cells in the models described above, results in loss of airway inflammation and associated pathology [544]. Indeed, the pathology in this "long model" described above was associated with recruitment of antigen-specific T cells to the airways.

A variation of the long airways model where one OVA immunisation and one OVA airways challenge is administered, the "short model", did not induce any BAL eosinophilia. This is in contrast to the long model where one OVA challenge results in recruitment of eosinophils to the airway.

Lung inflammation caused by LPS has been used as a model of acute lung injury, mirroring some features of acute respiratory distress syndrome in humans [357]. To determine if LPS would be sufficient to induce eosinophilia it was added to OVA and given as the airways challenge in the short model. When OVA plus LPS was introduced into this model as the airway challenge, instead of OVA alone, a significant BAL eosinophilia was induced.

Analysis of inflammation in lung histological sections also showed a trend towards increased inflammation in animals challenged with OVA plus LPS. This was accompanied by a significant increase in BAL IL-5 production compared with OVA alone as seen previously in the long model. No difference was seen between antibody levels when OVA and OVA+LPS were compared. The increase in IL-5 production with the addition of LPS to OVA in the airways challenge may help to induce more eosinophilia in the short model. Antigen-specific T cells were measured in the DLN and BAL fluid by flow cytometry and OVA+LPS treatment resulted in accumulation of T cells in the BAL and lung sections.

Overall, these results suggest that the addition of LPS to a model that previously showed no airway inflammation or associated pathology has been switched to a model with pathology. This is associated with an increase in IL-5 production and recruitment of antigen-specific T cells to the BAL. This recruitment of T cells also suggests a role for them in the effector phase of the response, after the initial sensitisation.

The use of LPS allowed the development of a shorter model that produced the same end result as the long model with only an OVA airways challenge. This raised the question of whether we were looking at two different routes to the same endpoint or whether they had different pathologies. To address this question an initial screen of co-stimulatory molecules, cytokine and chemokine expression on OVA-specific T cells, was performed by quantitative RT-PCR to phenotype the two models. It was found that although the two different models produced the same results, the route by which it was reached was different. These preliminary results emphasize the need for caution when comparing different studies from different groups, even if the end results seem comparable.

These experiments have optimised and characterised robust models of murine airway inflammation. The use of adoptively transferred OVA-specific T cells allows the identification and quantification of antigen-specific cells in the lymph nodes, lungs and BAL over a timecourse, without obviously affecting the disease outcome. These models will enable investigation into the effects of exogenous inflammation (LPS), an anti-inflammatory therapy (anti-TNF alpha) and to track cell divisions and interactions in cellular compartments over the course of the disease.

7.3 Using novel models to investigate the location of T cell division

We next went on to use these models to examine the location and timing of T cell division in the lung, DLN and PLN in the airways model. The recirculation of T cells through secondary lymphoid organs and peripheral and non-lymphoid tissues is fundamental to the maintenance of immune surveillance. It is believed that naïve T cells recirculate randomly through secondary lymphoid sites until they are stimulated by antigen conveyed there from tissues by antigen presenting cells [495, 496]. During the ensuing T cell activation, naïve T cells are imprinted with a template of the site in which they were initially stimulated [497]. These cells then differentiate to acquire a tissue specific homing phenotype via the expression of different receptor and signalling molecules, which facilitate return to that site [498]. The development of specific cell-mediated pulmonary immunity requires an afferent phase, during which inhaled antigens are captured, processed and presented to T cells in the context of MHCII on antigen presenting cells [502] and an efferent phase in which T cells are trafficked through lymph nodes to sites of inflammation [545]. Thus, T cells are potentially involved in two phases of airway inflammation.

Previous studies depleting $CD4^+$ T cells in a murine model of airways inflammation before antigen challenge have shown reduction in AHR response to acetylcholine challenge and reduced eosinophilia, IgE and IL-13 production [505]. A study using A/J mice in an OVA induced model of airways inflammation with administration of anti-CD4 mAb prior to airways challenge caused reduction in AHR and lung eosinophilia [124] and lung eosinophilia was also prevented in a murine model of airways inflammation in CD4 deficient mice [363]. These studies suggest an important role for T cells in the induction and maintenance of immune responses to antigen. Gaining an insight into the location and timing of T cell division and activation will enable more accurate therapeutic intervention in airways inflammation. The information obtained in these experiments may allow T cells to be targeted before they become activated or to stop cell migration to lymph nodes and subsequent reduce the ensuing immune response to encountered antigen.

To determine the site of T cell division in airway inflammation, antigen-specific T cells were tracked in different compartments throughout the course of the disease and proliferating cell nuclear antigen (PCNA) expression was measured on antigen-experienced T cells as an indication of their recent division. When T cell division was quantified by LSC in the long model it was found that there was an early wave of T cell division in the lung at day one post airways challenge, followed by similar in the DLN at day three post challenge. At day five post challenge there was another wave of antigen-specific T cell division seen in the lung tissue. The early T cell division may have been caused by uptake and presentation of antigen by resident dendritic cells, which have been shown to be present in the lung [518]. The location of these dendritic cells allows them to have direct contact with incoming antigens. It is known that there is a dendritic cell network situated immediately above and

beneath the basement membrane of the upper and lower airways enabling these cells to sample the epithelium for inhaled antigens [225, 509, 521]. It has also been shown in experimental models of respiratory infection that a population of memory T cells are established in the lung parenchyma and the lung airways [522, 523]. Airway resident memory cells contribute significantly to recall responses by providing immediate effector activity at the site of antigen entry [524], showing that there is local antigen presentation in the lung [525].

Upon antigen encounter, respiratory tract DCs migrate to the draining mediastinal lymph nodes where they control the activation and differentiation of antigen-specific T cells [526]. Activated effector T cells will migrate back to the site of inflammation and will be (re)stimulated locally by activated DCs [525]. It has been shown that different subsets of DCs are responsible for antigen transportation in response to different types of antigen [527]. A study by De heer *et al.*, showed both myeloid and plasmacytoid DCs take up antigen within the draining lymph node in response to OVA inhalation, but only the myeloid subset was shown to present antigen to naïve $CD4^+$ T cells [231]. This movement and activation of T cells corresponds to the peak of antigen-specific T cell division seen in the DLN at day three post airways challenge. The subsequent wave of T cell expansion seen in the lung may be due to T cells returning to the site of inflammation where they initially encountered antigen after being imprinted in the DLN. It has been hypothesised that during activation naïve T cells are imprinted with a template of the site in which they were initially stimulated [497]. These cells then differentiate to acquire a tissue-specific homing phenotype which facilitates there return to that site [481, 498]. The idea that antigen-specific T cells can home back to the site of initial antigen encounter parallels the data shown in this study in which

there is a second wave of T cell division seen in the lung tissue at day five post airways challenge. The two stages of T cell division in the lung mimics the data shown by Catron *et al.*, who showed that as well as an initial T cell response to antigen, a population of "late-arriving" T cells are present after antigen challenge that may be central memory cells and were shown to proliferate more extensively than the resident T cells [513]. These cells may be critical for the chronic pathology that is seen in asthma patients. This data gives an insight into the timing and location of Ag-specific T cell division in airways inflammation and will be important for rational design and application of therapeutics.

7.4 Using novel models to assess the impact of TLR ligands on allergic airways inflammation

The adoptive transfer models developed enabled investigation into the effects of exogenous sources of inflammation on allergic airways inflammation. Epidemiological studies have suggested that overall prevalence of atopy and asthma in a population is inversely related to the level of endotoxin in the environment [391, 395, 396] but in patients known to have asthma, the level of endotoxin in house dust is positively related to disease severity [394, 397, 399, 400]. The data presented here show conclusively that in a murine model of eosinophilic airways inflammation, concurrent administration of LPS or BLP with antigen-challenge exacerbates the subsequent inflammation in previously sensitised mice. Importantly, these data also show that where the protocol is suboptimal for inducing airway eosinophilia, the addition of TLR ligands can promote an inflammatory outcome. Furthermore the route of administration of TLR agonist is critical as systemic injection of the same agents lead to suppression of airway inflammation. Comparing the data presented

here, with those of other studies suggests that not only is route of administration important but also dose and timing, going some way to explain the conflicting results seen in animal and human studies [332, 407].

The effect of TLR agonists, LPS and BLP, administered locally and systemically at the time of airways challenge was investigated. The protocols used for these studies use mice previously sensitised to the allergen, which may represent a more clinically relevant model of disease, as most patients will already have been sensitised when they seek treatment. This approach differs from many other studies that have looked at TLR administration at the time of sensitisation [401, 407-409, 433-435]. The results suggest that exposure to microbial products lead to the worsening of airways inflammation in the model. This is supportive of studies done in humans [436-440], which showed that inhalation of LPS by allergic asthmatics induced airways inflammation, including increased eosinophilia in the airways. The experiments imply that this may be due to an accumulation of antigen-specific T cells in the airways, which may affect the underlying adaptive immune response. This may also lead to increased sensitisation to other allergens, as Th2 cells have been shown to facilitate priming of naïve T cells to unrelated antigen through IL-4 secretion [441]. This has been seen in atopic patients that accumulate allergens over time [442, 443].

Throughout this study LPS has been used in conjunction with OVA and ALUM which are known to induce a Th2 phenotype. LPS was shown to exacerbate this response. It has been shown that innate immune responses to LPS can modulate adaptive immune responses to allergens, and that low doses of LPS can induce Th1 type responses and high doses of LPS

can induce Th2 responses [576] It is also known that signalling through TLR is required for adaptive Th1 responses and unclear if it is needed for Th2 priming [577[. This suggests that although the addition of LPS in the adoptive transfer model used in this study exacerbated the Th2 response, it is possible that if OVA was given with a Th1 antigen that Th1 responses would be augmented as well as LPS is not classically a Th2 inducer.

When compared to other studies, when LPS is given locally during antigen challenge, my results are in concurrence, showing exacerbation of airways inflammation [332, 407, 433]. However, there are also conflicting studies showing amelioration of the inflammatory response [410, 433, 444, 445]. The use of systemic LPS to suppress the allergic response agrees with the data presented in this thesis [432, 444]. When LPS has been administered during sensitisation either locally or systemically most studies have shown a reduction in airways inflammation [423, 432, 446, 447]. In spite of this, caution must be taken when comparing these studies as different routes of administration, doses, timing and species have been used throughout the publications.

Several mechanisms have been proposed to explain LPS induction of eosinophilia and proinflammatory cytokines. DCs are well recognized to play a central role in inflammatory reactions elicited by LPS [421]. When DCs are activated by LPS through TLR4, they become mature and acquire an increased ability to prime T cells [422]. A study by Eisenbarth *et al.*, reported that low level inhaled LPS signaling through TLR4 is necessary to induce Th2 responses to inhaled antigens in a mouse model of allergic sensitization. The mechanism by which LPS signaling results in Th2 sensitization was shown to involve the

activation of antigen-containing dendritic cells [407]. However, a recent study proposed that LPS-induced MCs activation and modulation with increased production of Th2 cytokines, such as IL-5 and IL-13, appear to control the severity of eosinophilic airway inflammation [423]. It is known that IL-5 and IL-13 play a crucial role in the induction and the severity of eosinophilic infiltrate in airway inflammation in the lung [362]. IL-5 is a key factor for eosinophilia and could be responsible for some tissue damage in chronic asthma [424].

In the context of clinical disease, these results suggest that exposure to bacterial products would be detrimental to patients with asthma, a conclusion that may seem self-evident. There are however two further important interpretations that can be made from these results. Firstly, the suggestion that microbial products and TLR ligands could be used in a therapeutic capacity in asthma [408, 546] should be considered carefully. Although some studies have shown promising results, whether the outcome after administration of these agents is suppression or exacerbation of inflammation is clearly dependent on a number of factors and requires further clarification before any therapeutic potential can be realised. Moreover the results reported here show that the suppressive effect following systemic administration was transient reducing the possible utility of these agents as long-term treatments. The second conclusion from these experiments is that the pro-inflammatory effect of local administration of LPS or BLP was associated with the accumulation of Ag-specific T cells in the airway.

7.5 Using novel models to examine mode of action of potential therapeutics

The use of the antagonist etanercept in the airways model allowed investigation into the mode of action of this therapy. It was found that TNFα blockade caused reduction in airways inflammation and associated pathology possibly via its ability to inhibit draining lymph node hyperplasia, suggesting a possible novel mode of action for anti-TNFα therapies.

Information on the critical locations of cells and cellular interactions should provide new ways of targeting the pathways involved. Recent studies have shown that MC-derived TNFα may regulate local lymph node enlargement in response to infection [305]. Given the importance of MCs in asthma [113, 205, 207] and recent interest in TNFα in airways disease [277, 459, 547], the effect of TNFα blockade in the role of lymph node hypertrophy in airways inflammation was investigated. When lymph node hypertrophy was inhibited by TNFα blockade in the airways model, it was associated with a reduction in draining lymph node cellularity, BAL eosinophilia and IL-5, and antigen-specific IgE production. This supported the notion that suppression of the lymph node response may be a novel route by which anti-TNF agents may act [305]. This hypothesis could also be applied to other diseases where TNFα blockade has been proposed [456].

One means by which TNFα may be exerting its effects is by blocking lymph node hypertrophy. It is known that naïve T cells continually enter and exit lymphoid organs in a recirculation process that is essential for immune surveillance. During an immune response

the egress process can be shut down transiently [474]. When this occurs locally it increases lymphocyte numbers in the responding lymphoid organ. Several mediators of the innate immune system are known to cause shutdown, including TNF α, although the mechanisms are still unclear [475-477]. TNFα has also been shown to be capable of altering the number and specificity of lymphocytes recirculating through stimulated lymph nodes by selectively altering the entry of lymphocytes into the efferent lymphatics of inflamed lymph nodes *in vivo* [478-480].

As noted earlier, lymphocyte trafficking is not a random event as although naïve T cells appear to circulate without preference for any one group of lymph nodes, memory T cells preferentially return to the tissues associated with the lymph node groups in which they became sensitised: as process known as lymphocyte homing [481]. This process is controlled by adhesion receptors on the lymphocyte binding to vascular addressins selectively expressed on the endothelium [482, 483]. In this thesis the role of L-selectin, which is expressed on most lymphocytes, and is the key receptor that initiates lymphocyte capture events in high endothelial venules in secondary lymphoid organs, and has been shown to be important in airways inflammation, was examined in anti-TNFα experiments. It was found that cell there was no difference in L-selectin expression on antigen-specific T cells between the anti-TNFα treated and control mice, suggesting that inhibition of lymph node hypertrophy was not mediated by L-selectin in these experiments.

7.6 Future studies

The study of cell trafficking is fascinating not just because of its obvious relevance to the treatment of diseases, but also because an increasing number of technological advances are

allowing us to visualise processes as never before [355]. Identifying and quantifying cells in different anatomical locations, as has been carried out here, will become the most basic of information obtained.

