Studying the effect of cigarette smoke exposure on murine models of allergic asthma

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Abstract

Exposure to pollution and active or passive smoking have been associated with a worsened asthma severity and a reduced response to treatment. These poorly controlled asthmatics are responsible for the majority of the economic burden of the disease but *how* pollution and/or cigarette smoke (CS) impacts on the disease is not well understood.

The aim of this thesis was to develop a murine model of allergic asthma where CS exposure results in a change in model phenotype and the sensitivity of the response to pharmacological intervention.

Two preclinical models of allergic asthma were utilised: the ovalbumin (OVA) model which had previously been established in-house, and the house dust mite (HDM) model which I developed in this thesis. As topical HDM exposure is known to cause innate inflammation I developed an allergic model where HDM challenge resulted in inflammation only in the mice which had been previously sensitised to HDM. The allergic inflammation in this model was accompanied by allergic airway hyper responsiveness, however the LAR was not observed in this model.

CS exposure did not have a dramatic impact on the cellular inflammation in either the OVA- or the HDM-driven model, nor did it impact upon the anti-inflammatory effects of oral steroid treatment with the exception of the addition of a steroid-insensitive neutrophil population. However CS exposure attenuated the AHR observed in the OVA and the HDM models. Finally cigarette smoke exposure not only enhanced the OVA-induced LAR but also rendered this response completely insensitive to oral steroid treatment. Further investigation into the effects of CS in these two models may provide clues as to the mechanisms behind the effect of smoking on asthma in the clinic. The CS-enhanced LAR model could be invaluable in understanding the clinical phenotype of treatment resistance in smoking asthmatics.

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Statement

The immunoglobulin measurements described in Chapter 5 were performed with the assistance and guidance of Sorif Uddin at GSK, Stevenage UK. The morphological diagrams of inflammatory cells (Figure 2.1) were created by Tom Malloy and the diagram of the smoke exposure system (Figure 2.2) was created by Dr Suffwan Eltom. All work described in this thesis is original work by the author, unless otherwise referenced. This PhD project was funded by an MRC and GSK CASE studentship.

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5-HT	5-hydroxytryptamine
AAT	α_1-antitrypsin
ACh	Acetylcholine
AHR	Airway hyperresponsiveness
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP-1	Adaptor protein 1
APC	Allergen presenting cell
ASC	Apoptotic speck protein containing a caspase recruitment domain (PYCARD): an inflammasome adaptor protein
(h) ASM	(Human) Airway smooth muscle
(h) ASMC	(Human) Airway smooth muscle cell
ATP	Adenosine triphosphate
ATS	American thoracic society
AUC	Area under the curve
β_2 (agonist)	Beta (2) adrenergic receptor agonist
BAL (F)	Bronchoalveolar lavage (fluid)
BSA	Bovine serum albumin
C3a	Complement component 3a
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CCR	CC or β chemokine receptor
CCL	CC or β chemokine ligand
CFA	Complete Freund's adjuvant
c-kit	Proto-oncogene c-Kit or mast/stem cell growth factor receptor
COPD	Chronic obstructive pulmonary disease

COX	Cyclooxygenase
CS	Cigarette smoke
CXCR	CXC or α chemokine receptor
CXCL	CXC or α chemokine
CD	Cluster of differentiation
DC	Dendritic cell
Der f	Dermatophagoides farinae
Der p	Dermatophagoides pteronyssinus
DNA	Deoxyribonucleic acid
EAR	Early asthmatic response
ECM	Extracellular matrix
ECP	Eosinophilic cation protein
EDN	Eosinophil-derived neurotoxin
ELISA	Enzyme-linked immunosorbent assay
EPO	Eosinophil peroxidase
ETS	Environmental tobacco smoke
FACS	Fluorescence-activated cell sorting
FeNO	Fraction of exhaled nitric oxide
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
FcεR	Fc-epsilon receptor (receptor for IgE)
FcγR	Fc-gamma receptor (receptor for IgG
FOXP3	Forkhead box P3
GATA	Trans-acting T-cell-specific transcription factor 3
GC	Glucocorticoid
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GR	Glucocorticoid receptor

GRE	Glucocorticoid response element
HAT	Histone acetyltransferase
HBEC	Human bronchial epithelial cell
HDAC	Histone deacetylase
HDM	House dust mite
HRP	Horseradish peroxidase
I-CAM	Intercellular adhesion molecule
ICS	Inhaled corticosteroid
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of κB
IκK	IκB kinase
IL	Interleukin
iNK	Invariant natural killer T cells
iNANC	Inhibitory nonadrenergic noncholinergic
iNOS	Inducible nitric oxide synthase
i.n	Intranasal
i.p	Intraperitoneal
i.t	Intratracheal
JNK	c-Jun N-terminal kinases
KHS	Krebs-Henseleit solution
IP (10)	CXC chemokine (interferon gamma induced protein 10)
LABA	Long acting β_2 agonist
LAMA	Long acting muscarinic antagonist
LAR	Late asthmatic response
LFA-1	Lymphocyte function-associated antigen -1
LPS	Lipopolysaccharide
LT	Leukotriene

M (2 or 3)	Muscarinic receptor (2 or 3)
mAB	Monocloncal antibody
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCh	Methacholine
MCP	Monocyte chemotactic protein
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinases
MPP	Multipotent progenitor cell
mRNA	Messenger ribonucleic acid
MTS	Mainstream tobacco smoke
MyD88	Myeloid differentiation primary response gene
NE	Neutrophil elastase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NLR	NOD-like receptor
NLRP3	Nucleotide-binding domain, leucine-rich repeats containing family, pyrin domain-containing-3
NO	Nitric oxide
OCS	Oral corticosteroid
OVA	Ovalbumin
PAF	Platelet activating factor
PAMP	Pathogen-associated molecular pattern
PAR	Protease activated receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PCLS	Precision-cut lung slices
PEF	Peak expiratory flow
Penh	Enhanced pause
PI3K	Phosphatidylinositide 3-kinases
PK	Protein kinase
PM	Particulate matter
PRR	Pathogen recognition receptor
PG	Prostaglandin
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RAST	Radioallergosorbent test
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SABA	Short acting β_2 agonist
SBM	Subbasement membrane
SCF	Stem cell factor
SEM	Standard error of the mean
SMA	Smooth muscle actin
SNP	Single nucleotide polymorphism
STAT	Signal Transducer and Activator of Transcription
Syk	Spleen tyrosine kinase
T1/ST2	IL-1 family receptor
TARC	Thymus and activation-regulated chemokine
T-bet	T-box transcription factor
TCR	T cell receptor
TF	Transcription factor
TGF	Transforming growth factor
Th (cell)	T helper (cell)

TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
Treg	Regulator T cell
TSLP	Thymic stromal lymphopoietin
TSP	Total suspended particulate
TxA_2	Thromboxane A_2
UA	Uric acid
V-CAM	Vascular cell adhesion molecule 1
WBP	Whole body plethysmography

Chapter 1. Introduction

1.1. Asthma

Asthma is a chronic inflammatory respiratory disorder which results in episodes of wheezing, breathlessness, chest tightness and coughing. These symptoms are often more pronounced early in the morning or at night. Airflow obstruction in asthma is variable and usually reversible spontaneously or with treatment. Triggers for asthma symptoms include exposure to allergens; exercise and cold temperatures; viral and bacterial infections; or exposure to pollution such as cigarette smoke.

There are two main types of asthma: atopic or non-atopic. In this thesis I will concentrate on atopic asthma, a type-1 hypersensitivity reaction. As the name suggests this is associated with atopy (increased production of total and allergen-specific immunoglobulin E (IgE) (Pearce *et al.* 1999)). Atopy is associated with other common diseases including allergic rhinitis and atopic dermatitis (eczema) (Arshad *et al.* 2001) and these three diseases, known as the atopic triad often occur together. The primary cause of atopic asthma is sensitisation to an allergen and in asthma the common etiological allergens include house dust mites (HDM), fungal species such as Aspergillus fumigatus, animal dander and plant pollen. One of the most prevalent allergens in asthma is HDM which has been shown to induce positive skin prick test responses in approximately 80% of asthmatic children (Smith *et al.* 1969). How HDM induces sensitisation and allergic responses will be discussed in more detail below.

In an article reviewing the risk factors for an individual to develop asthma Peter Sly provided a succinct explanation of the etiology of asthma, which I quote below:

> "Asthma can be thought of as a developmental disease, in which the normal development of the respiratory and immune systems is altered by the impacts of environmental exposures acting on underlying genetic predispositions." (Sly 2011)

It is thought that a mixture of genetic factors such as pre-disposition to atopy or AHR, and environmental factors such as exposure to indoor allergens; indoor and outdoor pollution; and passive or active smoking may determine the development of asthma in an individual.

Atopy or increased production of allergen-specific IgE is a key risk factor for asthma (Pearce *et al.* 1999; Arshad *et al.* 2001; Arbes *et al.* 2007). A large national study in the US estimated asthma prevalence in the general population to be 5.2% (Arbes *et al.* 2007) while general levels of atopy in western countries were estimated to be around 30% - 54% (Pearce *et al.* 1999; Arbes *et al.* 2007); the proportion of asthma cases attributable to atopy

was estimated at being between 25 and 63% (Pearce *et al.* 1999). Another US study estimated atopy levels in asthmatics to be 79%, calculating the population-attributable risk (PAR) at 56% (Arbes *et al.* 2007). Notably living in a highly populated metropolitan area resulted in a higher PAR for atopy in asthma (Arbes *et al.* 2007). There is an association between atopy and asthma and a history of these conditions in the parents (Kuehr *et al.* 1992; Bergmann *et al.* 1997), thus a proportion of the risk of asthma development is attributable to genetic factors and heritability (Sandford *et al.* 1996).

According to the World Health Organisation approximately 300 million people worldwide are affected by asthma, which represents between 1 and 18% of the population of a given country (Masoli *et al.* 2004). There is a trend for increased prevalence in developed countries; the greatest prevalence was found in countries such as the UK, the USA, South America, Australia and New Zealand. Interestingly there is also a trend for increased prevalence within poorer populations than the affluent populations in those countries (Masoli *et al.* 2004). There is also evidence that a traditional lifestyle, rural environment and specifically farm living may be protective against asthma (von Mutius *et al.* 1992, 1994; Wichmann 1996; Yemaneberhan *et al.* 1997; Riedler *et al.* 2001; von Hertzen & Haahtela 2006; Peters *et al.* 2006; Debarry *et al.* 2007; von Mutius & Vercelli 2010), whereas affluence and urban lifestyles may promote development of atopy (Von Hertzen & Haahtela 2004). These phenomena can be in part explained by the hygiene hypothesis.

1.1.1. Hygiene hypothesis

The adaptive immune system is thought to function as a balance between Th1 and Th2 type immune responses (Martinez & Holt 1999). Th1 responses are classically associated with immunity to invading pathogens while Th2 responses are associated with the generation of atopy. Th2 and Th1 cells produce cytokines which reciprocally regulate each other's differentiation and cell functions (Mosmann & Coffman 1989; Mosmann & Sad 1996; Yssel *et al.* 2001), as explained in Figure 1.1. The hypothesis is that early life infection results in increased production and activation of Th1 cells which antagonise the generation of the Th2 responses that lead to atopy (Martinez 1994). A traditional lifestyle involved exposure to soil, waste, wood and animal excrement which would lead to high levels of exposure to microbial organisms and thus infections (Von Hertzen & Haahtela 2006). In a modern and urbanised environment these exposures are reduced. Childhood infection has been shown to inversely correlate with asthma and atopy: (Shaheen *et al.* 1996; Shirakawa *et al.* 1997; Matricardi *et al.* 1997). A lack of early life infection due to cleaner living environments and smaller family sizes may therefore promote the development of

atopy and asthma (Strachan 1989; Martinez 1994; Holt *et al.* 1997; Martinez & Holt 1999; Renz & Herz 2002). It may be that the immune system originates with a Th2 bias which is normally dampened through microbial infections, but in certain individuals the Th2 environment prevails leading to atopy (Barrios *et al.* 1996; Prescott *et al.* 1998*a*, *b*; Von Hertzen & Haahtela 2004).

The Th1 and Th2 paradigm is thought to be overly simplified now and the hypothesis has been recently revised with the suggestion that interleukin 10 (IL-10) production from regulatory T cells (Tregs) may also be involved in protection from the development of atopy (Yssel *et al.* 2001; Wills-Karp *et al.* 2001). IL-10, crucially involved in immune homeostasis, is normally induced by microbial infection and dampens immune responses through its multiple immunomodulatory functions (Moore *et al.* 1993). Parasite-induced production of IL-10 reduces sensitisation to allergens; a lack of infection results in lack of this feedback and causes predisposition to allergy (van den Biggelaar *et al.* 2000; Yazdanbakhsh *et al.* 2001; Wills-Karp *et al.* 2001).

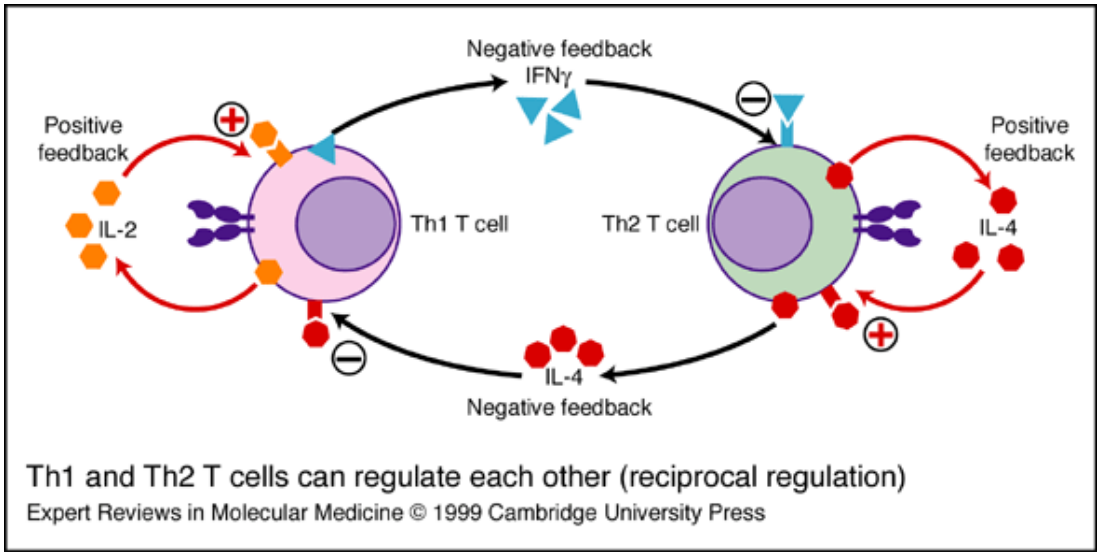


Figure 1.1: Reciprocal regulation by Th1 and Th2 cells (Bushell & Wood 1999)

1.1.2. Allergic sensitisation in asthma

The immune response in atopic asthma is made up of two parts: the initial sensitisation phase, followed by a challenge or allergic response phase in sensitised patients. When an allergen is encountered this is detected by an antigen presenting cell (APC) such as a dendritic cell (DC) in the lungs. These cells patrol the airway epithelium and submucosa, extending their dendrites into the airway to scan the lumen for invading pathogens. When a

DC detects an allergen it internalises it, processes it onto a major histocompatibility complex class II molecule (MHC-II) which it then expresses on the cell surface. The DC will also start to express T cell co-stimulatory molecules such as CD40, B7-1 (CD80), B7-2 (CD86) and ICAM-1 (Vermaelen *et al.* 2001). DCs expressing the MHC-II allergen complex will then migrate to the secondary lymphatic system transporting the antigen with them (Vermaelen *et al.* 2001). There it presents the allergen to naive B cells and T cells; this process is known as antigen presentation.

When the APC encounters a $CD4^+$ T cell expressing the relevant T cell receptor (TCR) for the allergen, a complex is formed between the MHC-II/allergen molecule and the T cell. The antigen must be presented to the relevant T cell expressing the correct TCR; however each TCR can recognise a vast number of different antigenic peptides in order for the immune system to provide effective immunity against all the possible antigens that may be encountered (Wooldridge *et al.* 2012). MHC-II/allergen complex formation, along with co-stimulatory signals from the APC such as CD80 (B7-1) and CD86 (B7-2) activates the T cell expressing the correct TCR to proliferate (clonal selection and expansion) and to differentiate into effector cells. Interleukin 2 (IL-2) is thought to promote T cell proliferation, and T cells only express the IL-2 receptor once activated.

Once activated mature $CD4^+$ T helper cells differentiate predominantly into a Th1 or Th2 effector cell phenotype, and this depends on the cytokine environment in which antigen presentation occurs. T cell differentiation into the Th2 phenotype is an important immunological feature of asthma which is likely to contribute to the pathogenesis of the disease by promoting allergic sensitisation to allergens. Th2 polarising signals such as interleukin 4 (IL-4) released from APCs during presentation promote Th2 polarisation while cytokines such as interferon-γ (IFN-γ) promote Th1 polarisation. CD86 expression is also likely to be important in induction of Th2 disease (Haczku *et al.* 1999). Th2 polarisation may not just occur in the lymphatic system as there is evidence to suggest that this process may also occur in the lung (Constant *et al.* 2002). Activated Th2 cells, through release of a plethora of cytokines, are then involved in directing cellular immune responses; their role and that of other T cell subsets in the pathogenesis of asthma will be explored further below.

B cells can also function as APCs; they are capable of internalising allergens and presenting them on their cell surface. CD40 ligand (CD40L) expressed on the surface of the T cell will bind to CD40 receptor (CD40R) on the B cell, if the T cell expresses the correct TCR for the allergen. This interaction combined with the release of IL-4 and interleukin 13 (IL-13) from the Th2 cell will lead to B cell activation and immunoglobulin class switching in the B cell from production of IgM to IgE. This ultimately culminates in the release of allergen-specific IgE. IL-4 and IL-13 are thought to be fundamentally important for IgE

production and the allergic response (Grünig *et al.* 1998; McKenzie *et al.* 1999). IgE molecules sensitise the individual to a subsequent allergen exposure by binding to high affinity Fcε receptors (FcεR1) expressed on mast cells and basophils, priming these cells to respond to a subsequent allergen exposure. IgE is an important mediator in allergic asthma and its role will also be discussed further below.

The principle steps of allergic sensitisation in asthma and the response to allergen exposure are outlined in Figure 1.2

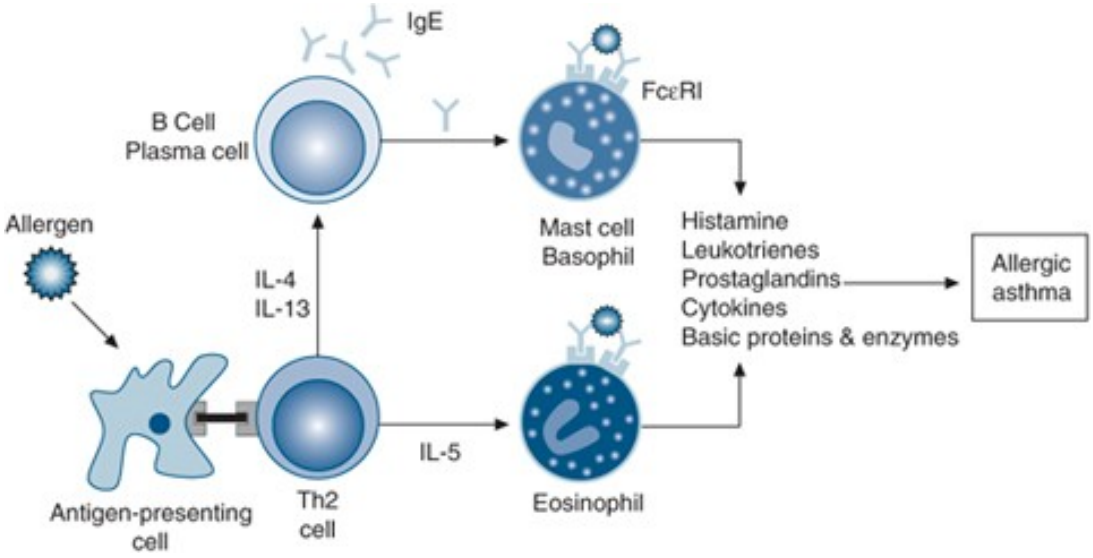


Figure 1.2: Allergic sensitisation and response in asthma (Holgate 2008)

1.1.3. Allergic responses in asthma

Once an individual has become sensitised, further allergen exposure will result in what is known as an allergic response. An asthmatic episode such as that triggered by exposure to allergen usually occurs in two phases: firstly the early asthmatic response (EAR) which is characterized by bronchoconstriction, mucus production and oedema and secondly the late asthmatic response (LAR). This phase is often prolonged, occurring several hours after allergen exposure and is often very severe. This phase is associated with cellular influx and mediator release into the lungs. The early response occurs immediately after allergen exposure while the late response tends to occur between 4 and 8 hours after exposure (Booij-Noord *et al.* 1971). Much of what is understood of these responses comes from allergen challenge studies performed in asthmatic patients (Crescioli *et al.* 1991; Rédier *et al.* 1992; Paggiaro *et al.* 1994; Hamilton *et al.* 1997, 1998; Inman *et al.* 2001; Gauvreau *et al.* 2002; Davis *et al.* 2009).

1.1.3.1. EAR

During sensitisation allergen-specific IgE is produced by B cells and then binds to high affinity IgE receptors (FcεR1) on mast cells and basophils. Upon allergen exposure, the allergen binds to these IgE molecules which results in IgE cross-linking, leading to cellular activation, degranulation and subsequent release of mediators. Mast cell degranulation results in release of preformed granule mediators such as histamine and 5-HT, and newly synthesised eicosanoid mediators including prostaglandins (PGD_2), and cysteinyl-leukotriene C_4, D_4 and E_4 (LTC_4, LTD_4 and LTE_4). These are thought to result in the early asthmatic response (EAR), characterised by a rapid bronchoconstriction, oedema (plasma extravasion) and mucus secretion; histamine and PGD_2 and LTC_4 are potent bronchoconstrictors (Juniper *et al.* 1978; Eiser *et al.* 1981; Weiss *et al.* 1982; Smith *et al.* 1985; Bisgaard *et al.* 1985; Casale *et al.* 1987; Wenzel *et al.* 1989; Jarjour *et al.* 1997; Ruck *et al.* 2001; Holgate *et al.* 2003). Upon allergen-induced cross linking of IgE on mast cells, mediators such as interleukin 6 (IL-6), macrophage inflammatory protein 1α (MIP-1α), tumour necrosis factor-α (TNF-α), IL-4, interleukin 5 (IL-5) and IL-13 are also synthesised and released, which are thought to drive the late phase response and the associated recruitment of inflammatory cells (Dullaers *et al.* 2012).

As this thesis will concern murine models of asthma, it is worth noting that different mediators have been implicated in mediating bronchoconstriction in different species. For example histamine and cysteinyl leukotrienes are potent bronchoconstrictors and asthmatic mediators in man and guinea pigs, mediating the response of isolated trachea to allergen (Adams & Lichtenstein 1979), whereas cysteinyl leukotrienes, 5-HT and products of the cyclooxygenase (COX) pathway have been shown to mediate allergen-induced bronchospasm in the rat (Dahlbäck *et al.* 1984; Hele *et al.* 2001). Histamine and cysteinyl leukotrienes are thought to play a less important role in murine asthma models (Weigand *et al.* 2009). The primary mediator of allergic bronchoconstriction in the mouse is 5-HT (Eum *et al.* 1999; Weigand *et al.* 2009), although others have suggested a role of both 5-HT and histamine in murine OVA-induced AHR (De Bie *et al.* 1998). In human airways the role of 5-HT as a contractile agent is more controversial, but it is unlikely to mediate allergen induced airway responses (Takahashi *et al.* 1995; Dupont *et al.* 1999).

1.1.3.2. LAR

Approximately 4-8 hours after allergen exposure the late response occurs in approximately 50% of asthmatic patients (Booij-Noord *et al.* 1971, 1972; Robertson *et al.* 1974). Aside from the bronchoconstriction this response is also characterised by influx of multiple inflammatory cells into the airways including neutrophils, eosinophils, mast cells

and T cells (Aalbers *et al.* 1993*a*, *b*; Hogan *et al.* 1998). The LAR may be a more relevant marker of asthma than the EAR, as firstly the bronchoconstriction tends to be more pronounced and long-lived, and secondly because the EAR is also observed in rhinitis patients (O'Byrne *et al.* 1987; Stevens & van Bever 1989; Muller *et al.* 1993). IgE, mast cells and mast cell mediators are heavily implicated in driving the LAR in human asthmatics (Booij-Noord *et al.* 1971; Cockcroft *et al.* 1979; Cockcroft & Murdock 1987; Yamada *et al.* 1992; Aalbers *et al.* 1993*a*; Hamilton *et al.* 1997, 1998; Fahy *et al.* 1997; Nabe *et al.* 2004; Singh *et al.* 2007; Moon *et al.* 2008; Davis *et al.* 2009).

It is likely that the LAR is at least in part driven by inflammation (Bousquet *et al.* 2000; Barnes 2008). This is largely based on the observation that the LAR is accompanied by pulmonary inflammation (Rossi *et al.* 1991; Cieslewicz *et al.* 1999; Gauvreau *et al.* 1999*b*, 2000) and that steroid treatment impacts on the LAR (Cockcroft & Murdock 1987; Paggiaro *et al.* 1994; Kidney *et al.* 1997; Cieslewicz *et al.* 1999; Inman *et al.* 2001; Gauvreau *et al.* 2002; Leigh *et al.* 2002; Duong *et al.* 2007). Eosinophilia, Th2 inflammation and the associated cytokines such as IL-5 and IL-13 are likely to play a role (Jarjour *et al.* 1997; Cieslewicz *et al.* 1999; Taube *et al.* 2002). However others have questioned the role of inflammation such as eosinophils in the LAR (Leckie *et al.* 2000). Basophils and mast cells enter the lung following topical OVA challenge in sensitised mice, but while basophils were found to be important for the EAR, these cell types were not responsible for the LAR (Nabe *et al.* 2013). Others have shown that histamine was released during the early and late allergic response, but that PGD_2 and tryptase (mast cell-specific mediators) were only present during the EAR, whereas late phase histamine release correlated with basophil numbers (Naclerio *et al.* 1985; Bascom *et al.* 1988; Charlesworth *et al.* 1989; Iliopoulos *et al.* 1992; Proud *et al.* 1992; Guo *et al.* 1994). Furthermore, 95% of the histamine positive, IgE bearing cells in the BALF (bronchoalevolar lavage fluid) during the LAR were shown to be basophils (Guo *et al.* 1994). This data suggests that basophils and basophil-derived mediators may be important for the LAR. However evidence from our lab suggests that the LAR (at least in the mouse and rat) may not be driven by the mediators characteristic of the early response (5-HT, histamine or cysteinyl leukotrienes), rather being induced by sensory nerve activation and a central neuronal reflex, culminating in cholinergic bronchoconstriction (Raemdonck *et al.* 2012). Activation of transient receptor potential cation channel A1 (TRPA1) receptors on sensory nerves was also implicated in the response (Raemdonck *et al.* 2012).

The allergen-induced late asthmatic response is also linked with development of AHR in asthmatic patients which can last for weeks or months after allergen exposure (Cartier *et al.* 1982; Cockcroft 2000). AHR in asthma will be discussed below.

1.1.4. Chronic pulmonary pathology of asthma

Apart from the responses to allergen exposure described above, repeated allergen exposure results in a chronic inflammatory state in the lungs. A large array of cells and mediators are implicated in the pathogenesis of asthma; the lungs of asthmatics are chronically inflamed, with infiltration of eosinophils, neutrophils, mast cells and activated ($CD4^+$) T cells of the Th2 phenotype in the epithelium and bronchial mucosa. Structural alterations of airway mucosa and increased basal membrane thickness are observed (Amin *et al.* 2000; Brightling *et al.* 2002) along with increased collagen deposition, subepithelial fibrosis; goblet cell and smooth muscle metaplasia. This chronic pulmonary inflammation and airway wall remodelling are mechanistically associated with the airway hyperresponsiveness (AHR) which also occurs in asthmatic patients. AHR results in an enhanced bronchospasm in asthmatic patients to spasmogens or a response to stimuli which in healthy individuals would not normally evoke bronchoconstriction. The chronic inflammation, eosinophilia and IgE mediated responses are typical of a Th2 type immune response. The precise mechanisms driving disease development and progression are poorly understood despite many decades of research in this field. Generation of the asthmatic phenotype involves a complex process of events but it is likely to occur as a result of prolonged activation of both the innate and adaptive immune systems directed towards the airways.

1.1.5. Cellular immune responses in asthma

The inflammatory response in asthma is complex, with many cell types and mediators being involved. Extensive clinical sampling of BALF and sputum from asthmatics has been performed, for example (Walker *et al.* 1991; Robinson *et al.* 1992, 1993*a*; Wenzel *et al.* 1997, 1999; Jatakanon *et al.* 1999; LouiS *et al.* 2000). Briefly, lungs of asthma patients are characterised by chronic airway inflammation including: mast cells, activated $CD4^+$ T helper cells and B cells; eosinophils, neutrophils and macrophages. All of these cells along with structural cells in the lung such as epithelial cells and airway smooth muscle cells are likely to contribute to the pathogenesis of the disease. Chemokines released from inflammatory cells and structural cells are involved in recruitment of inflammatory cells to the lungs, and cytokines and enzymes are likely to promote the tissue remodelling and AHR which are characteristic of the disease. The mast cell is classically described as being involved in orchestrating early allergic responses through the action of IgE produced by B cells, and both dendritic cells and epithelial cells are recently being given increased

responsibility for promoting allergic sensitisation in the disease. Th2 cells and their mediators are likely to drive and direct immune responses in asthma, while a role in AHR and in structural remodelling has been attributed to eosinophils; in asthma many of the cell types are likely to have overlapping functions. The following sections will outline the current theory as to the contribution of these various cell types to the pathogenesis of allergic asthma.

1.1.5.1. APCs

Antigen presenting cells (APC) specialize in detecting antigens and presenting them to naïve T cells. They do this by capturing the antigen, internalising and processing it, and then expressing it on their cell surface in conjunction with MHC-II molecules. APCs then promote T cell selection, proliferation and differentiation and through release of mediators can promote different T cell responses. Principal APCs are dendritic cells, macrophages and B cells.

1.1.5.1.1. DCs

Dendritic cells are the principal APC implicated in allergic sensitisation in asthma. They are likely to act as a link between the innate and adaptive immune response, as they can be activated by innate cytokines in the lung, and then go on to promote adaptive immune responses by activation of T cells. They are widely thought to possess Th2 polarising capabilities, although the mechanism behind DC-mediated Th2 activation is not fully understood (Kaiko & Foster 2011).