Host immunity and immunopathology are mediated largely by cytokines which are short-lived effector molecules rapidly secreted by different cell types in many different tissue compartments. Analysis of cytokine activities *in vivo* has been extrapolated from *in vitro* studies using isolated cells and through generation of cytokine knockout and transgenic overexpressing mice. Although many insights have been gained by these approaches, disadvantages are also apparent. *In vitro* analysis of cells removes them from their *in situ* environment and from potentially critical signals present in tissues, and exogenous cytokines are often provided in amounts that might not be physiological. Furthermore, knock out or reporter knock in mice preclude the ability to judge the contributions of the cytokine that has been deleted since it can no longer contribute to the immune response[548]. The development of fluorescent reporter genes allows cells that are activated, or producing specific cytokines, to be identified. Thus, cells from mice expressing the enhanced green fluorescent protein gene linked to IL-4 become fluorescent when IL-4 is produced, allowing the visualisation of a Th2-type response [548, 549]. Cross-breeding these mice with strains expressing transgenic T cell receptors would produce cells that allow an antigen-specific Th2 response to be monitored in real time in distinct compartments *in vivo*. The adoptive transfer techniques used in this thesis have recently been refined so that fewer cells can be transferred and as few as ten antigen-specific cells can be recovered and identified allowing a more physiological level of response to be tracked. Furthermore, not only can Ag-specific

T cells be tracked as demonstrated here but antigen processing and presentation can now be demonstrated [550].

Data on antigen presentation have come primarily from *in vitro* studies or examination of the late *in vivo* consequences of presentation, analyses that do not reveal the details of the early steps in this process in a physiological setting. Recently, however, new methods of static and dynamic imaging have begun to overcome the barriers to understanding these events, providing new insights into when and where antigen acquisition and processing takes place, as well as where T cells and APCs interact and for how long. Measurements of the rate and directionality of lymphocyte movement are emerging from the application of four-dimensional imaging techniques to isolated lymphoid tissues and even tissues in living animals.

Furthermore, the journey of antigen, from uptake to processing, and finally to presentation and activation of T cells can now be visualised [550]. Using the Eα-RFP system a fluorescently-labelled antigen can be identified (either free or within cells) until its fluorescence is lost through antigen processing. Presentation of the processed peptide (p Eα:I-A^b) in the context of MHC class II can then be detected by the use of a specific monoclonal Ab labelled with a second fluorochrome (Y-Ae) [551]. The picture is completed by the use of transgenic T cells (Tea T cells) with TCRs specific for the peptide-MHC complex [552]. The ability to identify every step in the sequence from antigen exposure through processing and presentation to T cell recognition provides a unique opportunity to study the cellular interactions that take place in antigen-induced allergic airways disease. Work from this thesis has demonstrated that $CD4^+$ T cells are essential

during the effector phase of the allergic airways response and suggest that they play an important role in the airways. Using the system described above, it should be possible to demonstrate antigen uptake and presentation ongoing in the airways during this response and possibly to detect effects on antigen presentation mediated by changes in the experimental conditions.

The use of the Eα-RFP allows the location and timing of antigen uptake and presentation to be tracked *in vivo* in disease models and has shown the importance of the interaction between T cells and DCs. Other studies have also shown an important role for dendritic cells in the airways, the implication being that antigen presentation by DCs to T cells takes place in the airways, rather than in secondary lymphoid organs, and is important during the effector arm of the response [230, 233, 553, 554]. Recently, a novel diptheria toxin (DT) based system was developed, which allows the short term inducible ablation of DC *in vivo* [555]. This system has been used to show that depletion of DCs results in a complete inability to cross-prime $CD8^+$ T cell responses against *Listeria monocytogenes* and *Plasmodium yoelii* [555] and to prime LCMV-specific $CD8^+$ T cell responses [556]. Analysis of the structure of lymphoid organs after $CD11c^+$ depletion by DT also resulted in complete and protracted depletion of marginal zone and matallophillic macrophages [557]. These mice will allow the contribution of DCs in diseases to be investigated.

Many chemokines have been implicated in allergic airways inflammation and strategies to inhibit T cell migration to the lung are directed to T cell adhesion or chemoattraction. Inhibiting the interaction between adhesion molecules and their ligands can target T cell adhesion. In this respect, the use of VLA-4 inhibitors may be relevant in asthma, as demonstrated by their effects in allergen-induced inflammatory responses in sheep [558].

OVA-sensitised and challenged ICAM-1 deficient mice have a dramatically reduced inflammatory influx into the airways plus attenuation of AHR compared to wild type mice [559]. However, no clinical data on blocking integrins or antibodies to ICAM-1 or VCAM-1 are available for asthma. Treatment with specific antagonists to chemokine receptors is in development for asthma. Pertussis toxin-treated Th2 cells have been described to be unable to traffic into the airways and to induce eosinophilic airway inflammation following OVA challenge in a mouse model of asthma [242]. Thus, chemokine receptor antagonism may become an important approach in the treatment of asthma. Therefore, although in the etanercept experiments L-selectin was not shown to be involved in the inhibition of lymph node hyperplasia, there are clearly many other chemokines involved in airways inflammation and migration of cells between lymph nodes and lungs that could be targeted as therapies for asthma. Further investigation of these molecules in the airways inflammation model used throughout this thesis would give valuable insight into the mechanisms involved in airways inflammation and possible therapeutic targets.

The experiments described using PCNA as a marker of recent cell division showed that there may be more than one phase of T cell division in different compartments that are potentially important in driving the subsequent immune responses. In order to effectively target therapies in diseases such as allergic airways inflammation it is important to understand the relevance of the T cell divisions at these timepoints and whether the T cells are functionally important in the draining lymph node or the lung. In recent years there has been increased interest in the use of FTY720 as a therapeutic drug in models of transplantation and autoimmunity and it has recently been shown to be efficacious in kidney transplantation [531-534]. FTY720 selectively and reversibly sequesters lymphocytes via the sphingosine

1-phosphate receptor, but not monocytes or granulocytes, from blood and spleen into secondary lymphoid organs, preventing their migration towards sites of inflammation [535-537]. More recently a study using FTY720 in a murine model of allergic airways inflammation showed administration of the drug before antigen airways challenge abrogated airways inflammation by inhibition of the migration of dendritic cells to draining lymph nodes, which in turn inhibited the formation of allergen-specific Th2 cells in lymph nodes [538]. This study again highlights the importance of using systems such as the adoptive transfer system, where tracking of cells of known specificity allows identification of both T cells and dendritic cells and their possible interactions in diseases.

Although the techniques described above will allow kinetic analysis of T cell and antigen-presenting cell interactions in disease models such as the adoptive transfer model described in this thesis, the emergence of new imaging techniques has allowed the behaviour of specific cells to be studied *in vivo* in real time [355, 513, 560-563]. The use of 2-photon microscopy has allowed the behaviour of cells with differing phenotypes to be characterised for the first time in intact organs.

There are many factors that are likely to shape the magnitude, character and functional output of the resulting immune response including the Ag dose, the type and availability of APCs bearing Ag, the presence and nature of costimulatory signals and the migratory behaviour of T cells and APC. The use of 2-photon microscopy can be used to determine many of these factors in real time, *in vivo*.

Two photon laser microscopy is based on the fact that the fluorophore is excited by near-simultaneous adsorption of two higher wavelength photons instead of a single low light

photon. These effects enable imaging at a depth of up to 300 microns and has improved biocompatibility compared to confocal imaging [564]. Since the first demonstration of multiphoton *in vivo* dynamic imaging of lymphocytes these approaches have given valuable insight into the mechanisms involved in immune responses [565].

Multiphoton microscopy on exposed or explanted LN has allowed imaging analysis of individual cellular interactions over several hours. The behaviour of individual T cells and T cell-DC interactions with and without antigen encounter has been studied. It was found that naïve T cells are incredibly motile, covering up to 25μm per minute, following a random walk rather than directed migration [563] and contacting multiple DCs per hour [560]. Once T cells encounter antigen they cluster around the APC inducing activation of the T cell and upregulation of CD69 [563, 566]. Subsequently, the T cells become motile again and make contact with further antigen-presenting DCs, enhancing their activation and allowing upregulation of the IL-2 receptor and production of IFNγ [567].

The involvement of chemokines and cytokines in the regulation and induction of the immune response has also been studied using multiphoton microscopy. Okada *et al.*, demonstrated that B cells upregulate CCR7 and migrate to the boundary of the follicle following activation [568]. Upon encountering the appropriate T cell, the B cell-T cell interaction can last for over 1 hour. Recently, this information has allowed researchers to investigate circumstances in which this normal immune response is altered, especially in the regulation of immune responses. Schneider *et al.* demonstrated that CTLA-4 increases T cell motility and prevents the formation of stable contacts between T cells and APC, providing a potential mechanism by which CTLA-4 may modulate the activation of T cells [569]. Thus, use of

multiphoton microscopy has recently allowed the detailed examination of cellular migration and interactions within the LN.

Multiphoton microscopy may be beneficial in studying the characteristics of cells in disease models, giving information on when and where cells interact and whether their characteristics affects the outcome of the disease. This method may give an insight into whether drugs are able to affect cell movement and speed. For example, studying the cells in draining lymph nodes in mice treated with etanercept could determine if anti-TNFα blockade affects the migration of cells from lymph nodes.

7.7 Summary

In summary, the data presented here has utilised a robust model of airways inflammation, using the transfer of naïve antigen-specific T cells which can be tracked and quantified in different anatomical locations (BAL, lung DLN and PLN) *in vivo*. It was found that T cell division occurs early in the lung after antigen inhalation, which may drive the division of T cells in the DLN resulting in eosinophilic inflammation. The data suggests that local antigen presentation in the lung may be important in driving the subsequent immune responses. The role of TLR2 and TLR4 in airways inflammation was investigated and it was found that bacterial products, such as BLP and LPS, could exacerbate eosinophilic inflammation in previously sensitised mice. It was also shown that the timing and route of administration of LPS/BLP could affect the outcome of the subsequent response. The model was also used to investigate the mechanism action of potential therapeutics. It was found that TNFα blockade caused a reduction in airways inflammation and associated pathology, possibly by blocking lymph node hypertrophy, suggesting a novel mode of action for anti-TNFα therapies.

REFERENCES

1. Schiller, J.S., P.F. Adams, and Z.C. Nelson, *Summary health statistics for the U.S. population: National Health Interview Survey, 2003*. Vital Health Stat 10, 2005(224): p. 1-104.
2. Cohn, L., J.A. Elias, and G.L. Chupp, *Asthma: mechanisms of disease persistence and progression*. Annu Rev Immunol, 2004. **22**: p. 789-815.
3. Holgate, S.T., *The epidemic of allergy and asthma*. Nature, 1999. **402**(6760 Suppl): p. B2-4.
4. *The Burden of Lung Disease*. 2001, The British Thoracic Society: London.
5. Mathers CD, L.D., *Updated projections of global mortality and burden of disease, 2002-2030: data sources, methods and results*. 2005, World Health Organization.
6. Masoli M, F.D., Holt S, Beasley R, *Global Burden Of Asthma*. 2004, Global Initiative for Asthma.
7. *Global Initiative for Chronic Obstructive Lung Disease, Executive Summary: Global Strategy for the diagnosis, management and prevention of Chronic Obstructive Pulmonary Disease*. 2001, National Heart, Lung and Blood Insitute, National Institutes of Health: Bethesda.
8. Burney, P.G., S. Chinn, and R.J. Rona, *Has the prevalence of asthma increased in children? Evidence from the national study of health and growth 1973-86*. Bmj, 1990. **300**(6735): p. 1306-10.
9. Upton, M.N., et al., *Intergenerational 20 year trends in the prevalence of asthma and hay fever in adults: the Midspan family study surveys of parents and offspring*. Bmj, 2000. **321**(7253): p. 88-92.

10. Burney, P., *The changing prevalence of asthma?* Thorax, 2002. **57 Suppl 2**: p. II36-II39.

11. Strachan, D.P., *Hay fever, hygiene, and household size.* Bmj, 1989. **299**(6710): p. 1259-60.

12. van Schayck, C.P. and H.A. Smit, *The prevalence of asthma in children: a reversing trend.* Eur Respir J, 2005. **26**(4): p. 647-50.

13. Netea, M.G., J.W. Van der Meer, and B.J. Kullberg, *Recognition of pathogenic microorganisms by Toll-like receptors.* Drugs Today (Barc), 2006. **42 Suppl A**: p. 99-105.

14. Anderson, K.V., L. Bokla, and C. Nusslein-Volhard, *Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product.* Cell, 1985. **42**(3): p. 791-8.

15. Hashimoto, C., K.L. Hudson, and K.V. Anderson, *The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein.* Cell, 1988. **52**(2): p. 269-79.

16. St Johnston, D. and C. Nusslein-Volhard, *The origin of pattern and polarity in the Drosophila embryo.* Cell, 1992. **68**(2): p. 201-19.

17. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.

18. Rock, F.L., et al., *A family of human receptors structurally related to Drosophila Toll.* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 588-93.

19. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5*. Nature, 2001. **410**(6832): p. 1099-103.

20. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors*. Annu Rev Immunol, 2003. **21**: p. 335-76.

21. Takeuchi, O., K. Hoshino, and S. Akira, *Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection*. J Immunol, 2000. **165**(10): p. 5392-6.

22. Zhang, D., et al., *A toll-like receptor that prevents infection by uropathogenic bacteria*. Science, 2004. **303**(5663): p. 1522-6.

23. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene*. Science, 1998. **282**(5396): p. 2085-8.

24. Lund, J.M., et al., *Recognition of single-stranded RNA viruses by Toll-like receptor 7*. Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5598-603.

25. Tabeta, K., et al., *Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection*. Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3516-21.

26. Lund, J., et al., *Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells*. J Exp Med, 2003. **198**(3): p. 513-20.

27. Netea, M.G., et al., *Recognition of fungal pathogens by Toll-like receptors*. Eur J Clin Microbiol Infect Dis, 2004. **23**(9): p. 672-6.

28. Campos, M.A., et al., *Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite*. J Immunol, 2001. **167**(1): p. 416-23.

29. Scanga, C.A., et al., *Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells.* J Immunol, 2002. **168**(12): p. 5997-6001.

30. Becker, I., et al., *Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2.* Mol Biochem Parasitol, 2003. **130**(2): p. 65-74.

31. Mun, H.S., et al., *TLR2 as an essential molecule for protective immunity against Toxoplasma gondii infection.* Int Immunol, 2003. **15**(9): p. 1081-7.

32. Hoffmann, J.A. and J.M. Reichhart, *Drosophila innate immunity: an evolutionary perspective.* Nat Immunol, 2002. **3**(2): p. 121-6.

33. Akira, S. and K. Takeda, *Functions of toll-like receptors: lessons from KO mice.* C R Biol, 2004. **327**(6): p. 581-9.

34. Ozinsky, A., et al., *The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13766-71.

35. Kawai, T., et al., *Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes.* J Immunol, 2001. **167**(10): p. 5887-94.

36. Wyllie, D.H., et al., *Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses.* J Immunol, 2000. **165**(12): p. 7125-32.