DCs originate in the blood where they are derived from bone marrow precursor cells, and are found in an immature form in the skin, the lining of the nose, the airways, and the GI tract; activation by an antigen leads to DC maturation. Detection of an antigen by a DC results in antigen presentation as described above. DCs recognise microbial motifs such as pathogen associated molecular patterns (PAMPS) via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) expressed on the DCs. This recognition of PAMPS is important for DC maturation as allergens do not activate DCs in the absence of these motifs. Indeed TLR deficient mice showed reduced DC function, reduced expression of CD86 and reduced production of Th2 cytokines from naive $CD4^+$ T cells. In addition low levels of LPS acting on TLR4 to activate DCs was essential for Th2 sensitisation to inhaled allergens (Dabbagh *et al.* 2002; Eisenbarth *et al.* 2002). Once a DC has been activated, antigen phagocytosis by DCs is then blocked to prevent activated DCs presenting self-antigens. A dendritic cell, activated by an allergen in the context of a PAMP, and expressing the MHC-II allergen complex, will then migrate to the secondary lymphatic system transporting the antigen with it (Vermaelen *et al.* 2001). There it presents the allergen to

naive B cells and $CD4^+$ T cells and promotes adaptive immune response through T cell selection, proliferation and differentiation. DC migration to draining mediastinal lymph nodes may be dependent on chemokine receptors CCR7 and CCR8 (Jakubzick *et al.* 2006; Hintzen *et al.* 2006). After allergen challenge, DCs accumulate in the airway mucosa within 4-5 hours following recruitment from the blood. These DCs are able to stimulate Th2 responses *ex vivo* (Xia *et al.* 1995; Jahnsen *et al.* 2001). DCs isolated from the lungs of allergen challenged rats show antigen presenting properties as early as 3 hours after challenge, and APC competent DCs are then found in the draining lymph nodes 24 hours after challenge. This suggests that antigen presenting DCs migrate from the lungs to the lymph nodes after allergen challenge (Xia *et al.* 1995).

Cytokines released by the DC can promote T cell polarisation towards the Th1 or Th2 phenotype, for example presentation to naïve $CD4^+$ T cells in conjunction with interleukin 12 (IL-12) promotes Th1 differentiation (Gately *et al.* 1998). However the mechanism behind DC induced Th2 cell differentiation is less well understood. It may be that DCs are preconditioned to activate either Th1 or Th2 type responses. Lymphoid derived DCs stimulate IFN-γ and IL-2 production from T cells while myeloid DCs induce production of IL-4 and IL-10; these cytokine profiles are associated with Th1 and Th2 phenotypes respectively (Pulendran *et al.* 1999; Pulendran 2004). Indeed myeloid airway DCs can induce Th2 sensitisation to HDM in mice which results in airway eosinophilia (Lambrecht *et al.* 2000) and depleting pulmonary DCs abolishes Th2 response in HDM model (Hammad *et al.* 2010). Intrinsic production of different mediator profiles by different DC subsets may influence the T cell outcome upon antigen presentation. DCs have been identified as DC1 and DC2 subtypes (Grouard *et al.* 1997; Olweus *et al.* 1997); the development of these cells from their respective precursors is described by (Liu 2001). It has been shown that DC1 DCs produce lots of IL-12 and as such promote Th1 responses while DC2 produce less IL-12 (Liu 2001); DCs deficient in IL-12 promote Th2 responses (Kaliński *et al.* 1997). Others have identified an additional APC (late-activator APCs) which migrate to the lung in response to influenza A virus, take up antigen and migrate to DLNs but on a slower timescale than conventional DCs. Presentation to T cells by these LAPCs results in solely Th2 polarisation, which may be mediated by direct-contact induced expression of GATA3 (Yoo *et al.* 2010) a Th2 related transcription factor (Zheng & Flavell 1997) .

However other evidence suggests that DCs are not intrinsically committed to Th1 or Th2 promoting phenotypes: IFN-γ stimulation at the point of DC activation was necessary for maturation into a Th1 promoting phenotype, while PGE_2 promoted generation of Th2 promoting DCs (Vieira *et al.* 2000). *In vitro* ingestion of different pathogens can promote maturation of DCs into different phenotypes resulting in different cytokine production profiles; this process may also be regulated by feedback cytokine production from T cells (d' Ostiani *et al.* 2000; Bozza *et al.* 2002; Edwards *et al.* 2002). Expression of additional molecules on DCs including OX40L, CD40 and B7 family members CD80 and CD86 may promote Th2 polarisation (Eisenbarth *et al.* 2003). Interaction of CD80 and CD86 on DCs with CD28 on T cells (Keane-Myers *et al.* 1997) and OX40/OX40L interaction have been shown to be important for Th2 sensitisation and features of asthma in murine models (Hoshino *et al.* 1998; Jember *et al.* 2001). Cytokines such as IL-4, IL-13, interleukin 18 (IL-18), interleukin 35 (IL-25) and IL-6 have also been implicated in promoting Th2 responses (Eisenbarth *et al.* 2003). DC skewing of T cell responses to the Th2 response may be a passive process relying on lack of Th1 signals IL-12 and CD40 or an active process dependent on OX40L, IL-25 and other Th2 cytokines (Eisenbarth *et al.* 2003).

Intrinsic differences in mediator levels in asthmatic individuals compared to normal individuals may influence the outcome of T cell polarisation by dendritic cells explaining the Th2 skewed immune responses in asthmatic patients. Blood cells from asthmatics produce less IFN γ and IL-12 (van der Pouw Kraan *et al.* 1997). DCs obtained from atopic individuals produce less IL-12 and IL-10 than DCs from normal individuals (Reider *et al.* 2002) and more Th2 skewing DC2s were observed in atopic patients (Reider *et al.* 2002). Allergen pulsed DCs from asthmatic or atopic patients induced proliferation of IL-4 , producing T cells, and production of IL-4, IL-5 and IL-10 from T cells, while IFN-γ producing T cells were produced by DCs from normal individuals (Bellinghausen *et al.* 2000; Graffi *et al.* 2002; Hammad *et al.* 2002).

Thus it may be there are two processes involved in regulating DC immune function. The first being a predetermined bias to Th1 or Th2 promoting phenotypes, and the second being through modulatory effects of microbes and mediators in the pulmonary environment (Pulendran 2004). Together this data suggests that DCs control T cell polarisation and that the environment in asthmatic individuals may regulate whether this generates a Th1 or Th2 response.

Not only are DCs important for allergic sensitisation, but they have also been shown to promote chronic inflammation in the disease (Lambrecht *et al.* 1998; Julia *et al.* 2002).

They may be key for stimulation of Th2 cells during on-going inflammation (Lambrecht *et al.* 1998; van Rijt *et al.* 2005) and are also important during the recall phase of asthma (van Rijt *et al.* 2005, 2011). As APCs during secondary responses to allergen, they are likely to present allergens to primed T cells and promote Th2 driven chronic eosinophilic inflammation.

1.1.5.2. T cells

T cells are lymphocytes which mature in the thymus and can be identified by their expression of TCR on the cell surface. There are multiple different subtypes of T cells including T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$). In asthma it is thought to be the $CD4^+$ T cell which is of principal importance. Antigens presented to naïve T cells via MHC II molecules on APCs are presented to $CD4^+$ T cells, while antigens presented on MHC I molecules will be presented to cytotoxic ($CD8^+$) T cells. APCs will only present the allergen to the T cell expressing the correct T cell receptor, a process known as clonal selection. During antigen presentation CD28 expressed on the T cell will interact with the APC via CD80 and CD86. This results in clonal expansion of the T cell. IL-2 release by the APC will promote proliferation of T cells, through activation of the IL-2 receptor which is only expressed on activated T cells.

1.1.5.2.1. CD4+ T cells

Different subtypes of $CD4^+$ T cells include Th1, Th2, Th17, and regulatory T cell (Tregs). Th1 cells are important in fighting invading pathogens and are involved in tissue damaging inflammatory responses; the archetypical cytokine produced by Th1 cells is IFN-γ. Th2 cells are important for promoting B cell production of immunoglobulins such as IgE and have been widely implicated for a role in allergic asthma, due to the importance of IgE in this disease. The characteristic cytokines of a Th2 response include IL-4, IL-5, IL-9 and IL-13. Th17 cells are increasingly being implicated in asthma and are characterised by interleukin 17 (IL-17) release. In contrast, Tregs play a role in suppression of immune responses. Which subtype the $CD4^+$ T cell differentiates into is controlled by the cytokine milieu present at the time of antigen presentation: IFN-γ and IL-12 drive Th1 differentiation while IL-4 drives differentiation into the Th2 subtype. The process of Th1 or Th2 cell differentiation following activation by DCs, and the resultant immune responses are outlined in more detail in Figure 1.3.

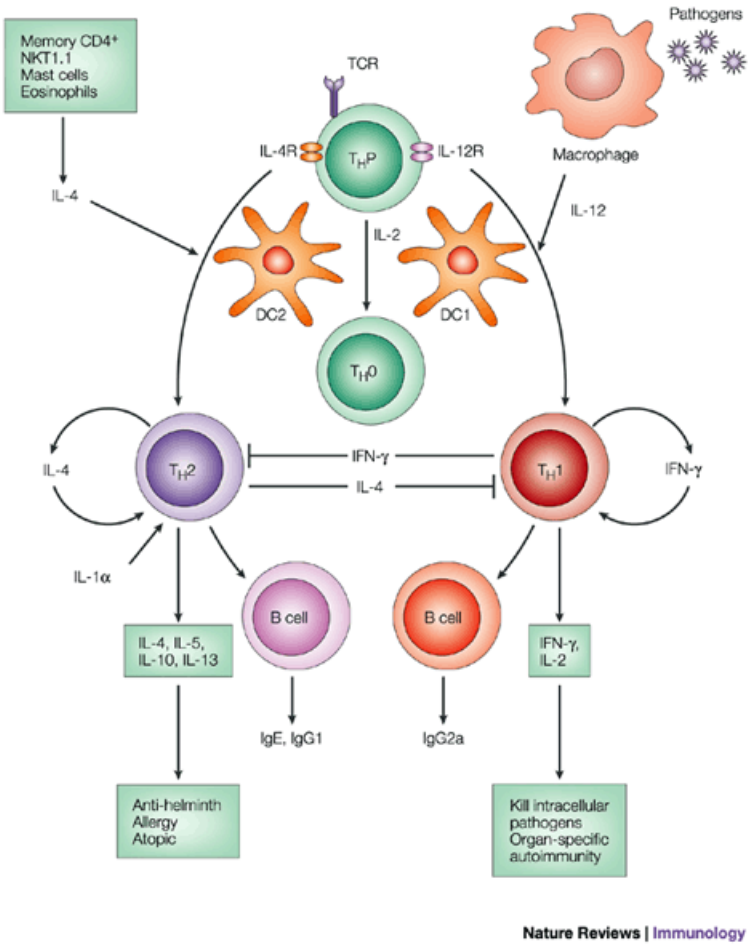


Figure 1.3: Dendritic cell mediated Th1 or Th2 proliferation and the resultant immune response (Liew 2002)

1.1.5.2.2. Th2 cells in asthma

The predominant role of T 'helper' cells is in directing and promoting the innate and adaptive immune responses. In asthma the Th2 cells are primarily differentiated into the Th2 phenotype, characterised by release of, among other mediators, IL-4, IL-5 and IL-13. This cytokine cocktail has the ability to promote generation of IgE from B cells, eosinophilic airway inflammation and AHR. There is a great deal of evidence for their involvement in asthma. BAL cells in asthmatic patients have been shown to contain a higher proportion of IL-2, IL-3, IL-4, IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) mRNA than in non-asthmatics (Robinson *et al.* 1992). The mRNA for IL4 and 5 was predominantly found in T cells, and this cytokine profile is consistent with a Th2 phenotype (Robinson *et al.* 1992). Cultured endobronchial biopsies from asthmatics express mRNA for IL-5 and IL-13, which were not found in normal tissues. In contrast cultured biopsies from normal individuals were found to express IFN-γ (Jaffar *et al.* 1999). Key Th2 cytokines IL-

4, IL-5 and IL-13 have widely established roles in allergic asthma. Il-5 is a selective eosinophil chemoattractant and activator and survival factor (Lopez *et al.* 1988; Yamaguchi *et al.* 1988, 1991; Wang *et al.* 1989). Thus Th2 cells and IL-5 are likely to be involved in driving eosinophilic inflammation which in turn is implicated in the AHR and airway remodelling observed in asthmatics. There is likely to be overlap between the function of IL-13 and IL-4, and cooperativity between IL-4 and IL-13 in inducing Th2 responses has been shown (McKenzie *et al.* 1999). IL-4 and IL-13 are thought to be fundamentally important for IgE production from B cells and the allergic response (Grünig *et al.* 1998; McKenzie *et al.* 1999), and in AHR in asthmatics (Walter *et al.* 2001; Brightling *et al.* 2002). Individually IL-13 has been shown to be important for AHR (Walter *et al.* 2001), inflammation, mucus hypersecretion, subepithelial fibrosis, eotaxin production and airway eosinophilia (Zhu *et al.* 1999; Mattes *et al.* 2001). Murine models have supported the role of $CD4^+$ T cells and their associated cytokines in the pathogenesis of asthma. IL-4, derived from T cells has also been shown to have a role in AHR in murine asthma models (Corry *et al.* 1996, 1998). In addition depletion of $CD4^+$ T cells type prior to allergen challenge in a murine model of airway eosinophilia resulted in loss of AHR and BAL eosinophilia (Gavett *et al.* 1994). $CD4^+$ T lymphocytes were again crucial for pulmonary eosinophil recruitment in a murine OVA model which was likely to be through production of V-CAM-1 and ICAM-1 (Gonzalo *et al.* 1996). Furthermore T cells and Th2 cytokines have also been implicated in allergic airway eosinophilia in a Brown Norway asthma model (Underwood *et al.* 2002).

Th2 cytokine-positive T cells and eosinophils are found in BAL and bronchial wash of asthmatic patients after allergen challenge and BAL/bronchial wash eosinophilia correlate with LAR, therefore Th2 cells may contribute to LAR through eosinophil recruitment (Robinson *et al.* 1993*a*).

1.1.5.2.3. Th1/Th2 paradigm in asthma

An important theory surrounding the pathogenesis of asthma is based on the Th1/Th2 paradigm. The cytokines important for differentiation of Th1 and Th2 cells – IFN-γ and IL-12, and IL-4 respectively – inhibit the differentiation of the other subtype (see Figure 1.1). For example, production of Th2 cytokines from T cells has been shown to be inhibited by IL-12 and IFN-γ (Varga *et al.* 2000). Thus Th1 and Th2 responses are mutually inhibitory. This idea makes up the basis of the hygiene hypothesis which I described earlier.

Th1 cell development is controlled by the transcription factor T-bet, which regulates production of IFN-γ (Szabo *et al.* 2000). In contrast Th2 cell development is controlled by the transcription factor GATA3 (Zheng & Flavell 1997). There is a hypothesis that naïve T cells generally tend towards the Th2 phenotype due to GATA3. This negatively regulates

Th1 differentiation via suppression of STAT4 – another transcription factor involved in Th1 responses – (Usui *et al.* 2006). T bet expression is induced early on in Th1 cell differentiation and can inhibit Th2 responses by inhibiting GATA3, and preventing its binding to DNA through direct protein-protein interaction, promoting Th1 responses (Hwang *et al.* 2005; Usui *et al.* 2006). Interestingly, T bet expression in T cells from asthmatics is reduced compared to non-asthmatics (Finotto *et al.* 2002) and deficiency of T-bet in mice resulted in spontaneous generation of asthma-like pathologies such as AHR to MCh, pulmonary eosinophilic inflammation and airway remodelling (Finotto *et al.* 2002). A lack of early life Th1 promoting infection or an inherent lack of Th1 promoting signals in asthmatics may therefore allow this original Th2 preference to predominate.

1.1.5.2.4. Epithelial-derived Th2 skewing innate cytokines

Although not an immune cell as such, airway epithelial cells are likely to be important in modulating airway immune responses by release of a plethora of cytokines and chemokines. Through activation by PAMPs and danger signals they may also play a role in promoting airway dendritic cell activity and ultimately in allergic sensitisation. Several novel epithelium-derived innate cytokines have been identified which may be generated in response to airway allergen exposure, or danger signals, and play a role in promoting DC function in the airways and in promoting Th2 type adaptive immune responses to allergen. These include thymic stromal lymphopoiettin (TSLP), interleukin 33 (IL-33) and interleukin 25 (IL-25).