37. Akira, S., *Toll-like receptors: lessons from knockout mice.* Biochem Soc Trans, 2000. **28**(5): p. 551-6.

38. Jack, R.S., et al., *Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection.* Nature, 1997. **389**(6652): p. 742-5.

39. Jiang, Q., et al., *Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B.* J Immunol, 2000. **165**(7): p. 3541-4.

40. Shimazu, R., et al., *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.* J Exp Med, 1999. **189**(11): p. 1777-82.

41. Kawai, T., et al., *Unresponsiveness of MyD88-deficient mice to endotoxin.* Immunity, 1999. **11**(1): p. 115-22.

42. Kaisho, T., et al., *Endotoxin-induced maturation of MyD88-deficient dendritic cells.* J Immunol, 2001. **166**(9): p. 5688-94.

43. Huang, Q., et al., *The plasticity of dendritic cell responses to pathogens and their components.* Science, 2001. **294**(5543): p. 870-875.

44. Flo, T.H., et al., *Differential expression of Toll-like receptor 2 in human cells.* J Leukoc Biol, 2001. **69**(3): p. 474-81.

45. Zarember, K.A. and P.J. Godowski, *Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines.* J Immunol, 2002. **168**(2): p. 554-61.

46. Fichorova, R.N., et al., *Response to Neisseria gonorrhoeae by cervicovaginal epithelial cells occurs in the absence of toll-like receptor 4-mediated signaling.* J Immunol, 2002. **168**(5): p. 2424-32.

47. Netea, M.G., et al., *Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system.* J Leukoc Biol, 2004. **75**(5): p. 749-55.

48. Weiss, D.S., et al., *Toll-like receptors are temporally involved in host defense.* J Immunol, 2004. **172**(7): p. 4463-9.

49. Ito, T., et al., *Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets.* J Exp Med, 2002. **195**(11): p. 1507-12.

50. Jarrossay, D., et al., *Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells.* Eur J Immunol, 2001. **31**(11): p. 3388-93.

51. Kadowaki, N., et al., *Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens.* J Exp Med, 2001. **194**(6): p. 863-9.

52. Krug, A., et al., *Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12.* Eur J Immunol, 2001. **31**(10): p. 3026-37.

53. Boonstra, A., et al., *Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation.* J Exp Med, 2003. **197**(1): p. 101-9.

54. Edwards, A.D., et al., *Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines.* Eur J Immunol, 2003. **33**(4): p. 827-33.

55. Sallusto, F., et al., *Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation.* Eur J Immunol, 1998. **28**(9): p. 2760-9.

56. Dieu, M.C., et al., *Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites.* J Exp Med, 1998. **188**(2): p. 373-86.

57. McWilliam, A.S., et al., *Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces.* J Exp Med, 1994. **179**(4): p. 1331-6.

58. Flores-Romo, L., *In vivo maturation and migration of dendritic cells.* Immunology, 2001. **102**(3): p. 255-62.

59. Woltman, A.M. and C. van Kooten, *Functional modulation of dendritic cells to suppress adaptive immune responses.* J Leukoc Biol, 2003. **73**(4): p. 428-41.

60. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.

61. Mazzoni, A. and D.M. Segal, *Controlling the Toll road to dendritic cell polarization.* J Leukoc Biol, 2004. **75**(5): p. 721-30.

62. Goldsby, R., et al., *Immunology.* Fifth ed. 2003: W H Freeman and company.

63. Kaech, S.M., et al., *Molecular and functional profiling of memory CD8 T cell differentiation.* Cell, 2002. **111**(6): p. 837-51.

64. McHeyzer-Williams, L.J. and M.G. McHeyzer-Williams, *Antigen-specific memory B cell development.* Annu Rev Immunol, 2005. **23**: p. 487-513.

65. Boes, M. and H.L. Ploegh, *Translating cell biology in vitro to immunity in vivo.* Nature, 2004. **430**(6996): p. 264-71.

66. Rudolph, M.G., R.L. Stanfield, and I.A. Wilson, *How TCRs bind MHCs, peptides, and coreceptors.* Annu Rev Immunol, 2006. **24**: p. 419-66.

67. Wang, S. and L. Chen, *T lymphocyte co-signaling pathways of the B7-CD28 family*. Cell Mol Immunol, 2004. **1**(1): p. 37-42.

68. Sharpe, A.H. and G.J. Freeman, *The B7-CD28 superfamily*. Nat Rev Immunol, 2002. **2**(2): p. 116-26.

69. Bonhagen, K., et al., *ICOS+ Th cells produce distinct cytokines in different mucosal immune responses*. Eur J Immunol, 2003. **33**(2): p. 392-401.

70. Steinman, R.M., *The dendritic cell system and its role in immunogenicity*. Annu Rev Immunol, 1991. **9**: p. 271-96.

71. Matzinger, P., *Tolerance, danger, and the extended family*. Annu Rev Immunol, 1994. **12**: p. 991-1045.

72. Hart, D.N., *Dendritic cells: unique leukocyte populations which control the primary immune response*. Blood, 1997. **90**(9): p. 3245-87.

73. Randolph, G.J., V. Angeli, and M.A. Swartz, *Dendritic-cell trafficking to lymph nodes through lymphatic vessels*. Nat Rev Immunol, 2005. **5**(8): p. 617-28.

74. Shortman, K. and Y.J. Liu, *Mouse and human dendritic cell subtypes*. Nat Rev Immunol, 2002. **2**(3): p. 151-61.

75. Banchereau, J., et al., *Immunobiology of dendritic cells*. Annu Rev Immunol, 2000. **18**: p. 767-811.

76. Ho, I.C. and L.H. Glimcher, *Transcription: tantalizing times for T cells*. Cell, 2002. **109 Suppl**: p. S109-20.

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

78. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.

79. Romagnani, S., *Lymphokine production by human T cells in disease states.* Annu Rev Immunol, 1994. **12**: p. 227-57.

80. Murphy, K.M. and S.L. Reiner, *The lineage decisions of helper T cells.* Nat Rev Immunol, 2002. **2**(12): p. 933-44.

81. O'Garra, A. and D. Robinson, *Development and function of T helper 1 cells.* Adv Immunol, 2004. **83**: p. 133-62.

82. Szabo, S.J., et al., *A novel transcription factor, T-bet, directs Th1 lineage commitment.* Cell, 2000. **100**(6): p. 655-69.

83. Szabo, S.J., et al., *Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells.* Science, 2002. **295**(5553): p. 338-42.

84. Zhang, D.H., et al., *Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene.* J Biol Chem, 1997. **272**(34): p. 21597-603.

85. Zheng, W. and R.A. Flavell, *The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells.* Cell, 1997. **89**(4): p. 587-96.

86. Ito, T., et al., *TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand.* J Exp Med, 2005. **202**(9): p. 1213-23.

87. Siveke, J.T. and A. Hamann, *T helper 1 and T helper 2 cells respond differentially to chemokines.* J Immunol, 1998. **160**(2): p. 550-4.

88. Austrup, F., et al., *P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflammed tissues.* Nature, 1997. **385**(6611): p. 81-3.

89. Sallusto, F. and A. Lanzavecchia, *Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression.* Immunol Rev, 2000. **177**: p. 134-40.

90. O'Byrne, P.M. and M.D. Inman, *Airway hyperresponsiveness.* Chest, 2003. **123**(3 Suppl): p. 411S-6S.

91. Cockcroft, D.W., et al., *Bronchial reactivity to inhaled histamine: a method and clinical survey.* Clin Allergy, 1977. **7**(3): p. 235-43.

92. Juniper, E.F., P.A. Frith, and F.E. Hargreave, *Airway responsiveness to histamine and methacholine: relationship to minimum treatment to control symptoms of asthma.* Thorax, 1981. **36**(8): p. 575-9.

93. Sulakvelidze, I., et al., *Increases in airway eosinophils and interleukin-5 with minimal bronchoconstriction during repeated low-dose allergen challenge in atopic asthmatics.* Eur Respir J, 1998. **11**(4): p. 821-7.

94. Gauvreau, G.M., R.M. Watson, and P.M. O'Byrne, *Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation.* Am J Respir Crit Care Med, 1999. **160**(2): p. 640-7.

95. Holgate, S.T., et al., *Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities.* J Allergy Clin Immunol, 2006. **117**(3): p. 496-506.

96. Davies, D.E., et al., *Airway remodeling in asthma: new insights.* J Allergy Clin Immunol, 2003. **111**(2): p. 215-25; quiz 226.

97. Carroll, N., et al., *The structure of large and small airways in nonfatal and fatal asthma.* Am Rev Respir Dis, 1993. **147**(2): p. 405-10.

98. Dunnill, M.S., G.R. Massarella, and J.A. Anderson, *A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema.* Thorax, 1969. **24**(2): p. 176-9.

99. Woodruff, P.G., et al., *Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression.* Am J Respir Crit Care Med, 2004. **169**(9): p. 1001-6.

100. Benayoun, L., et al., *Airway structural alterations selectively associated with severe asthma.* Am J Respir Crit Care Med, 2003. **167**(10): p. 1360-8.

101. Kuwano, K., et al., *Small airways dimensions in asthma and in chronic obstructive pulmonary disease.* Am Rev Respir Dis, 1993. **148**(5): p. 1220-5.

102. Ebina, M., et al., *Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study.* Am Rev Respir Dis, 1993. **148**(3): p. 720-6.

103. Pascual, R.M. and S.P. Peters, *Airway remodeling contributes to the progressive loss of lung function in asthma: an overview.* J Allergy Clin Immunol, 2005. **116**(3): p. 477-86; quiz 487.

104. Lazaar, A.L. and R.A. Panettieri, Jr., *Airway smooth muscle: a modulator of airway remodeling in asthma.* J Allergy Clin Immunol, 2005. **116**(3): p. 488-95; quiz 496.

105. Robinson, D.S., et al., *Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma.* N Engl J Med, 1992. **326**(5): p. 298-304.

106. Walker, C., et al., *Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage.* Am Rev Respir Dis, 1992. **146**(1): p. 109-15.

107. Walker, C., et al., *Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity.* J Allergy Clin Immunol, 1991. **88**(6): p. 935-42.

108. Beasley, R., et al., *Cellular events in the bronchi in mild asthma and after bronchial provocation.* Am Rev Respir Dis, 1989. **139**(3): p. 806-17.

109. Bradding, P. and S.T. Holgate, *Immunopathology and human mast cell cytokines.* Crit Rev Oncol Hematol, 1999. **31**(2): p. 119-33.

110. Okumura, S., et al., *Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and FcepsilonRI.* Blood, 2003. **102**(7): p. 2547-54.

111. Bradding, P., A.F. Walls, and S.T. Holgate, *The role of the mast cell in the pathophysiology of asthma.* J Allergy Clin Immunol, 2006. **117**(6): p. 1277-84.

112. Brightling, C.E., et al., *Eosinophilic bronchitis is an important cause of chronic cough.* Am J Respir Crit Care Med, 1999. **160**(2): p. 406-10.

113. Brightling, C.E., et al., *Mast-cell infiltration of airway smooth muscle in asthma.* N Engl J Med, 2002. **346**(22): p. 1699-705.

114. Brightling, C.E., et al., *Interleukin-4 and -13 expression is co-localized to mast cells within the airway smooth muscle in asthma.* Clin Exp Allergy, 2003. **33**(12): p. 1711-6.

115. Anderson, G.P., *The immunobiology of early asthma.* Med J Aust, 2002. **177 Suppl**: p. S47-9.

116. Gibson, P.G., et al., *Allergen-induced asthmatic responses. Relationship between increases in airway responsiveness and increases in circulating eosinophils, basophils, and their progenitors.* Am Rev Respir Dis, 1991. **143**(2): p. 331-5.

117. Metzger, W.J., et al., *Bronchoalveolar lavage of allergic asthmatic patients following allergen bronchoprovocation.* Chest, 1986. **89**(4): p. 477-83.

118. Wachs, M., et al., *Observations on the pathogenesis of nasal priming.* J Allergy Clin Immunol, 1989. **84**(4 Pt 1): p. 492-501.

119. Ying, S., et al., *Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells.* Eur J Immunol, 1997. **27**(12): p. 3507-16.

120. Xystrakis, E., S.E. Boswell, and C.M. Hawrylowicz, *T regulatory cells and the control of allergic disease.* Expert Opin Biol Ther, 2006. **6**(2): p. 121-33.

121. Hansen, G., et al., *Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation.* J Clin Invest, 1999. **103**(2): p. 175-83.

122. Randolph, D.A., et al., *Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma.* J Immunol, 1999. **162**(4): p. 2375-83.

123. Li, L., et al., *Th2-induced eotaxin expression and eosinophilia coexist with Th1 responses at the effector stage of lung inflammation.* J Immunol, 1998. **161**(6): p. 3128-35.

124. Gavett, S.H., et al., *Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia.* Am J Respir Cell Mol Biol, 1994. **10**(6): p. 587-93.

125. MacLean, J.A., R. Ownbey, and A.D. Luster, *T cell-dependent regulation of eotaxin in antigen-induced pulmonary eosinophila.* J Exp Med, 1996. **184**(4): p. 1461-9.

126. Robinson, D., et al., *Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma.* N Engl J Med, 1992. **326**(5): p. 298-304.

127. Christodoulopoulos, P., et al., *TH2 cytokine-associated transcription factors in atopic and nonatopic asthma: evidence for differential signal transducer and activator of transcription 6 expression.* J Allergy Clin Immunol, 2001. **107**(4): p. 586-91.

128. Gavett, S.H., et al., *Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice.* Am J Physiol, 1997. **272**(2 Pt 1): p. L253-61.

129. Kuperman, D., et al., *Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production.* J Exp Med, 1998. **187**(6): p. 939-48.

130. Finotto, S., et al., *Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet.* Science, 2002. **295**(5553): p. 336-8.

131. Shirai, T., et al., *Th1/Th2 profile in peripheral blood in atopic cough and atopic asthma.* Clin Exp Allergy, 2003. **33**(1): p. 84-9.

132. Bodey, K.J., et al., *Cytokine profiles of BAL T cells and T-cell clones obtained from human asthmatic airways after local allergen challenge.* Allergy, 1999. **54**(10): p. 1083-93.

133. Gelder, C.M., et al., *Cytokine expression in normal, atopic, and asthmatic subjects using the combination of sputum induction and the polymerase chain reaction.* Thorax, 1995. **50**(10): p. 1033-7.

134. Schleimer, R.P., et al., *IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1.* J Immunol, 1992. **148**(4): p. 1086-92.

135. Lampinen, M., et al., *Cytokine-regulated accumulation of eosinophils in inflammatory disease.* Allergy, 2004. **59**(8): p. 793-805.

136. Coyle, A.J., et al., *Interleukin-4 is required for the induction of lung Th2 mucosal immunity.* Am J Respir Cell Mol Biol, 1995. **13**(1): p. 54-9.

137. Corry, D.B., et al., *Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity.* J Exp Med, 1996. **183**(1): p. 109-17.

138. Brusselle, G., et al., *Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice.* Am J Respir Cell Mol Biol, 1995. **12**(3): p. 254-9.

139. Brusselle, G.G., et al., *Attenuation of allergic airway inflammation in IL-4 deficient mice.* Clin Exp Allergy, 1994. **24**(1): p. 73-80.

140. Finkelman, F.D., et al., *IL-4 is required to generate and sustain in vivo IgE responses.* J Immunol, 1988. **141**(7): p. 2335-41.

141. Kon, O.M., et al., *Randomised, dose-ranging, placebo-controlled study of chimeric antibody to CD4 (keliximab) in chronic severe asthma.* Lancet, 1998. **352**(9134): p. 1109-13.

142. Kon, O.M., et al., *The effects of an anti-CD4 monoclonal antibody, keliximab, on peripheral blood CD4+ T-cells in asthma.* Eur Respir J, 2001. **18**(1): p. 45-52.

143. Walter, D.M., et al., *Critical role for IL-13 in the development of allergen-induced airway hyperreactivity.* J Immunol, 2001. **167**(8): p. 4668-75.

144. Leigh, R., et al., *Is interleukin-13 critical in maintaining airway hyperresponsiveness in allergen-challenged mice?* Am J Respir Crit Care Med, 2004. **170**(8): p. 851-6.

145. Kuperman, D.A., et al., *Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma.* Nat Med, 2002. **8**(8): p. 885-9.

146. Whittaker, L., et al., *Interleukin-13 mediates a fundamental pathway for airway epithelial mucus induced by CD4 T cells and interleukin-9.* Am J Respir Cell Mol Biol, 2002. **27**(5): p. 593-602.

147. Yang, M., et al., *Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin.* Am J Respir Cell Mol Biol, 2001. **25**(4): p. 522-30.

148. Padilla, J., et al., *IL-13 regulates the immune response to inhaled antigens.* J Immunol, 2005. **174**(12): p. 8097-105.

149. Rothenberg, M.E., *Eosinophilia.* N Engl J Med, 1998. **338**(22): p. 1592-600.

150. Campbell, H.D., et al., *Molecular cloning, nucleotide sequence, and expression of the gene encoding human eosinophil differentiation factor (interleukin 5).* Proc Natl Acad Sci U S A, 1987. **84**(19): p. 6629-33.

151. Lampinen, M., S. Rak, and P. Venge, *The role of interleukin-5, interleukin-8 and RANTES in the chemotactic attraction of eosinophils to the allergic lung.* Clin Exp Allergy, 1999. **29**(3): p. 314-22.

152. Corrigan, C.J., et al., *CD4 T-lymphocyte activation in asthma is accompanied by increased serum concentrations of interleukin-5. Effect of glucocorticoid therapy.* Am Rev Respir Dis, 1993. **147**(3): p. 540-7.

153. Lee, J.J., et al., *Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma.* J Exp Med, 1997. **185**(12): p. 2143-56.

154. Foster, P.S., et al., *Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model.* J Exp Med, 1996. **183**(1): p. 195-201.

155. Cho, J.Y., et al., *Inhibition of airway remodeling in IL-5-deficient mice.* J Clin Invest, 2004. **113**(4): p. 551-60.

156. Leckie, M.J., et al., *Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response.* Lancet, 2000. **356**(9248): p. 2144-8.

157. Flood-Page, P.T., et al., *Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway.* Am J Respir Crit Care Med, 2003. **167**(2): p. 199-204.

158. Kobayashi, T., K. Iijima, and H. Kita, *Marked airway eosinophilia prevents development of airway hyper-responsiveness during an allergic response in IL-5 transgenic mice.* J Immunol, 2003. **170**(11): p. 5756-63.

159. Cohn, L., *Mucus in chronic airway diseases: sorting out the sticky details.* J Clin Invest, 2006. **116**(2): p. 306-8.

160. McHugh, S.M., et al., *Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern: comparison of rush and conventional immunotherapy.* Clin Exp Allergy, 1995. **25**(9): p. 828-38.

161. Smart, J.M., et al., *Polyclonal and allergen-induced cytokine responses in adults with asthma: resolution of asthma is associated with normalization of IFN-gamma responses.* J Allergy Clin Immunol, 2002. **110**(3): p. 450-6.

162. Cohn, L., et al., *T helper 1 cells and interferon gamma regulate allergic airway inflammation and mucus production.* J Exp Med, 1999. **190**(9): p. 1309-18.

163. Finotto, S., et al., *Development of Spontaneous Airway Changes Consistent with Human Asthma in Mice Lacking T-bet.* Science, 2002. **295**(5553): p. 336-338.

164. Krug, N., et al., *T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma.* Am J Respir Cell Mol Biol, 1996. **14**(4): p. 319-26.

165. Ford, J.G., et al., *Il-13 and IFN-gamma: interactions in lung inflammation.* J Immunol, 2001. **167**(3): p. 1769-77.

166. Hansen, G., et al., *CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation.* J Clin Invest, 2000. **105**(1): p. 61-70.

167. Randolph, D.A., et al., *Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation.* J Clin Invest, 1999. **104**(8): p. 1021-9.

168. Stephens, R., et al., *Antigen-nonspecific recruitment of Th2 cells to the lung as a mechanism for viral infection-induced allergic asthma.* J Immunol, 2002. **169**(10): p. 5458-67.

169. Thornton, A.M. and E.M. Shevach, *CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production.* J Exp Med, 1998. **188**(2): p. 287-96.

170. Thornton, A.M. and E.M. Shevach, *Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific.* J Immunol, 2000. **164**(1): p. 183-90.

171. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.

172. Jonuleit, H., et al., *Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood.* J Exp Med, 2001. **193**(11): p. 1285-94.

173. Ng, W.F., et al., *Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells.* Blood, 2001. **98**(9): p. 2736-44.

174. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.

175. Khattri, R., et al., *An essential role for Scurfin in CD4+CD25+ T regulatory cells.* Nat Immunol, 2003. **4**(4): p. 337-42.

176. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.

177. Chen, Y., et al., *Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis.* Science, 1994. **265**(5176): p. 1237-40.

178. Fukaura, H., et al., *Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients.* J Clin Invest, 1996. **98**(1): p. 70-7.

179. Groux, H., et al., *A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis.* Nature, 1997. **389**(6652): p. 737-42.

180. Jaffar, Z., T. Sivakuru, and K. Roberts, *CD4+CD25+ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype.* J Immunol, 2004. **172**(6): p. 3842-9.

181. Kearley, J., et al., *Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent.* J Exp Med, 2005. **202**(11): p. 1539-47.

182. Lewkowich, I.P., et al., *CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function.* J Exp Med, 2005. **202**(11): p. 1549-61.

183. Akbari, O., et al., *Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity.* Nat Med, 2002. **8**(9): p. 1024-32.

184. Stock, P., et al., *Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity.* Nat Immunol, 2004. **5**(11): p. 1149-56.

185. Xystrakis, E., et al., *Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients.* J Clin Invest, 2006. **116**(1): p. 146-155.

186. Filley, W.V., et al., *Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma.* Lancet, 1982. **2**(8288): p. 11-6.

187. de Andres, B., et al., *Release of O2- and LTC4 by murine eosinophils: role of intra- and extracellular calcium.* Immunology, 1990. **69**(2): p. 271-6.

188. Weller, P.F., et al., *Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C4.* Proc Natl Acad Sci U S A, 1983. **80**(24): p. 7626-30.

189. Huber, H.L. and K.K. Koessler, *The pathology of bronchial asthma.* Arch Intern Med, 1922. **30**: p. 689.

190. Bousquet, J., et al., *Eosinophilic inflammation in asthma.* N Engl J Med, 1990. **323**(15): p. 1033-9.

191. Fujimoto, K., et al., *Eosinophil cationic protein levels in induced sputum correlate with the severity of bronchial asthma.* Chest, 1997. **112**(5): p. 1241-7.

192. Bentley, A.M., et al., *Prednisolone treatment in asthma. Reduction in the numbers of eosinophils, T cells, tryptase-only positive mast cells, and modulation of IL-4, IL-5, and interferon-gamma cytokine gene expression within the bronchial mucosa.* Am J Respir Crit Care Med, 1996. **153**(2): p. 551-6.

193. Wills-Karp, M., et al., *Interleukin-13: central mediator of allergic asthma.* Science, 1998. **282**(5397): p. 2258-61.

194. Cohn, L., J.S. Tepper, and K. Bottomly, *IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells.* J Immunol, 1998. **161**(8): p. 3813-6.

195. Cohn, L., et al., *IL-4 promotes airway eosinophilia by suppressing IFN-gamma production: defining a novel role for IFN-gamma in the regulation of allergic airway inflammation.* J Immunol, 2001. **166**(4): p. 2760-7.

196. Lee, J.J., et al., *Defining a link with asthma in mice congenitally deficient in eosinophils.* Science, 2004. **305**(5691): p. 1773-6.

197. Humbles, A.A., et al., *A critical role for eosinophils in allergic airways remodeling.* Science, 2004. **305**(5691): p. 1776-9.

198. Shen, H.H., et al., *A causative relationship exists between eosinophils and the development of allergic pulmonary pathologies in the mouse.* J Immunol, 2003. **170**(6): p. 3296-305.

199. Woerly, G., et al., *Expression of Th1 and Th2 immunoregulatory cytokines by human eosinophils.* Int Arch Allergy Immunol, 1999. **118**(2-4): p. 95-7.

200. Shi, H.Z., et al., *Lymph node trafficking and antigen presentation by endobronchial eosinophils.* J Clin Invest, 2000. **105**(7): p. 945-53.

201. van Rijt, L.S., et al., *Airway eosinophils accumulate in the mediastinal lymph nodes but lack antigen-presenting potential for naive T cells.* J Immunol, 2003. **171**(7): p. 3372-8.

202. Kawakami, T. and S.J. Galli, *Regulation of mast-cell and basophil function and survival by IgE.* Nat Rev Immunol, 2002. **2**(10): p. 773-86.

203. Metcalfe, D.D., D. Baram, and Y.A. Mekori, *Mast cells.* Physiol Rev, 1997. **77**(4): p. 1033-79.

204. Williams, C.M. and S.J. Galli, *The diverse potential effector and immunoregulatory roles of mast cells in allergic disease.* J Allergy Clin Immunol, 2000. **105**(5): p. 847-59.

205. Djukanovic, R., et al., *Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry.* Am Rev Respir Dis, 1990. **142**(4): p. 863-71.

206. Pesci, A., et al., *Histochemical characteristics and degranulation of mast cells in epithelium and lamina propria of bronchial biopsies from asthmatic and normal subjects.* Am Rev Respir Dis, 1993. **147**(3): p. 684-9.

207. Kobayashi, T., et al., *An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model.* J Immunol, 2000. **164**(7): p. 3855-61.

208. Williams, C.M. and S.J. Galli, *Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice.* J Exp Med, 2000. **192**(3): p. 455-62.

209. Mayr, S.I., et al., *IgE-dependent mast cell activation potentiates airway responses in murine asthma models*. J Immunol, 2002. **169**(4): p. 2061-8.

210. Taube, C., et al., *Mast cells, Fc epsilon RI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant*. J Immunol, 2004. **172**(10): p. 6398-406.

211. Martin, T.R., et al., *Mast cell activation enhances airway responsiveness to methacholine in the mouse*. J Clin Invest, 1993. **91**(3): p. 1176-82.

212. Supajatura, V., et al., *Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity*

10.1172/JCI200214704. J. Clin. Invest., 2002. **109**(10): p. 1351-1359.

213. Nakajima, T., et al., *Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcepsilon receptor I cross-linking: an interspecies comparison*

10.1182/blood-2002-02-0602. Blood, 2002. **100**(12): p. 3861-3868.

214. Galli, S.J., S. Nakae, and M. Tsai, *Mast cells in the development of adaptive immune responses*. Nat Immunol, 2005. **6**(2): p. 135-42.

215. Fish, S.C., et al., *IgE generation and mast cell effector function in mice deficient in IL-4 and IL-13*. J Immunol, 2005. **174**(12): p. 7716-24.

216. Burrows, B., et al., *Association of asthma with serum IgE levels and skin-test reactivity to allergens*. N Engl J Med, 1989. **320**(5): p. 271-7.

217. Sunyer, J., et al., *Relationship between serum IgE and airway responsiveness in adults with asthma*. J Allergy Clin Immunol, 1995. **95**(3): p. 699-706.

218. Winchester, D.E., A. Jacob, and T. Murphy, *Omalizumab for asthma.* N Engl J Med, 2006. **355**(12): p. 1281-2.

219. Prussin, C., et al., *Omalizumab treatment downregulates dendritic cell FcepsilonRI expression.* J Allergy Clin Immunol, 2003. **112**(6): p. 1147-54.

220. Milgrom, H., et al., *Treatment of allergic asthma with monoclonal anti-IgE antibody. rhuMAb-E25 Study Group.* N Engl J Med, 1999. **341**(26): p. 1966-73.

221. Busse, W., et al., *Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma.* J Allergy Clin Immunol, 2001. **108**(2): p. 184-90.

222. Soler, M., et al., *The anti-IgE antibody omalizumab reduces exacerbations and steroid requirement in allergic asthmatics.* Eur Respir J, 2001. **18**(2): p. 254-61.

223. Djukanovic, R., et al., *Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma.* Am J Respir Crit Care Med, 2004. **170**(6): p. 583-93.

224. Mehlhop, P.D., et al., *Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma.* Proc Natl Acad Sci U S A, 1997. **94**(4): p. 1344-9.

225. Lambrecht, B.N., et al., *Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice.* J Immunol, 1998. **160**(8): p. 4090-7.

226. von Garnier, C., et al., *Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract.* J Immunol, 2005. **175**(3): p. 1609-18.

227. Lambrecht, B.N., *The dendritic cell in allergic airway diseases: a new player to the game.* Clin Exp Allergy, 2001. **31**(2): p. 206-18.

228. Graffi, S.J., et al., *Systemic administration of antigen-pulsed dendritic cells induces experimental allergic asthma in mice upon aerosol antigen rechallenge.* Clin Immunol, 2002. **103**(2): p. 176-84.

229. Vermaelen, K.Y., et al., *Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes.* J Exp Med, 2001. **193**(1): p. 51-60.

230. Vermaelen, K. and R. Pauwels, *Accelerated airway dendritic cell maturation, trafficking, and elimination in a mouse model of asthma.* Am J Respir Cell Mol Biol, 2003. **29**(3 Pt 1): p. 405-9.

231. de Heer, H.J., et al., *Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen.* J Exp Med, 2004. **200**(1): p. 89-98.

232. Lambrecht, B.N., et al., *Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation.* J. Clin. Invest., 2000. **106**(4): p. 551-559.

233. Huh, J.C., et al., *Bidirectional interactions between antigen-bearing respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC activation occurs in the airway mucosa but not in the lung parenchyma.* J Exp Med, 2003. **198**(1): p. 19-30.

234. van Rijt, L.S., et al., *In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma.* J Exp Med, 2005. **201**(6): p. 981-91.

235. De Sanctis, G.T., et al., *T-lymphocytes regulate genetically determined airway hyperresponsiveness in mice*. Nat Med, 1997. **3**(4): p. 460-2.