IL-25 belongs to the IL-17 family and is produced by Th2 polarized T cells (Fort *et al.* 2001). *In vivo* it is capable of causing airway eosinophilia and other pathological changes in the lung through production of Th2 cytokines IL-4, IL-5 and IL-13 from a non T or B cell origin (Fort *et al.* 2001). IL-25 production occurs after in vivo allergen challenge, and its receptor is expressed on Th2 cells. IL-25 is likely to promote Th2 differentiation through production of IL-4 and increased GATA3 expression (Angkasekwinai *et al.* 2007). To support a role for IL-25 in Th2 differentiation and cytokines production, IL-25 knockout mice have delayed Th2 cell cytokine production (Fallon *et al.* 2006).

Murine models have highlighted a role for IL-25 in allergic asthma. Airway instillation of mice lungs with IL-25 induced features of asthma including AHR, eosinophilia and production of Th2 cytokines and mucus (Sharkhuu *et al.* 2006). IL-25 expression is detected in epithelial cells following allergen challenge, and IL-25 overexpression results in mucus production and airway eosinophilia. In contrast IL-25 blockade results in a reduction of airway inflammation and Th2 cytokine production

(Angkasekwinai *et al.* 2007). IL-25 blockade during allergen challenge in a mouse model resulted in loss of AHR, whereas blockade during sensitisation resulted in reduced IL-5, IL-13 production, eosinophilia, goblet cell hyperplasia, AHR and IgE production (Ballantyne *et al.* 2007).This implicates the cytokine in driving allergic sensitisation but also the response to allergen challenge. IL-25 has been implicated in driving TSLP and IL-33 production in the lung and may drive remodelling and inflammation in a house dust mite model; IL-25 blockade reduced influx of eosinophils to the lung; production of IL-5 and IL-13; collagen deposition and ASM hyperplasia: and AHR (Gregory *et al.* 2013).

The IL-25 receptor (IL-17RB) is also expressed by naïve invariant natural killer T cells (iNK) which can produce Th2 cytokines in response to IL-25 stimulation (Stock *et al.* 2009). IL-25-induced AHR was found to be dependent on these IL-17R^{+} iNKT cells (Stock *et al.* 2009).

IL-33 is a member of the interleukin 1 (IL-1) signalling family which acts through the IL-1 receptor ST2. The T1/ST2 receptor has previously been implicated in Th2 inflammatory responses, eosinophilia, IgE and IL-5 production (Coyle *et al.* 1999). Increased levels of IL-33 expression are found in airway smooth muscle, airway epithelial cells and BAL fluid from asthmatics, and levels were particularly enhanced in severe asthmatics (Préfontaine *et al.* 2009, 2010). IL-33 causes release of Th2 cytokines IL-5 and IL-13 from Th2 cells, and induces expression of IL-4, IL-5 and IL-13 and increased IgE production *in vivo*. Pulmonary pathology such as eosinophilia and mucus production were also observed in response to IL-33 (Schmitz *et al.* 2005). IL-33 treatment stimulates mast cells or basophils to produce mediators such as IL-4, IL-13, GM-CSF, and regulated upon activation normal T cell expressed and secreted (RANTES) and IL-33 instillation into mice induces goblet cell hyperplasia and AHR through production of IL-4, IL-5 and IL-13, and this occurs in the absence of adaptive immune responses from B and T cells (Kondo *et al.* 2008). IL-33 has been shown to be able to polarise naïve CD4^{+} T cells to produce IL-5 but not IL-4 (Kurowska-Stolarska *et al.* 2008). IL-33 administration *in vivo* results in increased levels of these IL-5 producing T cells and exacerbates ovalbumin induced allergic airway inflammation; thus IL-33 may promote Th2 responses in the absence of IL-4 (Kurowska-Stolarska *et al.* 2008). IL-33, together with TSLP may induce mast cell maturation and production of Th2 cytokines and chemokines, implying a mechanism for mast cell activation and promotion of Th2 responses independently of IgE stimulation of FcεR1 (Allakhverdi *et al.* 2007*b*) (Allakhverdi *et al.* 2007*a*; Iikura *et al.* 2007; Ho *et al.* 2007).

In allergen-driven models, IL-33 was important for allergen induced airway inflammation *in vivo* in mice (Oboki *et al.* 2010). IL-33 was also shown to promote the

differentiation of eosinophils and as such to enhance eosinophil-mediated pathologies (Stolarski *et al.* 2010). In addition deficiency of IL-33 or blockade of the receptor for IL-33 has been shown to inhibit allergic airway inflammation and IL-5 production (Kurowska-Stolarska *et al.* 2008; Stolarski *et al.* 2010). IL-33 can be released from epithelial cells into the airway lumen in response to allergen exposure which is followed by the release of Th2 cytokines IL-5 and IL-13. The IL-33 release may be due to an initial release of the danger signal adenosipe triphosphate (ATP) and activation of P2Y2 purinergic receptor (Kouzaki *et al.* 2011). This ATP-driven release of IL-33 may be one way in which airborne allergens promote Th2 responses (Kouzaki *et al.* 2011).

Thymic stromal lymphopoietin (**TLSP**) is another cytokine produced by epithelial cells which may promote Th2 immunity and activate antigen presenting cells. Human mast cells, epithelial cells, ASM cells and skin keratinocytes have all been shown to produce TSLP (Soumelis *et al.* 2002). TSLP released from epithelial cells and mast cells in asthmatics (Ying *et al.* 2005) may promote DC maturation and cause them to recruit Th2 cells through release of TARC and MDC. These are Th2 chemoattractants and act on CCR4 expressed on Th2 cells; TLSP treated DCs promote proliferation of $CD4^+$ T cells and T cell production of IL-13, IL-5 and IL-4 but not IFN-γ (Soumelis *et al.* 2002; Barnes 2008). OX40 (or CD134, a member of the TNF receptor family) expression on $CD4^+$ memory cells and OX40-OX40L interactions are important for eosinophilia, AHR, and production of Th2 cytokines and mucus in response to allergen challenge (Salek-Ardakani *et al.* 2003). TSLP can induce the expression of OX40L on DCs and this process is important for the activation of naive $CD4^+$ T cells to produce Th2 cytokines (IL-4, 5 and 13) (Soumelis *et al.* 2002; Ito *et al.* 2005).

Several human studies and murine studies have implicated TSLP in asthma. Elevated levels of TLSP have been found in the BAL fluid (Nguyen *et al.* 2010) and in the airway epithelium (Shikotra *et al.* 2012) of asthmatic patients. Increased expression is particularly notable in severe asthmatics and TSLP levels correlated with airflow limitation (Shikotra *et al.* 2012). Increased levels of TSLP in asthmatic airways also correlates with production of Th2 cytokines and with disease severity (Ying *et al.* 2005). To further support this, SNPs in TSLP promoter regions which increase TSLP production via increasing binding of AP-1 are associated with increased asthma susceptibility (Harada *et al.* 2011). TSLP was found to inhibit IL-10 production from Tregs and thus reduce their suppressive abilities (Nguyen *et al.* 2010). Indeed the increased level of TSLP in asthmatics was associated with a reduced pulmonary Treg suppressive ability and production of IL-10 (an

inhibitory cytokine). From this data it is thought increased levels of TLSP may be responsible for dampened tolerance mechanisms in asthmatics (Nguyen *et al.* 2010).

In animal models overexpression of TSLP results in allergic airway inflammation; AHR, goblet cell hyperplasia and subepithelial fibrosis; and increased production of Th2 cytokines and IgE (Zhou *et al.* 2005*a*). Therefore TSLP is sufficient to induce allergic inflammation and asthma-like pathologies. Increased expression of TSLP is found in allergen-challenged mice (Zhou *et al.* 2005*a*; Shi *et al.* 2008), and allergen-induced disease was inhibited in mice deficient in the TSLP receptor or using a TSLP-R antibody (Zhou *et al.* 2005*a*; Shi *et al.* 2008). TSLP activated DCs to produce Th2 cell chemokine TARC (Zhou *et al.* 2005*a*). TSLP is important for driving early Th2 responses to allergen in mice (Jang *et al.* 2013) and TSLP-conditioned DCs caused production of IL-4 from T cells with a reduction in T cell production of IFN-γ (Shi *et al.* 2008). Levels of TSLP in the airways following allergen challenge correlated with airway eosinophilia and production of IL-5 (Shi *et al.* 2008). The role of TSLP in these models was in driving DC maturation, migration and promotion of OVA-specific T cells proliferation. Further roles of TSLP in allergic disease involve activation of mast cells to release Th2 cytokines such as IL-13 and subsequent promotion of allergic mast cell-driven responses in the lungs (Allakhverdi *et al.* 2007*a*).

In a chronic HDM-driven asthma model, inhibiting Th2 responses using an anti-TSLP mAB resulted in reduced allergic inflammation, AHR, remodelling effects and levels of TGF-β. Thus TSLP may also drive chronic remodelling in asthma (Chen *et al.* 2013).

1.1.5.2.5. Novel innate immune cells

It used to be assumed that the Th2 disease phenotype was predominantly mediated by the Th2 cell, however other cells and mediators are now thought to contribute to promoting the Th2 phenotype. Recently, 3 new innate lymphoid cell types have been described which may be involved in linking innate immune cytokines released from epithelial cells with Th2 immune responses (Kaiko & Foster 2011).

First of all (Moro *et al.* 2010) described innate natural helper cells, which were found to proliferate in response to IL-2 and to produce Th2 type cytokines such as IL-5 and IL-13 (Moro *et al.* 2010). This cell type is found widely dispersed in murine tissues and produces IL-13 in response to IL-33 and IL-25 (Price *et al.* 2010). Subsequently Bartemes et al also described IL-33 responsive lymphoid cells in the lungs of IL-33 challenged mice which were responsible for increased levels of IL-5, IL-13 and airway eosinophilia in the absence of an adaptive immune response from B or T cells (Bartemes *et al.* 2012). Allergen-induced production of IL-33 from epithelial cells, acting on ST2 and resulting in recruitment

of innate lymphoid cells which release IL-5 and IL-13 may be involved in allergen-induced asthma (Bartemes *et al.* 2012).

(Neill *et al.* 2010) identified another innate type 2 immune effector cell which they named nuocytes. These cells proliferate in response to known Th2 inducing mediators IL-25 and IL-33, and also produce IL-13 in response to helminth infection (Neill *et al.* 2010). Pulmonary IL-13^{+} nuocytes were observed in a murine OVA-driven asthma model, and were found in the BAL fluid of mice following IL-25 or IL-33 treatment. In addition transfer of nuocytes restored IL-25 induced AHR and BAL eosinophilia in IL-13-deficient mice which are normally resistant to these endpoints (Barlow *et al.* 2012). Thus IL-33 and IL-25 induced nuocytes in the lung may be important in allergic asthma (Barlow *et al.* 2012). Nuocytes and natural helper cells have similar phenotypes and may therefore actually be the same cell type (Kaiko & Foster 2011) .

A population of IL-25 dependent non-T/non-B cells have also been identified which produce IL-4, IL-5 and IL-13 and may play a role in clearance of helminth infection (Fallon *et al.* 2006). This cell type was later named multipotent progenitor cells (MPP) (Saenz *et al.* 2010). MPPs can develop into monocytic cell types or granulocytes and also act as APCs; in response to IL-25 these cells accumulate in lymphoid tissues and can promote generation of Th2 cytokine responses (Fallon *et al.* 2006; Saenz *et al.* 2010); they may therefore play a role in allergic asthma..

1.1.5.2.6. Th17 cells

As mentioned, the T cell traditionally implicated in asthma was the Th2 cell, however more recently Th17 cells and their mediators IL-17 and IL-22 are also being implicated in the both asthma and severe asthma (Lindén 2001; Hellings *et al.* 2003; Prause *et al.* 2004; Zhou *et al.* 2005*b*; Weaver *et al.* 2007; Ouyang *et al.* 2008; McKinley *et al.* 2008; Wakashin *et al.* 2008; Wilson *et al.* 2009; Kawaguchi *et al.* 2009; Wang *et al.* 2010; Souwer *et al.* 2010; Bajoriūnienė *et al.* 2012; Kudo *et al.* 2012). Mediators such as IL-1β, IL-6 and IL-23 are implicated in promotion of Th17 proliferation (Acosta-Rodriguez *et al.* 2007; Wilson *et al.* 2007). Th17 cells express the transcription factor RORγ and multiple mediators including IL-17A, IL-17F, IL-22, IL-26 and IFN-γ. Increased levels of IL-17 have been detected in sputum from asthmatics and this may play a role in neutrophil recruitment via inducing production of IL-8, a neutrophil chemoattractant (Bullens *et al.* 2006). IL-17 has also been shown to be important in T cell activation in antigen-specific immune responses, and for antigen dependent neutrophilia (Nakae *et al.* 2002, 2007). IL-17 positive cells are increased in the BAL and sputum of asthmatics, but interestingly both T cells and eosinophils were found to express IL-17 (Molet *et al.* 2001). Th17 cells have also been

implicated in steroid insensitive asthma (McKinley *et al.* 2008) which I will discuss in more detail below.

1.1.5.2.7. Regulatory T cells

Regulatory T cells are important in the suppression of immune responses and in self-tolerance which is likely to be through production of TGF-β and IL-10. These cells are under the control of the transcription factor forkhead box P3 (FOXP3) (Hori *et al.* 2003). Tregs have been shown to inhibit $CD4^+$ and $CD8^+$ T cell proliferation (Itoh *et al.* 1999), inhibit AHR, pulmonary recruitment of eosinophils, Th2 cell proliferation and expression of Th2 cytokines in a murine OVA model; this effect was dependent on IL-10 – a cytokine which is known to have immunosuppressive properties (Cottrez *et al.* 2000; Kearley *et al.* 2005). In support of this, characteristic features of allergic asthma such as airway inflammation, increased production of IgE, goblet cell metaplasia and mucus impaction have been observed in FOXP3 mutant mice (Lin *et al.* 2005*b*). Increased production of cytokines of both the Th1 and Th2 subtype was observed indicative of dysregulated cytokine production rather than Th2 skewing (Lin *et al.* 2005*b*), therefore Tregs may normally inhibit both Th1 and Th2 responses, not just the Th2 responses described in this chapter (Bellinghausen *et al.* 2003). Tregs can also inhibit established asthmatic airway disease and inhibit the generation of remodelling effects such as mucus hypersecretion and peribronchial collagen deposition (Kearley *et al.* 2008).

The above data may suggest that the immunomodulatory role of Tregs is missing in asthmatics. One study showed diminished capacity to inhibit T cell proliferation in Tregs from atopic individuals (Ling *et al.* 2004) but other studies have not shown defective Treg function in atopic individuals (Bellinghausen *et al.* 2003). Others have found reduced levels of Tregs in mild asthmatics; however levels were higher in severe asthmatics. In addition expression of FOXP3 levels were increased in severe asthmatics compared to mild asthmatics (Lee *et al.* 2007). There may therefore be a paradigm where asthma may represent an initial Treg defect but that FOXP3 and IL-10 are induced in more severe disease (Lee *et al.* 2007).