236. Lukacs, N.W., *Role of chemokines in the pathogenesis of asthma*. Nat Rev Immunol, 2001. **1**(2): p. 108-16.

237. Gerber, B.O., et al., *Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils*. Curr Biol, 1997. **7**(11): p. 836-43.

238. D'Ambrosio, D., et al., *Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells*. J Immunol, 1998. **161**(10): p. 5111-5.

239. Sallusto, F., et al., *Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes*. J Exp Med, 1998. **187**(6): p. 875-83.

240. Loetscher, P., et al., *CCR5 is characteristic of Th1 lymphocytes*. Nature, 1998. **391**(6665): p. 344-5.

241. Panina-Bordignon, P., et al., *The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics*. J Clin Invest, 2001. **107**(11): p. 1357-64.

242. Mathew, A., et al., *Cutting edge: Th2 cell trafficking into the allergic lung is dependent on chemoattractant receptor signaling*. J Immunol, 2002. **169**(2): p. 651-5.

243. Fulkerson, P.C., et al., *Pulmonary chemokine expression is coordinately regulated by STAT1, STAT6, and IFN-gamma*. J Immunol, 2004. **173**(12): p. 7565-74.

244. Ma, W., et al., *CCR3 is essential for skin eosinophilia and airway hyperresponsiveness in a murine model of allergic skin inflammation.* J Clin Invest, 2002. **109**(5): p. 621-8.

245. Humbles, A.A., et al., *The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness.* Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1479-84.

246. Ponath, P.D., et al., *Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils.* J Exp Med, 1996. **183**(6): p. 2437-48.

247. Romagnani, P., et al., *Tryptase-chymase double-positive human mast cells express the eotaxin receptor CCR3 and are attracted by CCR3-binding chemokines.* Am J Pathol, 1999. **155**(4): p. 1195-204.

248. Uguccioni, M., et al., *High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines.* J Clin Invest, 1997. **100**(5): p. 1137-43.

249. Conroy, D.M., et al., *CCR4 blockade does not inhibit allergic airways inflammation.* J Leukoc Biol, 2003. **74**(4): p. 558-63.

250. Cronshaw, D.G., et al., *Evidence that phospholipase-C-dependent, calcium-independent mechanisms are required for directional migration of T-lymphocytes in response to the CCR4 ligands CCL17 and CCL22.* J Leukoc Biol, 2006. **79**(6): p. 1369-80.

251. Lloyd, C.M., et al., *CC chemokine receptor (CCR)3/eotaxin is followed by CCR4/monocyte-derived chemokine in mediating pulmonary T helper lymphocyte*

type 2 recruitment after serial antigen challenge in vivo. J Exp Med, 2000. **191**(2): p. 265-74.

252. Vasquez, Y.R. and D. Spina, *What have transgenic and knockout animals taught us about respiratory disease?* Respir Res, 2000. **1**(2): p. 82-6.

253. Cieslewicz, G., et al., *The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration.* J Clin Invest, 1999. **104**(3): p. 301-8.

254. Tournoy, K.G., et al., *Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness.* Clin Exp Allergy, 2000. **30**(1): p. 79-85.

255. Kirkil, G., et al., *[Investigating the anti-inflammatory effect of dexamethasone in an asthma mouse model].* Tuberk Toraks, 2005. **53**(3): p. 245-51.

256. Hamelmann, E., et al., *Anti-interleukin 5 but not anti-IgE prevents airway inflammation and airway hyperresponsiveness.* Am J Respir Crit Care Med, 1999. **160**(3): p. 934-41.

257. Lee, N.A., E.W. Gelfand, and J.J. Lee, *Pulmonary T cells and eosinophils: coconspirators or independent triggers of allergic respiratory pathology?* J Allergy Clin Immunol, 2001. **107**(6): p. 945-57.

258. Persson, C.G. and J.S. Erjefalt, *Degranulation in eosinophils in human, but not in mouse, airways.* Allergy, 1999. **54**(11): p. 1230-2.

259. Malm-Erjefalt, M., C.G. Persson, and J.S. Erjefalt, *Degranulation status of airway tissue eosinophils in mouse models of allergic airway inflammation.* Am J Respir Cell Mol Biol, 2001. **24**(3): p. 352-9.

260. Clark, K., et al., *Eosinophil degranulation in the allergic lung of mice primarily occurs in the airway lumen.* J Leukoc Biol, 2004. **75**(6): p. 1001-9.

261. Hamelmann, E., et al., *Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography.* Am J Respir Crit Care Med, 1997. **156**(3 Pt 1): p. 766-75.

262. Sly, P.D., et al., *Penh is not a validated technique for measuring airway function in mice.* Am J Respir Crit Care Med, 2005. **172**(2): p. 256.

263. Schwarze, J., E. Hamelmann, and E.W. Gelfand, *Barometric whole body plethysmography in mice.* J Appl Physiol, 2005. **98**(5): p. 1955-7; author reply 1956-7.

264. Wagers, S., et al., *The allergic mouse model of asthma: normal smooth muscle in an abnormal lung?* J Appl Physiol, 2004. **96**(6): p. 2019-27.

265. Reinhart, K. and W. Karzai, *Anti-tumor necrosis factor therapy in sepsis: update on clinical trials and lessons learned.* Crit Care Med, 2001. **29**(7 Suppl): p. S121-5.

266. Tracey, K.J., et al., *Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia.* Nature, 1987. **330**(6149): p. 662-4.

267. Schwald, M., A. Schneider, and F.J. Meyer, *[Inhalative application devices for patients suffering from asthma bronchiale].* Ther Umsch, 2006. **63**(6): p. 383-90.

268. Nelson, H.S., *Is there a problem with inhaled long-acting beta-adrenergic agonists?* J Allergy Clin Immunol, 2006. **117**(1): p. 3-16; quiz 17.

269. *British Guideline on the Management of Asthma.* 2005, BTS/SIGN.

270. Wenzel, S., *Severe asthma in adults.* Am J Respir Crit Care Med, 2005. **172**(2): p. 149-60.

271. Di Giovine, F.S., G. Nuki, and G.W. Duff, *Tumour necrosis factor in synovial exudates.* Ann Rheum Dis, 1988. **47**(9): p. 768-72.

272. Maini, R.N., et al., *Beneficial effects of tumour necrosis factor-alpha (TNF-alpha) blockade in rheumatoid arthritis (RA).* Clin Exp Immunol, 1995. **101**(2): p. 207-12.

273. Moreland, L.W., et al., *Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein.* N Engl J Med, 1997. **337**(3): p. 141-7.

274. Weinblatt, M.E., et al., *A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate.* N Engl J Med, 1999. **340**(4): p. 253-9.

275. Lipsky, P.E., et al., *Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group.* N Engl J Med, 2000. **343**(22): p. 1594-602.

276. Prete, M., et al., *Biological therapy with monoclonal antibodies: a novel treatment approach to autoimmune disease.* Clinical and Experimental Medicine, 2005. **5**(4): p. 141-160.

277. Howarth, P.H., et al., *Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma.* Thorax, 2005. **60**(12): p. 1012-8.

278. Sany, J., *[Monoclonal antibodies in the treatment of rheumatoid arthritis: toward a therapeutic revolution].* C R Biol, 2006. **329**(4): p. 228-40.

279. Gauvreau, G.M., et al., *The effects of an anti-CD11a mAb, efalizumab, on allergen-induced airway responses and airway inflammation in subjects with atopic asthma.* J Allergy Clin Immunol, 2003. **112**(2): p. 331-8.

280. Carswell, E.A., et al., *An endotoxin-induced serum factor that causes necrosis of tumors*. Proc Natl Acad Sci U S A, 1975. **72**(9): p. 3666-70.

281. Cerami, A. and B. Beutler, *The role of cachectin/TNF in endotoxic shock and cachexia*. Immunol Today, 1988. **9**(1): p. 28-31.

282. Beutler, B., I.W. Milsark, and A.C. Cerami, *Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin*. Science, 1985. **229**(4716): p. 869-71.

283. Bazzoni, F. and B. Beutler, *The tumor necrosis factor ligand and receptor families*. N Engl J Med, 1996. **334**(26): p. 1717-25.

284. Pfeffer, K., *Biological functions of tumor necrosis factor cytokines and their receptors*. Cytokine Growth Factor Rev, 2003. **14**(3-4): p. 185-91.

285. Eck, M.J. and S.R. Sprang, *The structure of tumor necrosis factor-alpha at 2.6 A resolution. Implications for receptor binding*. J Biol Chem, 1989. **264**(29): p. 17595-605.

286. Black, R.A., et al., *A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells*. Nature, 1997. **385**(6618): p. 729-33.

287. Rothe, J., et al., *Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes*. Nature, 1993. **364**(6440): p. 798-802.

288. Chan, K.F., M.R. Siegel, and J.M. Lenardo, *Signaling by the TNF receptor superfamily and T cell homeostasis*. Immunity, 2000. **13**(4): p. 419-22.

289. Erickson, S.L., et al., *Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice*. Nature, 1994. **372**(6506): p. 560-3.

290. Gordon, J.R. and S.J. Galli, *Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses.* J Exp Med, 1991. **174**(1): p. 103-7.

291. Sedgwick, J.D., et al., *Tumor necrosis factor: a master-regulator of leukocyte movement*. Immunol Today, 2000. **21**(3): p. 110-3.

292. Paleolog, E.M., et al., *Functional activities of receptors for tumor necrosis factor-alpha on human vascular endothelial cells*. Blood, 1994. **84**(8): p. 2578-90.

293. Paleolog, E.M., et al., *Deactivation of vascular endothelium by monoclonal anti-tumor necrosis factor alpha antibody in rheumatoid arthritis*. Arthritis Rheum, 1996. **39**(7): p. 1082-91.

294. Saxne, T., et al., *Detection of tumor necrosis factor alpha but not tumor necrosis factor beta in rheumatoid arthritis synovial fluid and serum*. Arthritis Rheum, 1988. **31**(8): p. 1041-5.

295. Keffer, J., et al., *Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis*. Embo J, 1991. **10**(13): p. 4025-31.

296. Furst, D.E., et al., *Adalimumab, a fully human anti tumor necrosis factor-alpha monoclonal antibody, and concomitant standard antirheumatic therapy for the treatment of rheumatoid arthritis: results of STAR (Safety Trial of Adalimumab in Rheumatoid Arthritis)*. J Rheumatol, 2003. **30**(12): p. 2563-71.

297. Bathon, J.M., et al., *A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis.* N Engl J Med, 2000. **343**(22): p. 1586-93.

298. Elliott, M.J., et al., *Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis.* Lancet, 1994. **344**(8930): p. 1105-10.

299. Weinblatt, M.E., et al., *Adalimumab, a fully human anti-tumor necrosis factor alpha monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the ARMADA trial.* Arthritis Rheum, 2003. **48**(1): p. 35-45.

300. Bradding, P., et al., *Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines.* Am J Respir Cell Mol Biol, 1994. **10**(5): p. 471-80.

301. Thomas, P.S., *Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma.* Immunol Cell Biol, 2001. **79**(2): p. 132-40.

302. Bischoff, S.C., et al., *Mast cells are an important cellular source of tumour necrosis factor alpha in human intestinal tissue.* Gut, 1999. **44**(5): p. 643-52.

303. Lassalle, P., et al., *Modulation of adhesion molecule expression on endothelial cells during the late asthmatic reaction: role of macrophage-derived tumour necrosis factor-alpha.* Clin Exp Immunol, 1993. **94**(1): p. 105-10.

304. Schwingshackl, A., et al., *Human eosinophils release matrix metalloproteinase-9 on stimulation with TNF-alpha.* J Allergy Clin Immunol, 1999. **104**(5): p. 983-9.

305. McLachlan, J.B., et al., *Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection.* Nat Immunol, 2003. **4**(12): p. 1199-205.

306. Teichtahl, H., N. Buckmaster, and E. Pertnikovs, *The incidence of respiratory tract infection in adults requiring hospitalization for asthma.* Chest, 1997. **112**(3): p. 591-6.

307. Nicholson, K.G., J. Kent, and D.C. Ireland, *Respiratory viruses and exacerbations of asthma in adults.* Bmj, 1993. **307**(6910): p. 982-6.

308. Johnston, S.L., et al., *Community study of role of viral infections in exacerbations of asthma in 9-11 year old children.* Bmj, 1995. **310**(6989): p. 1225-9.

309. Fraenkel, D.J., et al., *Lower airways inflammation during rhinovirus colds in normal and in asthmatic subjects.* Am J Respir Crit Care Med, 1995. **151**(3 Pt 1): p. 879-86.

310. Grunberg, K., et al., *Experimental rhinovirus 16 infection. Effects on cell differentials and soluble markers in sputum in asthmatic subjects.* Am J Respir Crit Care Med, 1997. **156**(2 Pt 1): p. 609-16.

311. Bardin, P.G., et al., *Lower airways inflammatory response during rhinovirus colds.* Int Arch Allergy Immunol, 1995. **107**(1-3): p. 127-9.

312. Green, R.M., et al., *Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study.* Bmj, 2002. **324**(7340): p. 763.

313. Sigurs, N., et al., *Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7.* Am J Respir Crit Care Med, 2000. **161**(5): p. 1501-7.

314. Stein, R.T., et al., *Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years.* Lancet, 1999. **354**(9178): p. 541-5.

315. Seddon, P.C., et al., *Branhamella catarrhalis colonization in preschool asthmatics.* Pediatr Pulmonol, 1992. **13**(3): p. 133-5.

316. ten Brinke, A., et al., *Risk factors of frequent exacerbations in difficult-to-treat asthma.* Eur Respir J, 2005. **26**(5): p. 812-8.

317. ten Brinke, A., et al., *Chronic sinusitis in severe asthma is related to sputum eosinophilia.* J Allergy Clin Immunol, 2002. **109**(4): p. 621-6.

318. Cunningham, A.F., et al., *Chronic Chlamydia pneumoniae infection and asthma exacerbations in children.* Eur Respir J, 1998. **11**(2): p. 345-9.

319. Black, P.N., et al., *Serological evidence of infection with Chlamydia pneumoniae is related to the severity of asthma.* Eur Respir J, 2000. **15**(2): p. 254-9.

320. Lieberman, D., et al., *Atypical pathogen infection in adults with acute exacerbation of bronchial asthma.* Am J Respir Crit Care Med, 2003. **167**(3): p. 406-10.

321. Hahn, D.L., et al., *Evidence for Chlamydia pneumoniae infection in steroid-dependent asthma.* Ann Allergy Asthma Immunol, 1998. **80**(1): p. 45-9.

322. Romagnani, S., *Induction of TH1 and TH2 responses: a key role for the 'natural' immune response?* Immunol Today, 1992. **13**(10): p. 379-81.

323. Martinez, F.D. and P.G. Holt, *Role of microbial burden in aetiology of allergy and asthma.* Lancet, 1999. **354 Suppl 2**: p. SII12-5.

324. Maggi, E., et al., *Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones.* J Immunol, 1992. **148**(7): p. 2142-7.

325. Gereda, J.E., et al., *Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma.* Lancet, 2000. **355**(9216): p. 1680-3.