1.1.5.3. B cells

B cells are another type of lymphocyte which develop in the bone marrow, and then migrate to lymphoid tissue such as the lymph nodes. Their primary role is to produce antibodies to help fight against invading pathogens. However aberrant antibody production is one of the key pathogenic processes in allergic asthma. Similar to T cells, B cells express

a B cell receptor which is specific for a given antigen. Presentation of an antigen to B cells will result in B cell clonal selection and expansion which results in the generation of plasma cells and memory cells. B cells are also APCs; they are capable of internalising allergens and presenting them on their cell surface. When a B cell encounters the T cell expressing the correct TCR for the allergen, a CD40 ligand (CD40L) expressed on the surface of the T cell will bind to the CD40 receptor (CD40R) on the B cell. Direct interation of the B and T cells results in the formation of an immunological synapse. This interaction will stimulate the B cell to produce antigen-specific immunoglobulins. As described in the previous sections, Th2 cells in asthma are differentiated into the Th2 phenotype, which produce cytokines such as IL-4 and IL-13. These cytokines promote class switching of immunoglobulin production in B cells to production of IgE. IgE is an important mediator in allergic asthma, IgE molecules sensitise the individual to a subsequent allergen exposure by binding to high affinity Fcε receptors (FcεR1) expressed on mast cells and basophils, priming these cells to respond to a subsequent allergen exposure. Its role will be discussed in more detail below. There is also some evidence that B cell class switching and production of IgE in asthma not just occur in lymphoid tissue, but may also occur in the lung (Takhar *et al.* 2007).

1.1.5.4. Mast cells

Mast cells are granulocytes which develop in the bone marrow and migrate to tissues in an immature form, prior to maturation in situ. Cross linking of IgE on mast cell FcεR1 following allergen exposure results in release of preformed granule mediators such as histamine and 5-HT and newly formed lipid mediators such as prostanoids and leukotrienes. As I described above, several of these mediators are known to cause bronchoconstriction and oedema and thus the mast cell is important in mediating the immediate response to allergen exposure in asthma. IgE dependent mast cell activation (via FcεR1) has been shown to be important for AHR and allergic airway inflammation in murine asthma models (Mayr *et al.* 2002). Mast cell degranulation was observed within an hour of allergen challenge in a murine asthma model, and AHR occurred in the absence of lung or BALF inflammation, implicating the mast cell mediators in AHR (Hessel *et al.* 1995). In addition to early mast cell mediator effects such as the EAR, IgE stimulation of FcεR1 on mast cells has been shown to induce release of preformed and newly synthesised Th2 type mediators; IL-4 is found preformed in human mast cells, but IgE acting on FcεR1 can also induce synthesis of IL-4, IL-5 and TNFα. Mast cells have also been shown to release IL-3, and GM-CSF (Okayama et al. 1995, 2003; Wilson et al. 2000; Bradding et al. 2006; Chung et al. 1986; Plaut et al. 1989; Wodnar-Filipowicz et al. 1989; Gordon & Galli 1991; Bradding et al. 1992, 1994). The ability of mast cells to release these mediators implicates them in

promoting chronic inflammation and Th2 responses in asthma. Under IL-4 stimulation mast cells are able to express CD40L (for cell-cell contact) and may promote IgE production independently of T cells (Gauchat *et al.* 1993). However others have refuted this (Yanagihara *et al.* 1998).

In addition to inducing allergen driven bronchospasm, IgE mediated mast cell activation and degranulation in mice has been shown to induce AHR (Martin *et al.* 1993). The key role of mast cells in immediate bronchospasm and in AHR may be in part down to their localization alongside, and interaction with, airway smooth muscle and airway neurons. The process of mast cell maturation, differentiation and proliferation is under the control of the mast cell chemotactic factor SCF acting on c-kit receptors and SCF can be released by ASM cells (Kassel *et al.* 1999). Therefore ASM has the ability to induce migration of mast cells. Th2 cytokine stimulated ASM from asthmatics has been shown to induce mast cell migration through activation of the chemokine receptors CCR3 and CXCR1 (Sutcliffe *et al.* 2006). Alternatively, others have shown smooth muscle-induced mast cell migration to be induced by CXCL10 (IP-10) release and activation of CXCR3, the most abundantly expressed chemokine receptor on mast cells (Brightling *et al.* 2005). Activated mast cells are found in bronchial biopsies of asthmatic patients and express of IL-4 and IL-13 (Brightling *et al.* 2003; Shahana *et al.* 2005). Mast cells also associate with ASM from asthmatic patients and numbers of mast cells in the ASM correlates with AHR (Brightling *et al.* 2002). Mast cell localisation in the ASM bundles can promote differentiation of smooth muscle into a more contractile phenotype through release of β-tryptase which enhances TGF-β1 release from ASM, and by increasing α-SMA (smooth muscle actin) expression (Woodman *et al.* 2008). Mast cells can also stimulate smooth muscle migration and repair processes through release of CCL19 acting on CCR7 on ASM (Kaur *et al.* 2006). Direct cell-cell contact of human mast cells with ASM resulted in eotaxin production from hASM (Liu *et al.* 2006). Therefore smooth muscle in asthmatics is implicated in recruiting mast cells to the airways and together they may promote eosinophil migration, in a process which propagates inflammation and disease pathogenesis in asthmatic lungs. Eosinophils in the sputum and mast cells in the epithelium in asthmatics correlated with AHR and disease severity (Gibson *et al.* 2000)

Mast cells are also likely to interact with sensory and parasympathetic neurons and so in addition to release of bronchoactive mediators, and modulation of smooth muscle contractility, mast cells may also induce bronchoconstriction via activation or modulation of the function of neuronal pathways (Myers *et al.* 1991). Mast cells can be found in close association with parasympathetic ganglia, and these can be activated by mast cell-derived mediators such as histamine (Myers *et al.* 1991; Myers & Undem 1995). Mast cells were

localised in tracheal segments from OVA sensitised mice, and depolarization of parasympathetic neurons in mouse tracheal ganglia was observed in response to 5-HT and OVA (Weigand *et al.* 2009). Antigen induced bronchoconstriction in mice appeared to be dependent on serotonin release from mast cells and required intact cholinergic neuronal signalling (Cyphert *et al.* 2009). This implies that mast cell derived spasmogens contributes to antigen-induced tracheal contraction via activation of parasympathetic cholinergic neurons (Weigand *et al.* 2009).

1.1.5.5. Basophils

Basophils are bi-lobed granulocytes which mature in the bone marrow, and circulate in the peripheral blood. This is in contrast to mast cells which infiltrate peripheral tissues under normal conditions. Basophils release histamine in response to IgE stimulation of FcεR1 receptors (Seder *et al.* 1991; Schroeder *et al.* 1994), therefore they play a similar role to mast cells in allergic diseases, however the role of basophils in asthma is less well understood than that of mast cells. Basophils are found in the sputum of asthmatics and their presence correlates with disease severity (Kimura *et al.* 1975). Their function is regulated by IL-3 which acts as a basophil growth factor and in addition to histamine basophils release multiple cytokines including Th2 cytokines IL-4, IL-5 and IL-13; GM-CSF, RANTES, MCP-1 and lipid mediators (Kondo *et al.* 2008) and as such may be involved in allergic diseases (Voehringer *et al.* 2004; Min *et al.* 2004). They have been identified as important initiators of IgE mediated chronic inflammation (Obata *et al.* 2007).

High affinity FcεR1 is also expressed on the surface of basophils (Thompson *et al.* 1990) giving basophils the ability to respond to IgE. Under stimulation with IgE acting on FcεR1 receptors, or with IL-4, basophils have been shown to release Th2 mediators, IL-4 and IL-13, and also express CD40L, which in turn enables them to stimulate B cell proliferation and production of IgE (Seder *et al.* 1991; Brunner *et al.* 1993; Gauchat *et al.* 1993; Schroeder *et al.* 1994; Yanagihara *et al.* 1998; Denzel *et al.* 2008). There is also some evidence that basophils function as APCs: basophils rather than DCs have been shown to be the important APCs in differentiation of T cells to Th2, and they do this through expression of MHC-II (Perrigoue *et al.* 2009; Sokol *et al.* 2009). In response to allergen challenge basophils produce IL-4 and TSLP and migrate to draining lymph nodes. There they may regulate naïve $CD4^+$ T cell differentiation into Th2 cells via release of IL-4 and direct cell-cell contact, and may therefore be necessary for induction of allergen-induced Th2 response (Hida *et al.* 2005; Oh *et al.* 2007; Sokol *et al.* 2008).

Basophils have also been implicated in IgE mediated responses in the absence of mast cells and T cells (Mukai *et al.* 2005; Obata *et al.* 2007) and have been implicated in the

EAR and the LAR (Naclerio *et al.* 1985; Bascom *et al.* 1988; Charlesworth *et al.* 1989; Iliopoulos *et al.* 1992; Guo *et al.* 1994; Nabe *et al.* 2013). They may also be involved in IgG mediated allergic responses (Crosby *et al.* 2002; Tsujimura *et al.* 2008; Ishikawa *et al.* 2010). Finally, basophils have been implicated in IL-33-induced AHR and goblet cell hyperplasia in the absence of T or B cells (Kondo *et al.* 2008).

1.1.5.6. Eosinophils

For decades the eosinophil has been a prominant cell in the field of asthma; its presence in the lung is used routinely as a marker of allergic asthma, differentiating the disease from COPD, and levels are also used to evaluate the efficacy of therapeutic interventions in clinical studies. Eosinophils evolved as part of the innate immune system with the function of destroying invading helminth and parasitic infection. They develop in the bone marrow from common myeloid progenitor cells, a process which is regulated by IL-3, IL-5 and GM-CSF. These factors along with eotaxin and IL-13 are involved in eosinophil survival, recruitment to the lungs and release of eosinophil mediators (Lopez *et al.* 1988; Wang *et al.* 1989; Sher *et al.* 1990; Carlson *et al.* 1993; Takamoto & Sugane 1995; Rothenberg *et al.* 1997; Horie *et al.* 1997). IL-1β and TNF-α released after allergen challenge also be involved in eosinophil recruitment to the lungs by inducing the expression of endothelial adhesion molecules and stimulating eosinophil adhesion, rolling and transmigration (Broide *et al.* 2000, 2001).

Eosinophils are found in the induced sputum and bronchial biopsies of allergic asthmatics (Vieira & Prolla 1979; Gibson *et al.* 2000; Shahana *et al.* 2005) and are recruited to the lung following allergen challenge in both asthmatics, and in animal models of asthma (De Monchy *et al.* 1985; Sehmi *et al.* 1997; Zeibecoglou *et al.* 1999; Birrell *et al.* 2003; Swedin *et al.* 2010*a*). Sputum, airway and epithelial eosinophils correlate with sites of epithelial damage, and severity of asthma, the allergen-induced LAR, and AHR (Zeibecoglou *et al.* 1999; Gibson *et al.* 2000; Miranda *et al.* 2004; Shahana *et al.* 2005). Eosinophils are polymorphonuclear granulocytes which can release numerous toxic proteins: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO), and these eosinophil-derived mediators have been implicated in the pathogenesis of asthma. Increased levels of eosinophils and MBP were found in the BALF of symptomatic asthmatics and further increases were found in those which showed signs of AHR (Wardlaw *et al.* 1988). MBP has been shown to damage bronchial epithelium in vitro (Frigas *et al.* 1981), and major basic protein has been detected in the lungs of asthmatic patients at sites of epithelium damage (Filley *et al.* 1982). MBP,

ECP and EPO have also been shown to damage the epithelium of guinea pig trachea (Motojima *et al.* 1989). Eosinophils and ECP were found in the BAL fluid of asthmatic patients which exhibited an LAR after allergen challenge (De Monchy *et al.* 1985) and MBP released from eosinophils has been shown to increase airway reactivity in rats (Coyle *et al.* 1995). As I will describe in more detail below, epithelial damage may cause increased allergen responses due to increased passage of allergens across the epithelium.

In addition to the toxic granule proteins described above, eosinophils are capable of releasing numerous other mediators such as cysteinyl leukotrienes (LTC_4, D_4 and E_4), prostanoids such as PGE_2 and PGD_2, multiple cytokines including GM-CSF, IFN-γ, TNF-α, RANTES, Th2 cytokines IL-4, IL-5 and IL-13, and fibrotic mediators such as TGF-β, and matrix metalloproteinases. These mediators are likely to contribute to AHR, remodelling and promoting the chronic inflammatory state in the asthmatic lung. For example eosinophils release leukotriene C_4 (LTC_4) which is a potent bronchoconstrictor, thus eosinophils are able to induce direct effects on ASM effects and contribute to AHR (Weller *et al.* 1983). In addition eosinophils are likely to promote and maintain the Th2-type environment which is characteristic of allergic asthma due to their ability to release IL-4 (Nonaka *et al.* 1995) and IL-13 (Woerly *et al.* 2002).

Much of the evidence for a role of eosinophils in the pathogenesis of asthma has come from studies manipulating two of the main mediators which control the recruitment of eosinophils: eotaxin and IL-5. **Eotaxin** represents a family of three chemokines (1, 2, 3) which are produced by epithelial cells and other cell types including macrophages, mast cells, airway smooth muscle cells (Liu *et al.* 2006) and eosinophils (Zeibecoglou *et al.* 1999). Eotaxin expression and number of eotaxin positive cells were increased in induced sputum of asthmatic patients after allergen challenge (Zeibecoglou *et al.* 1999). Eotaxin signals through CCR3 receptor expressed on eosinophils (Sehmi *et al.* 1997) and is important for eosinophil recruitment into the lungs (Griffithsjohnson *et al.* 1993). The absence of eotaxin and subsequent reduction in eosinophils is associated with a lack of BHR in animal models of asthma (Gonzalo *et al.* 1996; Rothenberg *et al.* 1997; Humbles *et al.* 1997). **IL-5** is a Th2 cytokine which is important in eosinophil differentiation (Yamaguchi *et al.* 1988) and survival of mature eosinophils (Yamaguchi *et al.* 1991) and has been shown to be important in eosinophil mobilisation from bone marrow (Palframan *et al.* 1998). IL-5 inhalation in asthmatic patients resulted in eosinophilia and ECP in induced sputum, and AHR (Shi *et al.* 1998). In IL-5 deficient mice, a lack of pulmonary eosinophilia, AHR to MCh and lung damage are observed in OVA sensitised and challenged mice (Foster *et al.* 1996; Shen *et al.* 2003). Eosinophil transfer to IL-5 deficient, OVA sensitised and challenged mice resulted in restoration of increased levels of BAL Th2 cytokines and AHR

(Shen *et al.* 2003). Further supporting this, ablation of pulmonary eosinophils using a CCR3 monoclonal antibody resulted in the loss of allergen-induced AHR, (Justice *et al.* 2003), and in congenital eosinophil deficient mice, allergen challenge failed to induce AHR or mucus accumulation (Lee *et al.* 2004*a*). Double knockout of IL-5 and eotaxin resulted in loss of both tissue eosinophilia and AHR, associated with impaired IL-13 production from Th2 cells. Eosinophils may therefore be involved in regulation of Th2 cytokine production from Th2 cells, and this may be a mechanism for their role in AHR (Mattes *et al.* 2002).

The hypothesis that pulmonary inflammation, particularly eosinophilia, plays a key mechanistic role in allergic asthma is also supported by the fact that in most allergic asthmatic patients anti-inflammatory treatment with inhaled or oral steroids results in a reduction of asthma symptoms and this reduction is associated with a reduction of pulmonary inflammation (Djukanović *et al.* 1992, 1997). Thus it is highly plausible that the inflammation in asthmatics may be driving the disease pathogenesis.