326. Bach, J.F., *A Toll-like trigger for autoimmune disease*. Nat Med, 2005. **11**(2): p. 120-1.

327. Yazdanbakhsh, M., P.G. Kremsner, and R. van Ree, *Allergy, parasites, and the hygiene hypothesis*. Science, 2002. **296**(5567): p. 490-4.

328. Matricardi, P.M., et al., *Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study*. Bmj, 2000. **320**(7232): p. 412-7.

329. Schaub, B., R. Lauener, and E. von Mutius, *The many faces of the hygiene hypothesis*. J Allergy Clin Immunol, 2006. **117**(5): p. 969-77; quiz 978.

330. Heinzel, F.P., et al., *Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon*. Infect Immun, 1994. **62**(10): p. 4244-9.

331. Dabbagh, K., et al., *Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells*. J Immunol, 2002. **168**(9): p. 4524-30.

332. Tulic, M.K., et al., *Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide*. Am J Respir Cell Mol Biol, 2000. **22**(5): p. 604-12.

333. Murphy, K.M., A.B. Heimberger, and D.Y. Loh, *Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo*. Science, 1990. **250**(4988): p. 1720-3.

334. Smith, K.M., F. McAskill, and P. Garside, *Orally tolerized T cells are only able to enter B cell follicles following challenge with antigen in adjuvant, but they remain unable to provide B cell help*. J Immunol, 2002. **168**(9): p. 4318-25.

335. Wang, Y. and C.T. McCusker, *Interleukin-13-dependent bronchial hyper-responsiveness following isolated upper-airway allergen challenge in a murine model of allergic rhinitis and asthma.* Clin Exp Allergy, 2005. **35**(8): p. 1104-11.

336. Leishman, A.J., P. Garside, and A.M. Mowat, *Immunological consequences of intervention in established immune responses by feeding protein antigens.* Cell Immunol, 1998. **183**(2): p. 137-48.

337. Leishman, A.J., P. Garside, and A.M. Mowat, *Induction of oral tolerance in the primed immune system: influence of antigen persistence and adjuvant form.* Cell Immunol, 2000. **202**(2): p. 71-8.

338. Smith, K.M., et al., *Th1 and Th2 CD4+ T cells provide help for B cell clonal expansion and antibody synthesis in a similar manner in vivo.* J Immunol, 2000. **165**(6): p. 3136-44.

339. Smith, K.M., et al., *In vivo generated Th1 cells can migrate to B cell follicles to support B cell responses.* J Immunol, 2004. **173**(3): p. 1640-6.

340. Pozarowski, P., E. Holden, and Z. Darzynkiewicz, *Laser scanning cytometry: principles and applications.* Methods Mol Biol, 2006. **319**: p. 165-92.

341. Boyce, J.A. and K.F. Austen, *No audible wheezing: nuggets and conundrums from mouse asthma models.* J Exp Med, 2005. **201**(12): p. 1869-73.

342. Schuyler, M. and J. Wilder, *T lymphocyte subpopulations in Immunotoxicology*, ed. I. Kimber and M.K. Selgrade. 1998, New York: John Wiley and Sons. 233-252.

343. Barnes, P.J., *Transcription factors in airway diseases.* Lab Invest, 2006.

344. Kips, J.C., *Cytokines in asthma.* Eur Respir J Suppl, 2001. **34**: p. 24s-33s.

345. Murray, L.A., et al., *Role of chemokines in severe asthma.* Curr Drug Targets, 2006. 7(5): p. 579-88.

346. Smit, J.J. and N.W. Lukacs, *A closer look at chemokines and their role in asthmatic responses.* Eur J Pharmacol, 2006. **533**(1-3): p. 277-88.

347. Pease, J.E., *Asthma, allergy and chemokines.* Curr Drug Targets, 2006. 7(1): p. 3-12.

348. Garside, P., et al., *Visualization of specific B and T lymphocyte interactions in the lymph node.* Science, 1998. **281**(5373): p. 96-9.

349. Jaffar, Z., K.S. Wan, and K. Roberts, *A key role for prostaglandin I2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen.* J Immunol, 2002. **169**(10): p. 5997-6004.

350. Lee, S.C., et al., *Regulation of pulmonary T cell responses to inhaled antigen: role in Th1- and Th2-mediated inflammation.* J Immunol, 1999. **162**(11): p. 6867-79.

351. Byersdorfer, C.A. and D.D. Chaplin, *Visualization of early APC/T cell interactions in the mouse lung following intranasal challenge.* J Immunol, 2001. **167**(12): p. 6756-64.

352. Cohn, L., et al., *Th2-induced airway mucus production is dependent on IL-4Ralpha, but not on eosinophils.* J Immunol, 1999. **162**(10): p. 6178-83.

353. Cohn, L., et al., *Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production.* J Exp Med, 1997. **186**(10): p. 1737-47.

354. Mathew, A., et al., *Signal transducer and activator of transcription 6 controls chemokine production and T helper cell type 2 cell trafficking in allergic pulmonary inflammation.* J Exp Med, 2001. **193**(9): p. 1087-96.

355. Catron, D.M., et al., *Visualizing the first 50 hr of the primary immune response to a soluble antigen.* Immunity, 2004. **21**(3): p. 341-7.

356. Fisher, C.E., et al., *FITC-induced murine pulmonary inflammation: CC10 up-regulation and concurrent Shh expression.* Cell Biol Int, 2005. **29**(10): p. 868-76.

357. Szarka, R.J., et al., *A murine model of pulmonary damage induced by lipopolysaccharide via intranasal instillation.* J Immunol Methods, 1997. **202**(1): p. 49-57.

358. Brewer, J.M., et al., *Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling.* J Immunol, 1999. **163**(12): p. 6448-54.

359. Lima, C., et al., *Modulation of the induction of lung and airway allergy in the offspring of IFN-gamma-treated mother mice.* J Immunol, 2005. **175**(6): p. 3554-9.

360. Voynow, J.A., et al., *Neutrophil elastase induces mucus cell metaplasia in mouse lung.* Am J Physiol Lung Cell Mol Physiol, 2004. **287**(6): p. L1293-302.

361. Howarth, P.H., *The airway inflammatory response in allergic asthma and its relationship to clinical disease.* Allergy, 1995. **50**(22 Suppl): p. 13-21.

362. Herrick, C.A. and K. Bottomly, *To respond or not to respond: T cells in allergic asthma.* Nat Rev Immunol, 2003. **3**(5): p. 405-12.

363. Gonzalo, J.A., et al., *Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors.* J Clin Invest, 1996. **98**(10): p. 2332-45.

364. Garlisi, C.G., et al., *T cells are necessary for Th2 cytokine production and eosinophil accumulation in airways of antigen-challenged allergic mice.* Clin Immunol Immunopathol, 1995. **75**(1): p. 75-83.

365. Hawrylowicz, C.M., *Regulatory T cells and IL-10 in allergic inflammation.* J Exp Med, 2005. **202**(11): p. 1459-63.

366. Knott, P.G., P.R. Gater, and C.P. Bertrand, *Airway inflammation driven by antigen-specific resident lung CD4(+) T cells in alphabeta-T cell receptor transgenic mice.* Am J Respir Crit Care Med, 2000. **161**(4 Pt 1): p. 1340-8.

367. Ramos-Barbon, D., et al., *Antigen-specific CD4+ T cells drive airway smooth muscle remodeling in experimental asthma.* J Clin Invest, 2005. **115**(6): p. 1580-9.

368. Umetsu, D.T., O. Akbari, and R.H. Dekruyff, *Regulatory T cells control the development of allergic disease and asthma.* J Allergy Clin Immunol, 2003. **112**(3): p. 480-7; quiz 488.

369. Leong, K.P. and D.P. Huston, *Understanding the pathogenesis of allergic asthma using mouse models.* Ann Allergy Asthma Immunol, 2001. **87**(2): p. 96-109; quiz 110.

370. Piggott, D.A., et al., *MyD88-dependent induction of allergic Th2 responses to intranasal antigen.* J Clin Invest, 2005. **115**(2): p. 459-67.

371. Herz, U., et al., *The relevance of murine animal models to study the development of allergic bronchial asthma.* Immunol Cell Biol, 1996. **74**(2): p. 209-17.

372. Krinzman, S.J., et al., *T cell activation in a murine model of asthma.* Am J Physiol, 1996. **271**(3 Pt 1): p. L476-83.

373. Elwood, W., et al., *Characterization of allergen-induced bronchial hyperresponsiveness and airway inflammation in actively sensitized brown-Norway rats.* J Allergy Clin Immunol, 1991. **88**(6): p. 951-60.

374. Lloyd, C.M. and S.M. Rankin, *Chemokines in allergic airway disease.* Curr Opin Pharmacol, 2003. **3**(4): p. 443-8.

375. Ip, W.K., et al., *Plasma concentrations of soluble CTLA-4, CD28, CD80 and CD86 costimulatory molecules reflect disease severity of acute asthma in children.* Pediatr Pulmonol, 2006. **41**(7): p. 674-82.

376. Rutkowski, R., et al., *[CD28 and CTLA-4 costimulatory molecules expression on T lymphocytes and NK cells in nonallergic bronchial asthma].* Pol Merkuriusz Lek, 2006. **20**(116): p. 139-44.

377. Barnes, P.J., *Th2 cytokines and asthma: an introduction.* Respir Res, 2001. **2**(2): p. 64-5.

378. Ngoc, P.L., et al., *Cytokines, allergy, and asthma.* Curr Opin Allergy Clin Immunol, 2005. **5**(2): p. 161-6.

379. Li, X.M., et al., *Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice.* J Immunol, 1996. **157**(8): p. 3216-9.

380. Lack, G., et al., *Nebulized IFN-gamma inhibits the development of secondary allergic responses in mice.* J Immunol, 1996. **157**(4): p. 1432-9.

381. Hamelmann, E. and E.W. Gelfand, *IL-5-induced airway eosinophilia--the key to asthma?* Immunol Rev, 2001. **179**: p. 182-91.

382. Weltman, J.K. and A.S. Karim, *IL-5: biology and potential therapeutic applications.* Expert Opin Investig Drugs, 2000. **9**(3): p. 491-6.

383. Mattes, J. and P.S. Foster, *Regulation of eosinophil migration and Th2 cell function by IL-5 and eotaxin.* Curr Drug Targets Inflamm Allergy, 2003. **2**(2): p. 169-74.

384. Foster, P.S., et al., *Interleukins-4, -5, and -13: emerging therapeutic targets in allergic disease.* Pharmacol Ther, 2002. **94**(3): p. 253-64.

385. Grunig, G., et al., *Requirement for IL-13 independently of IL-4 in experimental asthma.* Science, 1998. **282**(5397): p. 2261-3.

386. Huang, S.K., et al., *IL-13 expression at the sites of allergen challenge in patients with asthma.* J Immunol, 1995. **155**(5): p. 2688-94.

387. Humbert, M., et al., *Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma.* J Allergy Clin Immunol, 1997. **99**(5): p. 657-65.

388. Pope, S.M., et al., *The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia.* J Immunol, 2005. **175**(8): p. 5341-50.

389. Johnston, S.L., *Influence of viral and bacterial respiratory infections on exacerbations and symptom severity in childhood asthma.* Pediatr Pulmonol Suppl, 1997. **16**: p. 88-9.

390. Kraft, M., *The role of bacterial infections in asthma.* Clin Chest Med, 2000. **21**(2): p. 301-13.

391. Braun-Fahrlander, C., et al., *Environmental exposure to endotoxin and its relation to asthma in school-age children.* N Engl J Med, 2002. **347**(12): p. 869-77.

392. von Mutius, E., *Influences in allergy: epidemiology and the environment.* J Allergy Clin Immunol, 2004. **113**(3): p. 373-9; quiz 380.

393. Riedler, J., et al., *Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey.* Lancet, 2001. **358**(9288): p. 1129-33.

394. Rizzo, M.C., et al., *Endotoxin exposure and symptoms in asthmatic children.* Pediatr Allergy Immunol, 1997. **8**(3): p. 121-6.

395. von Mutius, E., et al., *Exposure to endotoxin or other bacterial components might protect against the development of atopy.* Clin Exp Allergy, 2000. **30**(9): p. 1230-4.

396. Bottcher, M.F., et al., *Endotoxin levels in Estonian and Swedish house dust and atopy in infancy.* Clin Exp Allergy, 2003. **33**(3): p. 295-300.

397. Tavernier, G.O., et al., *Endotoxin exposure in asthmatic children and matched healthy controls: results of IPEADAM study.* Indoor Air, 2005. **15 Suppl 10**: p. 25-32.

398. Michel, O., *Systemic and local airways inflammatory response to endotoxin.* Toxicology, 2000. **152**(1-3): p. 25-30.

399. Michel, O., et al., *Severity of asthma is related to endotoxin in house dust.* Am J Respir Crit Care Med, 1996. **154**(6 Pt 1): p. 1641-6.

400. Michel, O., et al., *Domestic endotoxin exposure and clinical severity of asthma.* Clin Exp Allergy, 1991. **21**(4): p. 441-8.

401. Kline, J.N., et al., *Variable airway responsiveness to inhaled lipopolysaccharide.* Am J Respir Crit Care Med, 1999. **160**(1): p. 297-303.

402. Michel, O., et al., *Dose-response relationship to inhaled endotoxin in normal subjects.* Am J Respir Crit Care Med, 1997. **156**(4 Pt 1): p. 1157-64.

403. Boehlecke, B., et al., *Low-dose airborne endotoxin exposure enhances bronchial responsiveness to inhaled allergen in atopic asthmatics.* J Allergy Clin Immunol, 2003. **112**(6): p. 1241-3.

404. Michel, O., J. Duchateau, and R. Sergysels, *Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects.* J Appl Physiol, 1989. **66**(3): p. 1059-64.

405. Feleszko, W., J. Jaworska, and E. Hamelmann, *Toll-like receptors--novel targets in allergic airway disease (probiotics, friends and relatives).* Eur J Pharmacol, 2006. **533**(1-3): p. 308-18.

406. Schnare, M., et al., *Toll-like receptors control activation of adaptive immune responses.* Nat Immunol, 2001. **2**(10): p. 947-50.

407. Eisenbarth, S.C., et al., *Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen.* J Exp Med, 2002. **196**(12): p. 1645-51.

408. Gerhold, K., et al., *Endotoxins prevent murine IgE production, T(H)2 immune responses, and development of airway eosinophilia but not airway hyperreactivity.* J Allergy Clin Immunol, 2002. **110**(1): p. 110-6.

409. Kuipers, H., et al., *Lipopolysaccharide-induced suppression of airway Th2 responses does not require IL-12 production by dendritic cells.* J Immunol, 2003. **171**(7): p. 3645-54.

410. Velasco, G., et al., *Toll-like receptor 4 or 2 agonists decrease allergic inflammation.* Am J Respir Cell Mol Biol, 2005. **32**(3): p. 218-24.

411. Patel, M., et al., *TLR2 Agonist Ameliorates Established Allergic Airway Inflammation by Promoting Th1 Response and Not via Regulatory T Cells.* J Immunol, 2005. **174**(12): p. 7558-63.