Despite this, others have suggested that eosinophils may not be as important in asthma as once thought. Firstly some patients with asthma have little evidence of pulmonary eosinophil infiltration or airway inflammation (Wenzel *et al.* 1999; Wenzel 2005). Others have suggested that AHR does not correlate with levels of inflammation including BAL, sputum or bronchial biopsy eosinophils, or total and allergen-specific IgE (Crimi *et al.* 1998; Wilder *et al.* 1999). In studies AHR was not modulated by reduction of eosinophil levels (Hessel *et al.* 1997) or in eosinophil deficient mice (Humbles *et al.* 2004). However eosinophils were implicated in airway remodelling such as allergen-induced subepithelial collagen deposition in a chronic OVA model (Humbles *et al.* 2004). In the clinic a humanised monoclonal antibody against IL-5 (mepolizumab) effectively ablated both blood eosinophils and allergen-induced sputum eosinophilia in asthmatics, yet failed to affect either allergen-induced AHR or LAR (Leckie *et al.* 2000). Further to this mepolizumab significantly reduced blood and sputum eosinophils, but failed to impact on clinical outcomes (Flood-Page *et al.* 2007). A recent meta-analysis of multiple placebo-controlled mepolizumab trials concluded that while the treatment reduced blood and sputum eosinophilia, and exacerbation rate, and improved quality of life scores in eosinophilic asthmatics, the treatment failed to impact upon lung function based endpoints (Liu *et al.* 2013).

While eosinophils are clearly a feature of animal models of asthma and are present in the lungs of asthmatic patients (where features such as remodelling, and AHR are also observed) their specific role is still controversial.

1.1.5.7. Macrophages

Macrophages develop in tissue such as the lungs from monocytes generated in the bone marrow. Their primary role is as phagocytes in the innate immune system; to engulf and digest pathogens and cell debris. Not only does this protect the body from damage but it also cleans up dead and dying cells. As inflammatory mediators, macrophages release multiple cytokines and chemokines such as IL-1β, TNF-α, IL-8, IL-6 and MIP-1α which may promote on-going inflammation in asthmatics. Along with pro-inflammatory cytokines, macrophages also contain many enzymes known as MMPs which are involved in tissue destruction and repair responses. Macrophages are one of the predominant cell types in sputum from asthmatic patients (Woodruff *et al.* 2001) and the presence of IL-1β in BALF from symptomatic asthmatics compared to stable asthmatics implicates activation of macrophages in the lungs of these patients (Broide *et al.* 1992). Activation of alveolar macrophages and subsequent release of chemoattractants such as LTB4, IL-8 and MIP-2 may then recruit neutrophils to the lungs. Macrophages can release prostaglandins, cysteinyl leukotrienes, PAF and other proinflammatory mediators; these mediators have been implicated in airway remodelling, AHR, bronchoconstriction and mucus production in asthma so macrophage activation may well contribute to these asthma features (Henderson *et al.* 1996, 2002; Holgate *et al.* 2003).

Macrophages may also be involved in the adaptive immune system. Macrophages are APCs and as such can ingest allergens and present them to T cells. Macrophages in asthmatics express the FcεRs and they may provide pro-inflammatory signals in response to allergen (Melewicz *et al.* 1982; Borish *et al.* 1991), thus contributing to the allergic responses observed in asthma. Indeed alveolar macrophages can release LTC_4 in response to IgE or IgE-allergen immune complexes (Rankin *et al.* 1982, 1984).

1.1.5.8. Neutrophils

Neutrophils are polymorphonuclear granulocytes which make up part of the innate immune system, acting as phagocytes and destroying invading pathogens. They respond rapidly as a first line defence against infections by migrating to the site of infection under the control of chemokines such as LTB_4 and IL-8. They also release phagosomes which contain high levels of reactive oxygen species which promote their ability to fight pathogens in a process known as respiratory burst. They are also capable of secreting granules containing myeloperoxidases, neutrophil elastases and collagen. They are thought to be important cell types in COPD but their role in asthma is less well characterised. They are highly abundant in the sputum of some asthmatic individuals (Woodruff *et al.* 2001) and are recruited to the airways rapidly following allergen exposure in asthmatics and in animal

models of asthma (Koh *et al.* 1993; Nocker *et al.* 1999; Tomkinson *et al.* 2001; Underwood *et al.* 2002). Disruption of the epithelial barrier induced by neutrophil elastase and other proteases (Ginzberg *et al.* 2001) may promote exposure to allergens. Release of neutrophil elastase following allergen challenge inversely correlated with patients' lung function, therefore neutrophils in the lungs and elastase release may contribute to loss of lung function in asthmatics (Monteseirín *et al.* 2003). Mediators released by neutrophils include MMPs, IL-6, IL-8, IL-1β, IL-1α and TNF-α (Tiku *et al.* 1986; Goh *et al.* 1989; Cicco *et al.* 1990; Dubravec *et al.* 1990; Strieter *et al.* 1992) which may promote on going chronic inflammation in the disease.

Neutrophils are also highly likely to be involved in exacerbations of asthma (Fahy *et al.* 1995); increased levels of neutrophils and NE are found in exacerbated patients which are commonly caused by respiratory infections. Neutrophils may also be responsible for steroid resistant inflammation in asthma exacerbations (Ito *et al.* 2008). There is substantial evidence that neutrophils may be involved in severe asthma and this is discussed in more detail below.

1.1.6. Immunoglobulins

Immunoglobulins are antibodies produced by B cells which recognise specific regions of antigens and pathogens. There are several types of immunoglobulins, including IgA, IgD, IgE, IgG and IgM, and they are all implicated in different types of immune responses; several of these can be found in high levels in the lung (Burnett 1986). IgE has low abundance in normal individuals but is important in type 1 hypersensitivity responses which cause atopic disease such as asthma, rhinitis, atopic dermatitis and allergies to food. In contrast IgG (split into subtypes 1-4) is highly abundant in the blood and its main role is in protecting the body from invading pathogens. Immature B cells express IgM while mature B cells produce IgM and IgD which are expressed on their cell surface. Activation of B cells by antigen presentation promotes secretion of immunoglobulins. Presentation of antigens to B cells in conjunction with Th2 cytokines such as IL-4 promotes B cell class switching to produce IgE (Snapper *et al.* 1988; Finkelman *et al.* 1988, 1989), and this is a key mediator of Th2 type responses. Other features of the immune system such as the presence of different APCs and supporting T cells may influence the type of antibody response which occurs in B cells (Burnett 1986). Once produced by B cells, allergen-specific IgE binds to high affinity FcεR1 receptors on immune cells such as mast cells and basophils, priming them to respond to allergen exposure. Allergen binding onto mast cell or basophil-bound IgE results in IgE cross-linking, activation of these cells, and the mast cell mediated responses described above. Aside from the well documented expression of high affinity IgE receptors on mast cells and basophils, the FcεR1 receptor is also found on basophils, mast cells, pDCs, mDCs

and eosinophils, (Maurer *et al.* 1996; Novak *et al.* 2004; Rosenwasser 2011; Dullaers *et al.* 2012) giving IgE the ability to activate these other key asthmatic cellular mediators. In addition the low affinity IgE receptor (FcεR11 or CD23) is also expressed on multiple asthma relevant cell types such as B cells, T cells and NK cells; macrophages and eosinophils; and structural cells such as ASM cells and epithelial cells (Dullaers *et al.* 2012). Both membrane-bound and soluble CD23 is implicated in regulation of IgE synthesis. Soluble CD23 can bind to complement mediators such as CD21, resulting in increased IL-4-mediated production of IgE (Henchoz *et al.* 1994). In contrast, IgE binding to membrane bound CD23 on B cells results in negative feedback to inhibit IgE production (Sherr *et al.* 1989; Flores-Romo *et al.* 1993; Yu *et al.* 1994). In turn soluble CD23 can bind to IgE, competing for IgE binding on membrane bound CD23 which causes a lack of this IgE mediated inhibitor feedback (Rosenwasser 2011). This is of particular relevance since HDM proteases may cleave CD23 from cell membranes and therefore may inhibit the CD23-mediated negative feedback that dampens IgE production (Schulz *et al.* 1995, 1997; Hewitt *et al.* 1995).

Decades of research have implicated IgE-mediated responses in asthma so IgE is now a key target for asthma therapy. Although much is known of the role of IgE in immediate allergic responses to allergen in asthma its involvement in chronic inflammation or AHR is less well understood. Blood IgE levels are associated with asthma (Burrows *et al.* 1989) and production of antigen-specific IgE is observed after sensitisation in murine asthma models (Hessel *et al.* 1995). However studies utilising IgE directed therapy such as the monoclonal anti-IgE antibody omalizumab (rhuMab-E25) have shown mixed results. Anti IgE therapy such as omalizumab which inhibits binding of IgE to mast cells via the FCεR1 receptor inhibited the allergen-induced early and late asthmatic responses (Boulet *et al.* 1997*a*; Fahy *et al.* 1997), however its efficacy in chronic features of asthma is less clear. Omalizumab improved asthma symptoms and lung function, reduced exacerbations of asthma and reduced the use of ICS and rescue medication in severe asthmatics (Busse *et al.* 2001). Reduction of ICS dose and reduced frequency of asthma exacerbations was also observed in asthmatic children (Milgrom *et al.* 2001). The overall efficacy of anti-IgE therapy across several studies including randomized placebo-controlled double-blind trials (Boulet *et al.* 1997*a*; Fahy *et al.* 1997, 1999; Milgrom *et al.* 1999, 2001; Busse *et al.* 2001; Holgate *et al.* 2004; Vignola *et al.* 2004; Djukanović *et al.* 2004) has been reviewed, with the conclusion that although improvements in asthma symptoms and quality of life were modest, the treatment does improve exacerbation rates in severe asthmatics (Avila 2007).

More recent evidence has suggested a role for IgG subclasses in asthma. Exposure to indoor allergens such as HDM or cats was associated with IgG and IgG4 production in both

asthmatics and non-asthmatics (Platts-Mills *et al.* 2001) and increased levels of IgGs and IgG1 are found in BALF of asthmatic children and adults (Out *et al.* 1991; Kitz *et al.* 2000). Increased risk of asthma in children was associated with both IgE and IgG but not IgG alone (Lau *et al.* 2005). As well as their role in asthma, IgE and mast cells are classically implicated in anaphylaxis and the two conditions may have overlapping pathogenesis. It has been suggested that mast cells and IgE may be dispensable for anaphylaxis, with a role for IgG1 being favored (Jacoby *et al.* 1984; Oettgen *et al.* 1994; Dombrowicz *et al.* 1997; Miyajima *et al.* 1997; Strait *et al.* 2006) with basophils and PAF also being important (Tsujimura *et al.* 2008). It is likely that IgE mediates the allergen-induced early response (Boulet *et al.* 1997*a*), however development of an LAR after allergen challenge was associated with increased serum levels of both total IgE and IgG (Pelikan & Pelikan-Filipek 1986*a*) and another small study suggested that IgG1 rather than IgE was predictive of a patient developing an LAR after HDM challenge (Ito *et al.* 1986). Thus in addition to IgE, IgG may well be involved in driving the LAR.

IgG acts on FcγRs, which are expressed on DCs, and on innate cells such as monocytes, macrophages, basophils, eosinophils, neutrophils, B cells, NK cells and mast cells, and thus may be involved in the integration of the innate and adaptive immune system (Williams *et al.* 2012). Inhalation of anti-OVA-IgG-immune complexes can induce allergic airway inflammation and eosinophilia, and Th2 cytokine production through proliferation of antigen specific T cells (Hartwig *et al.* 2010) and this may be through activation of FcγRs on DCs (Hartwig *et al.* 2010). This response failed to occur in FcγR deficient mice. In addition FcγRIII deficient DCs failed to induce Th2 cell differentiation *in vitro* and Th2 inflammation *in vivo*, and a lack of Th2-driven airway inflammation was observed in FcγRIII deficient mice (Bandukwala *et al.* 2007) in response to allergen sensitisation and challenge (Hartwig *et al.* 2010). Further to this a model has been recently proposed whereby IgG promotes secondary Th2 responses to allergen exposure by binding to FcγRs on innate immune cells (Williams *et al.* 2012). Indeed FcγR knockout mice had reduced IgG mediated cellular immune responses (Clynes & Ravetch 1995; Kagari *et al.* 2003; Zhang *et al.* 2004). However these FcγR receptors may also have inhibitory actions as increased Th2 responses were observed in FcγRIIb knockout mice in a model of allergic airway inflammation (Takai *et al.* 1996; Smith & Clatworthy 2010).

1.1.7. AHR and airway remodelling

Airway hyperresponsiveness (AHR) is a cardinal feature of asthma. Airways of asthmatic patients respond more strongly to bronchoconstrictors and other stimuli which

would normally not cause bronchoconstriction in normal individuals. This results in excessive bronchoconstriction and airflow limitation, which asthmatics experience as shortness of breath and chest tightness. Increased airway smooth muscle levels via hypertrophy and hyperplasia (Lambert *et al.* 1993; Ebina *et al.* 1993) and airway wall swelling (oedema) (Kimura *et al.* 1992) have been implicated in this excessive airway narrowing (Chung *et al.* 1999). AHR has been found in young infants that go on to develop asthma; AHR at 1 month was independently and significantly associated with parameters of asthma at 6 years old including doctor diagnosed asthma and reduced FEV_1 (Palmer *et al.* 2001). In allergic asthmatics AHR is associated with allergen-induced AHR and this can last for several weeks or even months after exposure to allergen (Cartier *et al.* 1982). In addition, increased bronchial reactivity or AHR may promote increased subsequent response to allergen (Cockcroft 1983).

Ordinarily a deep inspiration following inhalation of a bronchoconstrictive stimuli results in a stretching of the airways which may result in bronchodilation (Nadel & Tierney 1961; Fish *et al.* 1981) and deep inspiration was also protective against spasmogen-induced bronchoconstriction (Kapsali *et al.* 2000). A loss of bronchodilation following deep inspiration is another feature of asthma (Fish *et al.* 1981; Kapsali *et al.* 2000). This can cause an excessive airway narrowing which may also contribute to breathlessness in asthmatics, and may be due to airway inflammation (Pliss *et al.* 1989; Brusasco *et al.* 1992; Sont *et al.* 1995; Chung *et al.* 1999). AHR is likely to be driven by inflammation and this hypothesis is supported by the fact that inhaled corticosteroids and oral corticosteroids reduce AHR in asthmatics (Djukanović *et al.* 1992, 1997; Laitinen *et al.* 1992; Chalmers *et al.* 2002; Clearie *et al.* 2012). Mast cell localisation in ASM bundles is observed in asthmatics and release of mast cell derived mediators may contributes to AHR in asthma (Brightling *et al.* 2002; Siddiqui *et al.* 2008). In contrast, mast cell are not observed associated with ASM in eosinophilic bronchitis where airway dysfunction does not occur (Brightling *et al.* 2002). Other mediators which may cause AHR or have been implicated in its development include eosinophilia, products of eosinophil degranulation, and key Th2 cytokines such as IL-4, IL-5 and IL-13 (Grünig *et al.* 1998; Walter *et al.* 2001; Taube *et al.* 2002). Eosinophilic inflammation correlates with asthma severity as assessed by lung function analysis (Bousquet *et al.* 1990) and several lines of evidence suggest eosinophils and their associated granule proteins are involved in AHR in asthma. AHR and BAL eosinophilia/ MBP are associated in asthmatics. MBP applied to the lungs results in bronchospasm and AHR, and when applied to epithelium causes an increase in the reactivity of ASM to spasmogens (Gleich & Adolphson 1993). Eosinophil derived MBP has been shown to induce airway hyperreactivity in rats, guinea pigs and primates (Gundel *et al.* 1991; Uchida *et al.* 1993;

Lefort *et al.* 1996) and this may be through an effect on the inhibitory M2 muscarinic receptors (Jacoby *et al.* 1993; Evans *et al.* 1997). Eosinophils and their production of IL-5 may be important for Th2 driven IL-13 production and thus may impact on AHR through this pathway (Mattes *et al.* 2002). However others have questioned the role of the eosinophil-derived protein MBP in AHR (Denzler *et al.* 2000) and dissociated AHR from eosinophilia (Tournoy *et al.* 2000; Birrell *et al.* 2003). Innate inflammatory mediators such as TNF-α have also been implicated in driving AHR in asthmatics (Sukkar *et al.* 2001; Chen *et al.* 2003).

Chronic inflammation in asthma is likely to drive airway remodelling in asthma, and together they are likely to drive AHR. As I have mentioned substantial airway remodelling occurs in asthmatic patients. This includes subepithelial fibrosis, increased collagen deposition, and hypertrophy and hyperplasia of airway smooth muscle (Davies *et al.* 2003). These features are likely to result in the characteristic AHR observed in asthmatic patients and are largely driven by the chronic airway inflammation observed in the disease. Adventitial, submucosal and smooth muscle area and mass were all increased in asthmatics (Lambert *et al.* 1993; Kuwano *et al.* 1993) along with thickening of the airway wall and the basement membrane (Paganin *et al.* 1992; Lynch *et al.* 1993; Cutz 2002); these changes are most pronounced in those who died from fatal asthma (Carroll *et al.* 1993). Changes in airway structure have been observed in both large and small airways in asthmatics (Dunnill *et al.* 1969; Hossain 1973; Saetta *et al.* 1991). Both smooth muscle hypertrophy and hyperplasia have been reported in asthma patients and both of these processes are likely to contribute to the increased smooth muscle mass (Ebina *et al.* 1993). The increase in smooth muscle mass is one of the most important remodelling events that contributes to increased bronchoconstrictive response to spasmogens observed in asthmatics (Lambert *et al.* 1993). Indeed presence of increased ASM in preschool wheezers rather than basement membrane thickness, eosinophils in the airway mucosa or mast cell localisation with ASM were associated with development of asthma at school age (O'Reilly *et al.* 2012). However changes in the contractile properties of airway smooth muscle (ASM) such as increased velocity and extent of muscle shortening have also been documented (Ma *et al.* 2002). Levels of the ASM contractile protein α-SMA were associated with increased AHR (Slats *et al.* 2008). To explain the increase in smooth muscle observed in asthmatics, *in vitro* cultured ASM from asthmatics shows increased proliferation compared to ASM from normal individuals (Johnson *et al.* 2001).

Aside from smooth muscle remodelling other important remodelling processes which are characteristic of asthma include an increase in collagen deposition in the airway wall and subepithelial fibrosis (Davies *et al.* 2003). Hyperplasia of connective tissue in the

mucosa and bronchial lumen of patients who died from asthma have been noted. Myofibroblasts were detected interspersed with collagen and elastic fibres (Gabbrielli *et al.* 1994). These changes may contribute to increased rigidity of asthmatic airways and to the loss of airflow reversibility observed in some asthmatics (Gabbrielli *et al.* 1994). Subepithelial fibrosis and collagen deposition are observed in bronchial biopsies from asthmatics; fibrosis was found to correlate with AHR and loss of FEV_1 (Boulet *et al.* 1997*b*; Hoshino *et al.* 1998). Further changes in fibroblast numbers, collagen deposition and ASM size are also observed in bronchial biopsies from severe asthmatics, and fibroblast numbers and ASM cell size correlated with a reduced FEV_1 (Benayoun *et al.* 2003). Increased collagen and tenascin thickness (extracellular matrix components) were observed in asthmatic airways along with increased MMP-9 to TIMP-1 ratio; these factors where associated with myofibroblasts and eosinophils respectively (Hoshino *et al.* 1998) which suggests that these cell types are likely to be involved in the remodelling which occurs in asthmatics. Murine asthma models have also supported a role for eosinophils, transforming growth factor-β (TGF-β) and IL-5 in airway remodelling and AHR in asthmatics (Tanaka *et al.* 2001; Cho *et al.* 2004; Humbles *et al.* 2004). Budesonide administration prevented remodelling through (TGF-β) signalling, further implicating this cytokine in the remodelling events in this model (McMillan *et al.* 2005). Interaction between the damaged airway epithelium and associated myofibroblasts; release of proinflammatory cytokines, growth factors and repair enzymes leads to activation of fibroblasts and increased ECM deposition, which may propagate airway remodelling (Holgate *et al.* 2000). Mast cells have also been implicated in airway remodelling due to their ability to release MMPs which are likely mediators of remodelling and repair (Dahlen *et al.* 1999; Wenzel *et al.* 2003).

Although airflow obstruction in asthma is classically thought to be reversible there may be some element of irreversible airflow obstruction, and this is likely to be due to airway remodelling including increased ASM mass, collagen deposition (Peat *et al.* 1987; Redington & Howarth 1997; Lange *et al.* 1998, 2006). Goblet cell hyperplasia also occurs in the airways of asthmatics (Davies *et al.* 2003), which causes the increased levels of mucus production observed in the disease, and this feature is also likely to contribute to airflow obstruction. These changes may already occur in the very young, as airway remodelling has been detected in preschool wheezers (Saglani *et al.* 2007).

In asthma, several other mechanisms may be involved in the regulation of airway tone. ACh, histamine, leukotrienes, PGD_2, PGF2α and 5-HT have all been reported to be involved in airway smooth muscle contraction and regulation of airway tone. Inflammatory

mediators present in the asthmatic lung including histamine, prostanoids, thromboxane A2, bradikinin and 5-HT may indirectly or directly stimulate bronchoconstriction by acting on their respective receptors on ASM. Upregulation of these mediators in the asthmatic lung may serve to potentiate ASM responses to other spasmogens, contributing to AHR. Nervous control of the airways is predominantly mediated by the parasympathetic nervous system, of which acetylcholine (ACh) is the primary neurotransmitter. ACh mediates bronchoconstriction and mucus secretion by acting on muscarinic (M_3) receptors on smooth muscle and mucus glands. Sensory neurons (e.g. C fibres) innervate the airway, responding to mechanical or chemical stimuli. These can be activated by a variety of inflammatory mediators or stimuli such as cold air, resulting in reflexive activation of bronchoconstriction, mucus secretion and cough. A consequence of prolonged asthmatic inflammation is epithelial shedding, exposing sensory nerve (C fibres) in the lung. This exposes them to the multiple mediators present in the asthmatic lung; several of which are capable of both stimulating, and sensitising sensory neurons. Cationic eosinophil products have been shown to sensitise pulmonary sensory nerves (Gu & Lee 2001). Indeed HDM, a key cause of asthma, has been shown to damage airway epithelium (Herbert *et al.* 1995; Wan *et al.* 1999*a*, 2000; Heijink *et al.* 2010). In addition, airway inflammation has been implicated in facilitating regulation of neuronal ACh release, and in turn this may regulate airway remodelling (Gosens *et al.* 2006). Inflammatory mediators (TxA_2, PGD_2 and tachykinins) are thought to enhance cholinergic neurotransmission, ACh release and therefore enhance cholinergic reflexes (Barnes 1996). Antigen sensitisation and challenge have been shown to facilitate transmission in autonomic and sensory nerves (Undem *et al.* 1991). Finally dysfunction of M2 autoreceptors which limit bronchoconstriction has also been implicated in asthma (Minette *et al.* 1989). Thus there is considerable interaction between airway nerves, ASM, and airway inflammation, which is likely to drive the airway remodelling and AHR in asthma. The full details of these interactions are not yet fully understood.

1.2. Treatment of asthma

Treatment of asthma utilises two main approaches. Firstly bronchodilators are used for symptomatic relief and prevention of the reversible airflow obstruction and breathing difficulties observed in asthma. For this short and long acting β_2-agonists such as salbutamol or salmeterol/formoterol respectively are most commonly used. Secondly, the chronic inflammation in asthma is controlled by anti-inflammatory treatments, for which the gold standards are inhaled corticosteroids such as budesonide or fluticasone, or oral corticosteroids such as prednisolone.

Short acting B_2-agonists (SABAs) ameliorate allergen-induced EAR (Cockcroft & Murdock 1987) but not the LAR or allergen-induced increased reactivity to histamine (Cockcroft & Murdock 1987). Long acting B_2-agonists (LABAs) inhibit allergen-induced LAR and EAR (Pedersen *et al.* 1993). On the other hand corticosteroids such as beclamethasone or prednisolone inhibited the allergen-induced LAR and allergen-induced AHR to histamine but not the EAR (Booij-Noord *et al.* 1971; Cockcroft & Murdock 1987; Paggiaro *et al.* 1994; Kidney *et al.* 1997; Inman *et al.* 2001; Leigh *et al.* 2002). Although the therapies are widely prescribed and well tolerated, there is some evidence that LABAs taken on their own may increase the risk of mortality in asthma (Castle *et al.* 1993; Nelson *et al.* 2006); however it is thought that combining the LABA with inhaled corticosteroids (ICS) reduces this risk (Weatherall *et al.* 2010). The combination of ICS and LABAs has been shown to be effective against clinical outcomes and asthma control in asthmatics and has beneficial effects over ICS alone (Aubier *et al.* 1999; Zetterström *et al.* 2001; Lalloo *et al.* 2003). In addition there is evidence that β_2-agonists and corticosteroids may promote each other's action. Steroids may prevent down regulation and desensitisation of β_2 adrenoceptors (Mak *et al.* 1995, 2002) and β_2-agonists may increase the efficacy of glucocorticoids and have some anti-inflammatory efficacy themselves (Eickelberg *et al.* 1999; Pang & Knox 2001; Usmani *et al.* 2005) providing further rationale for the combined treatment. This has however been contradicted as another study has suggested that β_2-agonists may act to attenuate glucocorticoid efficacy (Adcock *et al.* 1996*a*).

In asthma a step-wise approach to treatment is utilised. Patients with mild intermittent symptoms are treated with inhaled short acting β_2-agonists such as salbutamol for symptomatic relief. More severe cases are treated with low dose ICS, and subsequently with a combination of low dose ICS (such as fluticasone, beclamethasone or budesonide) and LABA (such as salmeterol or formoterol); higher doses of ICS are utilised in severe asthma. OCS (such as prednisolone or dexamethasone) and add-on therapy such as theophylline, leukotriene antagonists (such as monteleukast) or anticholinergics (such as tiotropium) are then added into the treatment regime in severe asthma if symptoms are poorly controlled with ICS and LABAs. Figure 1.4 shows a diagrammatic representation of treatment approaches for the various degrees of asthma severity.

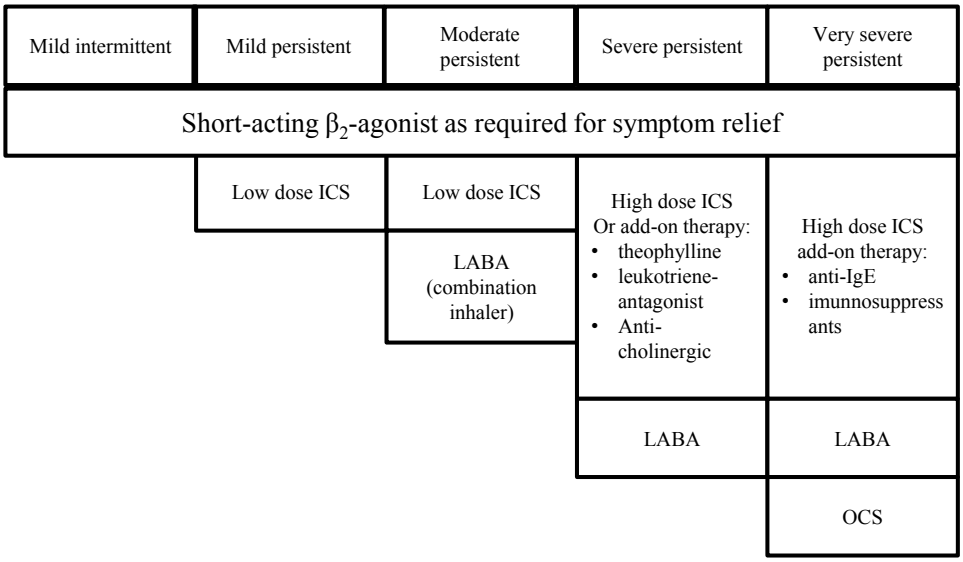


Figure 1.4: Step-wise approach to asthma treatment (adapted from Bateman *et al.* (2008) and Barnes (2010*b*)

B_2-agonists are thought to relax airway smooth muscle through stimulation of the β_2 adrenoceptor and a subsequent increase in cyclic adenosine monophosphate (cAMP) production and protein kinase A (PKA) or PKG activity, culminating in phosphorylation-induced changes in the smooth muscle contractile machinery. There is however some evidence that the relaxant effects of these compounds may be mediated through PKA/PKG-independent mechanisms (Torphy *et al.* 1982; Torphy 1994; Spicuzza *et al.* 2001).

Both topical and systemic steroids are highly effective at inhibiting pulmonary inflammation and the combination of this and their effect on AHR has promoted the hypothesis that inflammation drives AHR in asthma. Prednisolone treatment has been shown to reduce airway reactivity to methacholine as well as reduce the number of BAL eosinophils and BAL cells containing mRNA for IL-4 and IL-5 and increase the number of BAL cells positive for IFN-γ (Robinson *et al.* 1993*b*). Steroids may therefore modulate the pulmonary cytokine profile leading to a reduction in local eosinophilia. There are several mechanisms thought to be involved in the anti-inflammatory effects of glucocorticoids (GCs). These include: inhibition of inflammatory gene expression through binding to negative regulatory elements on DNA; positively regulating expression of inhibitory proteins; and transrepression of transcription factor-induced inflammatory gene expression (Pascual & Glass 2006). The former two require direct binding of glucocorticoid receptors (GR) to DNA, whereas transrepression involves protein-protein interactions with transcription factors (TFs) and associated proteins; this either involves preventing the TF

binding to the DNA, or preventing binding of transcription accessory molecules to the DNA-bound TF.

Steroids bind to the glucocorticoid receptors (GR) in the cytoplasm of various cell types. Upon binding the GR will dimerize and migrate to the nucleus where it binds to a glucocorticoid response element (GRE): a specific location on the promoter region of steroid responsive genes. GR binding to the GRE causes an increase in transcription of anti-inflammatory proteins such as annexin-1, IL-10 and IκB-α; however this is unlikely to be the main mechanism behind their efficacy; rather suppression of inflammatory gene synthesis through interaction with transcription factors is likely to be more important (Barnes & Adcock 2003). In states of inflammation activation of pro-inflammatory transcription factors such as NF-κB and AP-1 results in increased expression of multiple inflammatory proteins such as cytokines, chemokines, adhesion molecules and receptors. TFs utilise two main mechanisms to increase transcription of these mediators. Transcription factors bind to promoter regions of DNA found alongside genes; they recruit co-activator molecules to the DNA transcription factor complex, and aid the binding of RNA polymerase which catalyses DNA transcription. Secondly they may act by modulating chromatin structure. In basal conditions, DNA is wound around core histones (a complex known as chromatin); this is a closed structure which results in minimal gene expression. Binding of a transcription factor such as NF-κB and associated co-activators results in histone acetylation by histone acetyltransferase (HAT). This results in chromatin taking on an open structure with less DNA winding which enables increased binding of the DNA transcription complex, and ultimately an increase in transcription. Several studies have documented protein-protein interactions between AP-1 and glucocorticoids (Jonat *et al.* 1990; Schüle *et al.* 1990; Yang-Yen *et al.* 1990) which may lead to inhibition of AP-1:DNA binding and result in reduced inflammatory gene expression. Nuclear receptors such as GRs may also control gene transcription through inhibiting JNK-mediated AP-1 activation which is important for recruitment of the transcriptional co-activator CBP (Arias *et al.* 1994; Bannister *et al.* 1995; Caelles *et al.* 1997; Bruna *et al.* 2003).