412. Herbert, A., et al., *Reduction of alveolar-capillary diffusion after inhalation of endotoxin in normal subjects.* Chest, 1992. **102**(4): p. 1095-8.

413. Michel, O., et al., *Inflammatory response to acute inhalation of endotoxin in asthmatic patients.* Am Rev Respir Dis, 1992. **146**(2): p. 352-7.

414. Van der Zwan, J.C., et al., *Bronchial obstructive reactions after inhalation with endotoxin and precipitinogens of Haemophilus influenzae in patients with chronic non-specific lung disease.* Clin Allergy, 1982. **12**(6): p. 547-59.

415. Wenzel, S.E., et al., *Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics.* Am J Respir Crit Care Med, 1999. **160**(3): p. 1001-8.

416. Jatakanon, A., et al., *Neutrophilic inflammation in severe persistent asthma.* Am J Respir Crit Care Med, 1999. **160**(5 Pt 1): p. 1532-9.

417. Douwes, J., et al., *Non-eosinophilic asthma: importance and possible mechanisms.* Thorax, 2002. **57**(7): p. 643-8.

418. Kamath, A.V., et al., *Is the neutrophil the key effector cell in severe asthma?* Thorax, 2005. **60**(7): p. 529-30.

419. Peden, D.B., et al., *Eosinophil influx to the nasal airway after local, low-level LPS challenge in humans.* J Allergy Clin Immunol, 1999. **104**(2 Pt 1): p. 388-94.

420. Delayre-Orthez, C., et al., *Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse.* Clin Exp Allergy, 2004. **34**(11): p. 1789-95.

421. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.

422. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.

423. Nigo, Y.I., et al., *Regulation of allergic airway inflammation through Toll-like receptor 4-mediated modification of mast cell function.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2286-91.

424. Renauld, J.C., *New insights into the role of cytokines in asthma.* J Clin Pathol, 2001. **54**(8): p. 577-89.

425. Sosne, G., et al., *Thymosin beta 4 promotes corneal wound healing and modulates inflammatory mediators in vivo.* Exp Eye Res, 2001. **72**(5): p. 605-8.

426. Oshiba, A., et al., *Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice.* J Clin Invest, 1996. **97**(6): p. 1398-408.

427. Coyle, A.J., et al., *Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody.* J Exp Med, 1996. **183**(4): p. 1303-10.

428. Reinhardt, R.L., et al., *Preferential accumulation of antigen-specific effector CD4 T cells at an antigen injection site involves CD62E-dependent migration but not local proliferation.* J Exp Med, 2003. **197**(6): p. 751-62.

429. Roman, E., et al., *CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function.* J Exp Med, 2002. **196**(7): p. 957-68.

430. Harris, N.L., et al., *Differential T cell function and fate in lymph node and nonlymphoid tissues.* J Exp Med, 2002. **195**(3): p. 317-26.

431. Krouwels, F.H., et al., *Cytokine production by T-cell clones from bronchoalveolar lavage fluid of patients with asthma and healthy subjects.* Eur Respir J Suppl, 1996. **22**: p. 95s-103s.

432. Iio, J., et al., *Lipid A analogue, ONO-4007, inhibits IgE response and antigen-induced eosinophilic recruitment into airways in BALB/c mice.* Int Arch Allergy Immunol, 2002. **127**(3): p. 217-25.

433. Delayre-Orthez, C., et al., *Exposure to endotoxins during sensitization prevents further endotoxin-induced exacerbation of airway inflammation in a mouse model of allergic asthma.* Int Arch Allergy Immunol, 2005. **138**(4): p. 298-304.

434. Blumer, N., et al., *Prenatal lipopolysaccharide-exposure prevents allergic sensitization and airway inflammation, but not airway responsiveness in a murine model of experimental asthma.* Clin Exp Allergy, 2005. **35**(3): p. 397-402.

435. Cochran, J.R., et al., *Influence of lipopolysaccharide exposure on airway function and allergic responses in developing mice.* Pediatr Pulmonol, 2002. **34**(4): p. 267-77.

436. Alexis, N.E. and D.B. Peden, *Blunting airway eosinophilic inflammation results in a decreased airway neutrophil response to inhaled LPS in patients with atopic asthma: a role for CD14.* J Allergy Clin Immunol, 2001. **108**(4): p. 577-80.

437. Alexis, N., et al., *CD14-dependent airway neutrophil response to inhaled LPS: role of atopy.* J Allergy Clin Immunol, 2001. **107**(1): p. 31-5.

438. Alexis, N.E., M.W. Eldridge, and D.B. Peden, *Effect of inhaled endotoxin on airway and circulating inflammatory cell phagocytosis and CD11b expression in atopic asthmatic subjects.* J Allergy Clin Immunol, 2003. **112**(2): p. 353-61.

439. Alexis, N.E., et al., *Acute LPS inhalation in healthy volunteers induces dendritic cell maturation in vivo.* J Allergy Clin Immunol, 2005. **115**(2): p. 345-50.

440. Alexis, N.E., et al., *Inhalation of low-dose endotoxin favors local T(H)2 response and primes airway phagocytes in vivo.* J Allergy Clin Immunol, 2004. **114**(6): p. 1325-31.

441. Eisenbarth, S.C., et al., *IL-4-dependent Th2 collateral priming to inhaled antigens independent of Toll-like receptor 4 and myeloid differentiation factor 88.* J Immunol, 2004. **172**(7): p. 4527-34.

442. Sigurs, N., et al., *Appearance of atopic disease in relation to serum IgE antibodies in children followed up from birth for 4 to 15 years.* J Allergy Clin Immunol, 1994. **94**(4): p. 757-63.

443. Purello-D'Ambrosio, F., et al., *Prevention of new sensitizations in monosensitized subjects submitted to specific immunotherapy or not. A retrospective study.* Clin Exp Allergy, 2001. **31**(8): p. 1295-302.

444. Rodriguez, D., et al., *Bacterial lipopolysaccharide signaling through Toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity.* J Immunol, 2003. **171**(2): p. 1001-8.

445. Revets, H., et al., *Lipoprotein I, a TLR2/4 ligand modulates Th2-driven allergic immune responses.* J Immunol, 2005. **174**(2): p. 1097-103.

446. Wan, G.H., C.S. Li, and R.H. Lin, *Airborne endotoxin exposure and the development of airway antigen-specific allergic responses.* Clin Exp Allergy, 2000. **30**(3): p. 426-32.

447. Gerhold, K., et al., *Exposure to endotoxin and allergen in early life and its effect on allergen sensitization in mice.* J Allergy Clin Immunol, 2003. **112**(2): p. 389-96.

448. Butler, D.M., et al., *Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist.* Eur Cytokine Netw, 1995. **6**(4): p. 225-30.

449. Stokes, D.G. and J.M. Kremer, *Potential of tumor necrosis factor neutralization strategies in rheumatologic disorders other than rheumatoid arthritis.* Semin Arthritis Rheum, 2003. **33**(1): p. 1-18.

450. Rutgeerts, P., et al., *Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease.* Gastroenterology, 2004. **126**(2): p. 402-13.

451. Holgate, S., et al., *The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation.* J Allergy Clin Immunol, 2005. **115**(3): p. 459-65.

452. Costa, J.J., et al., *Human eosinophils can express the cytokines tumor necrosis factor-alpha and macrophage inflammatory protein-1 alpha.* J Clin Invest, 1993. **91**(6): p. 2673-84.

453. Thomas, P.S. and G. Heywood, *Effects of inhaled tumour necrosis factor alpha in subjects with mild asthma.* Thorax, 2002. **57**(9): p. 774-8.

454. Thomas, P.S., D.H. Yates, and P.J. Barnes, *Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects.* Am J Respir Crit Care Med, 1995. **152**(1): p. 76-80.

455. Rouhani, F.N., et al., *Effect of tumor necrosis factor antagonism on allergen-mediated asthmatic airway inflammation.* Respir Med, 2005. **99**(9): p. 1175-82.

456. Choo-Kang, B.S., et al., *TNF-blocking therapies: an alternative mode of action?* Trends Immunol, 2005. **26**(10): p. 518-22.

457. Shiow, L.R., et al., *CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs.* Nature, 2006. **440**(7083): p. 540-4.

458. Howarth PH, B.K., Arshad HS, Lau L, Buckley M, McConnell W, *Tumour Necrosis Factor (TNF alpha) as a novel therapeutic target in symptomatic corticosteroid dependant asthma.* Thorax, 2005. **60**(12): p. 1012-8.

459. Berry MA, H.B., Shelley M, Parker D, Shaw DE, Green RH, *Evidence of a role of tumour necrosis factor in refractory asthma.* N Engl J Med, 2006. **381**(6577): p. 77-80.

460. Ordonez, C.L., et al., *Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: Clinical and biologic significance.* Am J Respir Crit Care Med, 2000. **161**(4 Pt 1): p. 1185-90.

461. Luster, A.D., R. Alon, and U.H. von Andrian, *Immune cell migration in inflammation: present and future therapeutic targets.* Nat Immunol, 2005. **6**(12): p. 1182-90.

462. Madsen, J.R., *[Chronic inflammatory bowel disease--pathogenic concepts and therapeutic perspectives].* Ugeskr Laeger, 2000. **162**(10): p. 1361-6.

463. Koller, M.D., *Targeted therapy in rheumatoid arthritis*. Wien Med Wochenschr, 2006. **156**(1-2): p. 53-60.

464. Boulos, P., et al., *Pharmacological treatment of ankylosing spondylitis: a systematic review*. Drugs, 2005. **65**(15): p. 2111-27.

465. Randolph, A.G., et al., *Extended haplotype in the tumor necrosis factor gene cluster is associated with asthma and asthma-related phenotypes*. Am J Respir Crit Care Med, 2005. **172**(6): p. 687-92.

466. Kim, J., et al., *Anti-tumor necrosis factor-alpha antibody treatment reduces pulmonary inflammation and methacholine hyper-responsiveness in a murine asthma model induced by house dust*. Clin Exp Allergy, 2006. **36**(1): p. 122-32.

467. Suryaprasad, A.G. and T. Prindiville, *The biology of TNF blockade*. Autoimmun Rev, 2003. **2**(6): p. 346-57.

468. Zuany-Amorim, C., *Induction of TNF-alpha autoantibody production by AutoVac TNF106: a novel therapeutic approach for the treatment of allergic diseases*. Int Arch Allergy Immunol, 2004. **133**(2): p. 154-63.

469. Choi, I.W., et al., *TNF-alpha induces the late-phase airway hyperresponsiveness and airway inflammation through cytosolic phospholipase A(2) activation*. J Allergy Clin Immunol, 2005. **116**(3): p. 537-43.

470. Broide, D.H., et al., *Inhibition of eosinophilic inflammation in allergen-challenged TNF receptor p55/p75--and TNF receptor p55-deficient mice*. Am J Respir Cell Mol Biol, 2001. **24**(3): p. 304-11.

471. Rudmann, D.G., et al., *Modulation of allergic inflammation in mice deficient in TNF receptors*. Am J Physiol Lung Cell Mol Physiol, 2000. **279**(6): p. L1047-57.

472. Sandborn, W.J., et al., *An engineered human antibody to TNF (CDP571) for active Crohn's disease: a randomized double-blind placebo-controlled trial.* Gastroenterology, 2001. **120**(6): p. 1330-8.

473. Busse, W.W., S. Banks-Schlegel, and S.E. Wenzel, *Pathophysiology of severe asthma.* J Allergy Clin Immunol, 2000. **106**(6): p. 1033-42.

474. Hall, J.G. and B. Morris, *The immediate effect of antigens on the cell output of a lymph node.* Br J Exp Pathol, 1965. **46**(4): p. 450-4.

475. Kalaaji, A.N., et al., *Recombinant bovine interferon-alpha I 1 inhibits the migration of lymphocytes from lymph nodes but not into lymph nodes.* Reg Immunol, 1988. **1**(1): p. 56-61.

476. Korngold, R., K.J. Blank, and D.M. Murasko, *Effect of interferon on thoracic duct lymphocyte output: induction with either poly I:poly C or vaccinia virus.* J Immunol, 1983. **130**(5): p. 2236-40.

477. Degre, M., *Influence of polyinosinic: polycytidylic acid on the circulating white blood cells in mice.* Proc Soc Exp Biol Med, 1973. **142**(4): p. 1087-91.

478. Young, A.J., et al., *A role for lymphatic endothelium in the sequestration of recirculating gamma delta T cells in TNF-alpha-stimulated lymph nodes.* Eur J Immunol, 2000. **30**(1): p. 327-34.

479. Kalaaji, A.N., K. McCullough, and J.B. Hay, *The enhancement of lymphocyte localization in skin sites of sheep by tumor necrosis factor alpha.* Immunol Lett, 1989. **23**(2): p. 143-7.

480. Colditz, I.G. and D.L. Watson, *The effect of cytokines and chemotactic agonists on the migration of T lymphocytes into skin.* Immunology, 1992. **76**(2): p. 272-8.

481. Wardlaw, A.J., C. Guillen, and A. Morgan, *Mechanisms of T cell migration to the lung*. Clin Exp Allergy, 2005. **35**(1): p. 4-7.

482. Picker, L.J. and E.C. Butcher, *Physiological and molecular mechanisms of lymphocyte homing*. Annu Rev Immunol, 1992. **10**: p. 561-91.

483. Mackay, C., *Lymphocyte migration. A new spin on lymphocyte homing*. Curr Biol, 1995. **5**(7): p. 733-6.

484. Gebb, S.A., et al., *Sites of leukocyte sequestration in the pulmonary microcirculation.* J Appl Physiol, 1995. **79**(2): p. 493-7.

485. Yamaguchi, K., et al., *Leukocyte kinetics in the pulmonary microcirculation: observations using real-time confocal luminescence microscopy coupled with high-speed video analysis.* Lab Invest, 1997. **76**(6): p. 809-22.

486. Nishio, K., et al., *Differential contribution of various adhesion molecules to leukocyte kinetics in pulmonary microvessels of hyperoxia-exposed rat lungs.* Am J Respir Crit Care Med, 1998. **157**(2): p. 599-609.

487. Keramidaris, E., et al., *L-selectin and intercellular adhesion molecule 1 mediate lymphocyte migration to the inflamed airway/lung during an allergic inflammatory response in an animal model of asthma.* J Allergy Clin Immunol, 2001. **107**(4): p. 734-8.

488. Tang, M.L., et al., *Intrinsic differences in L-selectin expression levels affect T and B lymphocyte subset-specific recirculation pathways.* J Immunol, 1998. **160**(10): p. 5113-21.

489. Tang, M.L., et al., *L-selectin is involved in lymphocyte migration to sites of inflammation in the skin: delayed rejection of allografts in L-selectin-deficient mice.* J Immunol, 1997. **158**(11): p. 5191-9.

490. Steeber, D.A., et al., *Efficient lymphocyte migration across high endothelial venules of mouse Peyer's patches requires overlapping expression of L-selectin and beta7 integrin.* J Immunol, 1998. **161**(12): p. 6638-47.

491. Spertini, O., et al., *Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion.* J Immunol, 1991. **147**(8): p. 2565-73.

492. Fiscus, L.C., et al., *L-Selectin is required for the development of airway hyperresponsiveness but not airway inflammation in a murine model of asthma.* J Allergy Clin Immunol, 2001. **107**(6): p. 1019-24.

493. von Andrian, U.H., *Intravital microscopy of the peripheral lymph node microcirculation in mice.* Microcirculation, 1996. **3**(3): p. 287-300.

494. von Andrian, U.H. and T.R. Mempel, *Homing and cellular traffic in lymph nodes.* Nat Rev Immunol, 2003. **3**(11): p. 867-78.

495. Bradley, L.M. and S.R. Watson, *Lymphocyte migration into tissue: the paradigm derived from CD4 subsets.* Curr Opin Immunol, 1996. **8**(3): p. 312-20.

496. Mackay, C.R., *Homing of naive, memory and effector lymphocytes.* Curr Opin Immunol, 1993. **5**(3): p. 423-7.

497. Davenport, M.P., M.C. Grimm, and A.R. Lloyd, *A homing selection hypothesis for T-cell trafficking.* Immunol Today, 2000. **21**(7): p. 315-7.

498. Springer, T.A., *Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm.* Cell, 1994. **76**(2): p. 301-14.

499. Krug, N. and A.J. Frew, *The Th2 cell in asthma: initial expectations yet to be realised.* Clin Exp Allergy, 1997. **27**(2): p. 142-50.

500. Henney, C.S. and R.H. Waldman, *Cell-mediated immunity shown by lymphocytes from the respiratory tract.* Science, 1970. **169**(946): p. 696-7.

501. Ahlstedt, S., et al., *Immune responses in rats sensitized with aerosolized antigen. Antibody formation, lymphoblastic responses and mast cell and mucous cell development related to bronchial reactivity.* Int Arch Allergy Appl Immunol, 1983. **72**(1): p. 71-8.

502. Xia, W., C.E. Pinto, and R.L. Kradin, *The antigen-presenting activities of Ia+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen.* J Exp Med, 1995. **181**(4): p. 1275-83.

503. Nakajima, H., et al., *CD4+ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea.* Am Rev Respir Dis, 1992. **146**(2): p. 374-7.

504. Foster, P.S., et al., *CD4(+) T-lymphocytes regulate airway remodeling and hyper-reactivity in a mouse model of chronic asthma.* Lab Invest, 2002. **82**(4): p. 455-62.

505. Komai, M., et al., *Role of Th2 responses in the development of allergen-induced airway remodelling in a murine model of allergic asthma.* Br J Pharmacol, 2003. **138**(5): p. 912-20.

506. Schon-Hegrad, M.A., et al., *Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-*

bearing dendritic cells (DC) in the conducting airways. J Exp Med, 1991. **173**(6): p. 1345-56.

507. Gong, J.J., et al., *Intraepithelial airway dendritic cells: a distinct subset of pulmonary dendritic cells obtained by microdissection.* J Exp Med, 1992. **175**(3): p. 797-807.

508. Holt, P.G., M.A. Schon-Hegrad, and J. Oliver, *MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen presentation activity by endogenous macrophage populations.* J Exp Med, 1988. **167**(2): p. 262-74.

509. Holt, P.G., et al., *A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium.* Int Arch Allergy Appl Immunol, 1990. **91**(2): p. 155-9.

510. Macatonia, S.E., et al., *Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies.* J Exp Med, 1987. **166**(6): p. 1654-67.

511. Reinhardt, R.L., et al., *Visualizing the generation of memory CD4 T cells in the whole body.* Nature, 2001. **410**(6824): p. 101-5.

512. Xu, B., et al., *Lymphocyte homing to bronchus-associated lymphoid tissue (BALT) is mediated by L-selectin/PNAd, alpha4beta1 integrin/VCAM-1, and LFA-1 adhesion pathways.* J Exp Med, 2003. **197**(10): p. 1255-67.

513. Catron, D.M., et al., *CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells.* J Exp Med, 2006. **203**(4): p. 1045-54.

514. Durham, S.R., S.J. Till, and C.J. Corrigan, *T lymphocytes in asthma: bronchial versus peripheral responses.* J Allergy Clin Immunol, 2000. **106**(5 Suppl): p. S221-6.

515. Kohlmeier, J.E. and D.L. Woodland, *Memory T cell recruitment to the lung airways.* Curr Opin Immunol, 2006. **18**(3): p. 357-62.

516. Lambrecht, B.N., R.A. Pauwels, and B. Fazekas De St Groth, *Induction of rapid T cell activation, division, and recirculation by intratracheal injection of dendritic cells in a TCR transgenic model.* J Immunol, 2000. **164**(6): p. 2937-46.

517. Hammad, H. and B.N. Lambrecht, *Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation.* J Allergy Clin Immunol, 2006. **118**(2): p. 331-6.

518. van Rijt, L.S. and B.N. Lambrecht, *Dendritic cells in asthma: a function beyond sensitization.* Clin Exp Allergy, 2005. **35**(9): p. 1125-34.

519. Hamid, Q.A. and L.A. Cameron, *Recruitment of T cells to the lung in response to antigen challenge.* J Allergy Clin Immunol, 2000. **106**(5 Suppl): p. S227-34.

520. Lambrecht, B.N., et al., *Sensitization to inhaled antigen by intratracheal instillation of dendritic cells.* Clin Exp Allergy, 2000. **30**(2): p. 214-24.

521. Jahnsen, F.L., et al., *Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge.* Thorax, 2001. **56**(11): p. 823-6.

522. Hogan, R.J., et al., *Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections.* J Immunol, 2001. **166**(3): p. 1813-22.

523. Marshall, D.R., et al., *Measuring the diaspora for virus-specific CD8+ T cells.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6313-8.

524. Hogan, R.J., et al., *Protection from respiratory virus infections can be mediated by antigen-specific CD4(+) T cells that persist in the lungs.* J Exp Med, 2001. **193**(8): p. 981-6.

525. Kuipers, H. and B.N. Lambrecht, *The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma.* Curr Opin Immunol, 2004. **16**(6): p. 702-8.

526. Maldonado, R.A., et al., *A role for the immunological synapse in lineage commitment of CD4 lymphocytes.* Nature, 2004. **431**(7008): p. 527-32.

527. Belz, G.T., et al., *Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus.* Proc Natl Acad Sci U S A, 2004. **101**(23): p. 8670-5.

528. Martinez, F.D., *Role of viral infections in the inception of asthma and allergies during childhood: could they be protective?* Thorax, 1994. **49**(12): p. 1189-91.

529. Venuprasad, K., S. Chattopadhyay, and B. Saha, *CD28 signaling in neutrophil induces T-cell chemotactic factor(s) modulating T-cell response.* Hum Immunol, 2003. **64**(1): p. 38-43.

530. Azzawi, M., et al., *Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma.* Am Rev Respir Dis, 1990. **142**(6 Pt 1): p. 1407-13.

531. Brinkmann, V., et al., *FTY720: altered lymphocyte traffic results in allograft protection.* Transplantation, 2001. **72**(5): p. 764-9.

532. Chiba, K., et al., *FTY720, a novel immunosuppressant possessing unique mechanisms. I. Prolongation of skin allograft survival and synergistic effect in combination with cyclosporine in rats.* Transplant Proc, 1996. **28**(2): p. 1056-9.

533. Matsuura, M., T. Imayoshi, and T. Okumoto, *Effect of FTY720, a novel immunosuppressant, on adjuvant- and collagen-induced arthritis in rats.* Int J Immunopharmacol, 2000. **22**(4): p. 323-31.

534. Suzuki, K., et al., *Prevention of experimentally induced autoimmune type I diabetes in rats by the new immunosuppressive reagent FTY720.* Transplant Proc, 1998. **30**(4): p. 1044-5.

535. Chiba, K., et al., *FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing.* J Immunol, 1998. **160**(10): p. 5037-44.

536. Brinkmann, V., et al., *FTY720: a novel transplantation drug that modulates lymphocyte traffic rather than activation.* Trends Pharmacol Sci, 2000. **21**(2): p. 49-52.

537. Yanagawa, Y., et al., *FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. II. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo.* J Immunol, 1998. **160**(11): p. 5493-9.

538. Idzko, M., et al., *Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function.* J Clin Invest, 2006. **116**(11): p. 2935-44.

539. Wills-Karp, M., *Immunologic basis of antigen-induced airway hyperresponsiveness.* Annu Rev Immunol, 1999. **17**: p. 255-81.

540. Cohn, L. and A. Ray, *T-helper type 2 cell-directed therapy for asthma.* Pharmacol Ther, 2000. **88**(2): p. 187-96.

541. Larche, M., D.S. Robinson, and A.B. Kay, *The role of T lymphocytes in the pathogenesis of asthma.* J Allergy Clin Immunol, 2003. **111**(3): p. 450-63; quiz 464.

542. Greenfeder, S., et al., *Th2 cytokines and asthma. The role of interleukin-5 in allergic eosinophilic disease.* Respir Res, 2001. **2**(2): p. 71-9.

543. Monaco, C., et al., *T cell-mediated signaling to vascular endothelium: induction of cytokines, chemokines, and tissue factor.* J Leukoc Biol, 2002. **71**(4): p. 659-68.

544. Choo-Kang, B., *Modulation of Inflammatory Pathways in Allergic Airways Inflammation*, in *Immunology, Infection and Inflammation*. 2006, University of Glasgow.

545. Buchanan, K.L. and J.W. Murphy, *Kinetics of cellular infiltration and cytokine production during the efferent phase of a delayed-type hypersensitivity reaction.* Immunology, 1997. **90**(2): p. 189-97.

546. Lundy, S.K., A.A. Berlin, and N.W. Lukacs, *Interleukin-12-independent down-modulation of cockroach antigen-induced asthma in mice by intranasal exposure to bacterial lipopolysaccharide.* Am J Pathol, 2003. **163**(5): p. 1961-8.

547. Babu, K.S., D.E. Davies, and S.T. Holgate, *Role of tumor necrosis factor alpha in asthma.* Immunol Allergy Clin North Am, 2004. **24**(4): p. 583-97.

548. Mohrs, M., et al., *Analysis of type 2 immunity in vivo with a bicistronic IL-4 reporter.* Immunity, 2001. **15**(2): p. 303-11.

549. Voehringer, D., K. Shinkai, and R.M. Locksley, *Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production.* Immunity, 2004. **20**(3): p. 267-77.

550. Itano, A.A., et al., *Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity.* Immunity, 2003. **19**(1): p. 47-57.

551. Rudensky, A., et al., *Sequence analysis of peptides bound to MHC class II molecules.* Nature, 1991. **353**(6345): p. 622-7.

552. Grubin, C.E., et al., *Deficient positive selection of CD4 T cells in mice displaying altered repertoires of MHC class II-bound self-peptides.* Immunity, 1997. **7**(2): p. 197-208.

553. Lambrecht, B.N. and H. Hammad, *Taking our breath away: dendritic cells in the pathogenesis of asthma.* Nat Rev Immunol, 2003. **3**(12): p. 994-1003.

554. van Rijt, L.S., et al., *Allergen-induced accumulation of airway dendritic cells is supported by an increase in CD31(hi)Ly-6C(neg) bone marrow precursors in a mouse model of asthma.* Blood, 2002. **100**(10): p. 3663-71.

555. Jung, S., et al., *In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens.* Immunity, 2002. **17**(2): p. 211-20.

556. Probst, H.C. and M. van den Broek, *Priming of CTLs by lymphocytic choriomeningitis virus depends on dendritic cells.* J Immunol, 2005. **174**(7): p. 3920-4.

557. Probst, H.C., et al., *Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells.* Clin Exp Immunol, 2005. **141**(3): p. 398-404.

558. Singh, J., et al., *Rational design of potent and selective VLA-4 inhibitors and their utility in the treatment of asthma.* Curr Top Med Chem, 2004. 4(14): p. 1497-507.

559. Tang, M.L. and L.C. Fiscus, *Important roles for L-selectin and ICAM-1 in the development of allergic airway inflammation in asthma.* Pulm Pharmacol Ther, 2001. **14**(3): p. 203-10.

560. Miller, M.J., et al., *T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node.* Proc Natl Acad Sci U S A, 2004. **101**(4): p. 998-1003.

561. Miller, M.J., et al., *Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes.* J Exp Med, 2004. **200**(7): p. 847-56.

562. Miller, M.J., et al., *Autonomous T cell trafficking examined in vivo with intravital two-photon microscopy.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2604-9.

563. Miller, M.J., et al., *Two-photon imaging of lymphocyte motility and antigen response in intact lymph node.* Science, 2002. **296**(5574): p. 1869-73.

564. Squirrell, J.M., et al., *Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability.* Nat Biotechnol, 1999. **17**(8): p. 763-7.

565. Witt, C.M. and K. Robbins, *Tracking thymocyte migration in situ.* Semin Immunol, 2005. **17**(6): p. 421-30.

566. Mempel, T.R., S.E. Henrickson, and U.H. Von Andrian, *T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases.* Nature, 2004. **427**(6970): p. 154-9.

567. Celli, S., Z. Garcia, and P. Bousso, *CD4 T cells integrate signals delivered during successive DC encounters in vivo*. J Exp Med, 2005. **202**(9): p. 1271-8.

568. Okada, T., et al., *Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells*. PLoS Biol, 2005. **3**(6): p. e150.

569. Schneider, H., et al., *Reversal of the TCR stop signal by CTLA-4*. Science, 2006. **313**(5795): p. 1972-5.

570. Rosentiel, P., et al., *NOD-like receptors and human diseases*. Microbes Infect, 2007. **9**(5): p 648-57.

571 Franchi, L., et al., *Intracellular NOD-like receptors in innate immunity, infection and disease*. Cell Microbiol, 2007. **Epub ahead of print.**

572 Wang, YH., et al., *IL-25 augments type 2 immune responses by enhancing the functions of TSLP-DC-activated Th2 memory cells*. J Exp Med, 2007. **204**(8).

573 Schmitz, J., et al., *IL-33, an IL-1 like cytokine that signals via the IL-1 receptor related protein ST-2 and induces T helper type-2 associated cytokines*. Immunity, 2005. **23**(5): p479-90.

574 Irifune, K., et al., *Adoptive transfer of T helper-cell type 1 clones attenuates an asthmatic phenotype in mice*. Eur Resp J, 2005. **25**(4): p 653-9.

575 Kita, H., et al., *Eosinophils and IgE recpetors; a continuing controversy*. Blood, 1997. **89**(10): p 3497-501.

576 Kim, YK., et al., *Airway exposure levels of lippopolysaccharide determine type 1 verses type 2 experimental asthma*. J Immunol, 2007. **178**(8): p 5375-82.

577 Eisenbarth SC., et al., *Lippopolysaccharide, toll-like receptor 4-dependent T helper cell type 2 responses in inhaled antigens*. J Exp Med, 2002. **196**(12): p1645-51.