Evidence of increased NF-κB activation has been documented in asthmatics (Hart *et al.* 1998). In basal states, NF-κB is sequestered in the cytoplasm via binding of IκBα which inhibits its transactivating ability (Auphan *et al.* 1995; Scheinman *et al.* 1995). NF-κB activation involves degradation of IκBα, which allows NF-κB to translocate to the nucleus. Glucocorticoids may inhibit NF-κB mediated gene expression by increasing transcription of IκBα and reducing its degradation. This prevents NF-κB migration into the nucleus and ultimately reduces inflammatory gene expression (Scheinman *et al.* 1995; Eberhardt *et al.* 2002). However other publications controvert the role of increased IκBα in the GC-induced

inhibition of NF-κB (Heck *et al.* 1997; Wissink *et al.* 1998). Others have shown that despite inhibiting inflammation and reducing AHR, corticosteroid treatment in asthmatics does not affect NF-κB: DNA binding (Hart *et al.* 2000) suggesting that protein-protein interactions as described for AP-1 may also be important in GR mediated inhibition of NF-κB. Indeed studies have shown direct protein-protein interaction between GR and the p65 subunit of NF-κB (Ray & Prefontaine 1994).

Another mechanism through which GCs may act to inhibit inflammation is through recruitment of histone deacetylases (HDACs) which are widely implicated in the effects of glucocorticoids on NF-κB mediated gene expression. Counteracting the activity of HATs, (HDACS) reduce DNA transcription by increasing DNA winding, and reducing DNA:TF binding. Steroids such as dexamethasone were shown to inhibit HAT activity and histone acetylation, and to induce HDAC recruitment. HDACs also induce GR deacetylation causing increased binding of GR to the NF-κB complex (Ito *et al.* 2000, 2006; Kagoshima *et al.* 2001). Finally steroids may also act via reducing the stability of mRNA (Lasa *et al.* 2001, 2002) such as that of COX-2, the enzyme responsible for the generation of multiple inflammatory mediators such as the prostanoids; reduced mRNA stability results in reduced gene translation to proteins.

1.3. Severe and steroid resistant asthma

Previous sections in this introduction have focused on mild-moderate asthma, where symptoms are generally well controlled. The majority of patients' symptoms are treated in the long term with SABAs or a combination of LABA and ICS as described above. However, in many cases the disease proves difficult to control with the maximum dose of standard medication, with patients exhibiting poor symptomatic control and a reduced response to steroid treatment. In these patients, symptoms can be both dramatic and debilitating, and in some cases can be fatal. It is important to note that severe asthma and treatment-resistant asthma do not necessarily correlate; severe asthmatics may not be resistant to treatment and treatment resistant asthmatics may not exhibit the severest symptoms – however the terms severe and difficult to treat asthma are often used interchangeably.

Estimating the levels of severe and therapy resistant asthma in the general population is difficult. The literature is complicated by varying criteria for inclusion in studies, and poor adherence to treatment regimens causing patients to present with apparent treatment-resistant disease. Classifications based on symptoms or lung function may result in discrepancy as the two features do not always correlate (Teeter & Bleecker 1998; Colice

2004; Bacharier *et al.* 2004). It has been estimated that approximately 25% of patients with severe asthma were steroid insensitive (Chan *et al.* 1998). Another study estimated that approximately 50% of asthmatics have uncontrolled asthma, while only 20% had well controlled asthma (Partridge *et al.* 2006), while others have more reservedly estimated that levels of severe asthma are only around 5% of those with asthma (Barnes 2008). Poor management or adherence to treatment regimens may skew the data; poor management of asthma and poor adherence have been associated with risk of fatal asthma episodes (Rea *et al.* 1986) while management of these issues has been reported to stabilise half of difficult to treat asthma cases (Heaney *et al.* 2003). Poor adherence may be responsible for poor control in half of the cases, and it is estimated that up to 80% of patients with difficult to treat asthma have poor adherence (Partridge *et al.* 2006; Gamble *et al.* 2009). A lack of serum steroid levels in severe asthma patients provides further evidence for a lack of adherence to asthma therapies (Robinson *et al.* 2003; Aburuz *et al.* 2007). Patients are often concerned about taking too much medication when they are feeling well, and up to 70% admitted to moderating their medication intake depending on their symptoms despite not being advised to do so by a practitioner (Partridge *et al.* 2006). Reasons for non-compliance with medical regimens may be lack of immediate benefit, concerns surrounding side effects – especially concerning corticosteroids, poor education, ethnicity and economic factors restricting access to healthcare (Griffith 1990). Psychosocial factors or a psychiatric diagnosis are also present in many severe asthmatics (approximately 40%) and have been associated with asthma deaths and near fatal asthma (Mohan *et al.* 1996; Kolbe *et al.* 2002; Heaney *et al.* 2003; Robinson *et al.* 2003). Unintentional under treatment is another factor which may lead to poor symptom control: both patients and physicians have failed to estimate the severity of asthmatic conditions (Teeter & Bleecker 1998; Boulet *et al.* 2002; Wolfenden *et al.* 2003). Due to these factors, the prevalence of treatment resistant asthma may be overestimated in some studies. However despite access to effective medication, the fact remains that asthma control across Europe and elsewhere such as North America and Asia is suboptimal (Rabe *et al.* 2000, 2004). The subpopulation of asthmatics with severe disease (estimated reservedly at around 5% of asthmatics (Barnes 2008)) accounts for a disproportionate amount of the health care costs associated with the disease (Serra-Batlles *et al.* 1998; Godard *et al.* 2002; Cisternas *et al.* 2003; Antonicelli *et al.* 2004). Estimates suggest severe asthmatics may be responsible for up to 70% of the medical costs associated with the disease (Weiss *et al.* 1992; Wenzel & Busse 2007). In addition symptoms in severe asthmatics are more likely to impact on work, possibly resulting in unemployment (Gaga *et al.* 2005). Therefore the severe treatment resistant asthma subset is an important unmet medical burden and improved disease therapies which are effective in the severe treatment resistant asthma subpopulation are an important goal for research. Further understanding of how severe asthma and steroid

insensitivity occurs would be useful to this process. The following outlines the current understanding of the pathophysiology of severe asthma and the mechanisms driving steroid insensitivity.

1.3.1. Immunological features of severe asthma

Factors which have been suggested to influence development and persistence of severe asthma include: rhinosinusitis, tobacco smoke, viruses, genetics, allergen exposure, obesity, adherence to treatment regimens, high level of co-morbidities; high doses of ICS and high use of rescue OCS; and a low FEV_1 (Heaney *et al.* 2003; Kupczyk & Wenzel 2012). Factors in the home environment may contribute to risk of symptoms in severe asthma (Strachan & Carey 1995) and respiratory infections are also likely to contribute to severe asthma episodes. As with mild asthma the phenotype of severe and therapy resistant asthma is highly variable between different individuals (Wenzel et al. 1999; Jatakanon et al. 1999; Louis et al. 2000; Gibson et al. 2000; Green et al. 2002; Miranda et al. 2004). This makes it difficult to fully understand the immunological mechanisms driving asthma severity. Several publications have evaluated severe/steroid resistant asthmatics and the factors which may predict development of these conditions (Irwin *et al.* 1993; Chan *et al.* 1998; ten Brinke *et al.* 2001; Phelan *et al.* 2002; Horak *et al.* 2003; Heaney *et al.* 2003; 'ENFUMOSA'' 2003; Robinson *et al.* 2003; Jenkins *et al.* 2003; Dolan *et al.* 2004; Bumbacea *et al.* 2004; Gaga *et al.* 2005; Kupczyk & Wenzel 2012).

Although asthma is typically thought to be characterised by reversible airflow obstruction, chronic airflow obstruction is often observed in severe asthmatics. Asthmatics have been shown to have a greater degree of lung function decline than normal individuals and this correlates with longer asthma duration and sputum eosinophil percentage. Decline in lung function in severe asthma may be augmented by smoking and a COPD-like phenotype (Peat *et al.* 1987; Lange *et al.* 1998; ten Brinke *et al.* 2001). Excessive airway narrowing is found in severe asthmatics which is characterised by a loss of plateau following bronchoconstrictor exposure which was not observed in mild asthmatics (Woolcock *et al.* 1984) along with a loss of bronchodilation following deep inspiration. Features that may contribute to this airway dysfunction and the resultant breathlessness in asthmatics include: increased airway smooth muscle via hypertrophy and hyperplasia (Lambert *et al.* 1993; Ebina *et al.* 1993), airway wall swelling (oedema) (Kimura *et al.* 1992) and the level of load on the airways (Macklem 1996); and finally airway inflammation (Pliss *et al.* 1989; Brusasco *et al.* 1992; Sont *et al.* 1995; Chung *et al.* 1999).

A common finding is that in contrast to milder asthmatics, airway inflammation such as eosinophils and activated T cells in severe asthmatics may not respond to steroid

treatment even at high doses (Schwartz *et al.* 1968; Leung *et al.* 1995). This treatment resistant inflammation may promote worsened symptoms as eosinophilic inflammation and ECP (present despite treatment with ICS and OCS) have been shown to correlate with asthma severity and AHR (Bousquet *et al.* 1990; Louis *et al.* 2000; Gibson *et al.* 2000). In another study IL-5 mRNA in bronchial mucosal biopsies was associated with severe disease as well as an increase in eosinophils and T cells, (Hamid *et al.* 1991).

A predominance of neutrophils has also been observed in the airway submucosa of asthmatics who died from sudden-onset fatal asthma (Sur *et al.* 1993), in asthmatics suffering severe acute exacerbations of asthma (Fahy *et al.* 1995) and in lavage, sputum and bronchial biopsy samples from severe asthmatics compared to mild/moderate asthmatics (Wenzel et al. 1997; Jatakanon et al. 1999). High levels of neutrophils are also found in the BALF of steroid resistant asthma patients (Tanizaki *et al.* 1993) and this is associated with steroid resistance in the patient (Green *et al.* 2002). Neutrophils and neutrophil markers fail to respond to steroid treatment, and in some cases may even be increased by it (Tanizaki *et al.* 1993; Culpitt *et al.* 1999; Gauvreau *et al.* 2002); steroids inhibit neutrophil apoptosis (Cox 1995). Neutrophil levels were highest in severe asthmatics taking high dose steroids (Wenzel *et al.* 1997) and persistent airway neutrophilia and eosinophilia despite high doses of steroid treatment correlate with disease severity (Louis *et al.* 2000). Thus inflammation can persist in severe asthmatics despite high doses of anti-inflammatory treatment and it is not clear whether this is due to the treatment or the underlying severity of disease (Wenzel *et al.* 1997).

Failure of neutrophil markers to be inhibited by steroid treatment may be linked to smoking status in asthmatics (Pedersen *et al.* 1996). Some have therefore suggested that the phenotype of inflammation in the lungs of severe asthmatics is more similar to a COPD-like phenotype (Barnes 2008) including increased neutrophilia and CXCL8 in the sputum (Jatakanon *et al.* 1999) along with an increase in oxidative stress markers and a poor response of the inflammation to steroid treatment (Cox *et al.* 1999). The increase in neutrophils in the lung may be due to Th17 cells and IL-17 release, which has been shown to induce release of CXCL8 from airway epithelial cells and the recruitment of airway neutrophils (Laan *et al.* 1999, 2001; Bullens *et al.* 2006). Indeed IL-17, IL-8 levels and neutrophilia in sputum of asthmatics were found to correlate (Bullens *et al.* 2006) and high levels of IL-17 and IL-8 were found in moderate and severe asthmatics despite steroid treatment.

The presence of specific cytokine patterns and eosinophilia in the airway and airway wall may be a sign of severe asthma (Bousquet *et al.* 1990; Hamid *et al.* 1991; Broide *et al.* 1992). TNF-α, GM-CSF, IL-6, and IL-1β were found in BALF of currently symptomatic asthmatics compared to stable asthmatics and a role of macrophages and T cells were

implicated in these episodes (Broide *et al.* 1992). In addition Th1 CD4+ T cells or a mixed Th1/Th2 phenotype (production of IL-4, IL-5 and IL-8) from both $CD8^+$ T cells and $CD4^+$ T cells related to disease severity (Cho *et al.* 2005; Kurashima *et al.* 2006; Barnes 2008). Bronchial $CD8^+$ T cells were found to correlate with lung function decline (van Rensen *et al.* 2005) and Th1 type $CD4^+$ T cells correlated with disease severity (Kurashima *et al.* 2006).

Wenzel et al. (1999) proposed two phenotypes of corticosteroid-dependent severe asthma namely: eosinophil (+) and eosinophil (-). Eosinophil (+) severe asthmatics also had increased levels of macrophages, lymphocytes and mast cells; and increased subbasement membrane (SBM) thickness, which correlated with eosinophilia (Wenzel et al. 1999). Interestingly FEV_1 was found to be lower in eosinophil (-) severe asthmatics, despite these patients having less SBM thickening (Wenzel et al. 1999). Severe asthma has also been classified according to disease onset – early onset and late onset – with the former being associated with a more atopic phenotype and the latter being associated with lower lung function. In either case persistent high levels of pulmonary eosinophils correlated with symptom severity (Miranda *et al.* 2004; Wenzel 2005). This classification and other data suggest that there may not be a simple correlation between age of onset or duration of disease and asthma severity (Horak *et al.* 2003; Jenkins *et al.* 2003; Wenzel 2005). Others have found severe asthmatics to have reduced atopy, reduced allergen-induced asthma features ('ENFUMOSA' 2003) and in some cases only minimal inflammation (Wenzel 2005). It may therefore be that the classical atopy and eosinophil mediated disease mechanisms involved in mild asthma are less important in severe asthma.

1.3.2. Therapy resistance

There are several different definitions of difficult to treat or treatment resistant asthma which are used regularly in the literature (Barnes *et al.* 1995; Woolcock 1996; 'Proceedings of the ATS workshop on refractory asthma' 2000; Heaney & Robinson 2005). An ATS workshop on refractory asthma in 2000 coined a definition of difficult to treat asthma whereby a patient must meet one of two major criteria and 2 of 7 minor criteria. The major criteria are that to achieve adequate control analogous with that seen in mild/moderate asthma the patient must be 1) treated with continuous or near continuous oral corticosteroids or 2) be treated with high doses of ICS. The minor criteria concern regular use of controller medication such as LABAs, daily or near daily use of SABAs, persistent airflow obstruction (FEV_1 < 80% predicted), urgent care asthma visits, bursts of oral steroids, deterioration of condition with reduction in ICS or OCS, and previous near fatal asthma events ('Proceedings of the ATS workshop on refractory asthma' 2000). In contrast others have

developed definitions based solely on poor efficacy of steroid treatment: for example "less than 15% improvement in baseline FEV_1 after a 14 day course of oral prednisolone (40mg/d) in patients who demonstrate more than 15% improvement in FEV_1 following inhaled β_2-agonist, salbutamol" (Barnes *et al.* 1995), or "persisting symptoms due to asthma despite high-dose inhaled steroids (2000µg beclamethasone diproprionate or equivalent) plus long acting β_2 agonist, with the requirement for either maintenance systemic steroids or at least 2 rescue courses of steroids over 12 months, and despite trials of add-ons such as leukotriene receptor antagonists or theophylline" (Heaney & Robinson 2005). Common to all of these definitions is the reliance upon the patient being under a tight management regimen, with a good level of adherence, with exposures to inducers and exacerbating factors eliminated and other disorders discounted. A true treatment resistant patient is symptomatic and experiences frequent exacerbations despite being adherent to their treatment and being on an appropriate treatment program including multiple asthma medications (Moore & Peters 2006). It is important to note that steroid resistance is 'relative' as a high dose of systemic steroid was more effective than a low dose therapy in severe life threatening asthma, improving PEF and preventing hospitalisations and emergency room visits (Ogirala *et al.* 1991). This is consistent with the dose related effects of steroid treatment in the clinic where inhaled fluticasone or budesonide treatment caused a dose-related improvement in lung function, PEF and FEV_1; reduction in rescue steroid use and a reduction in exacerbations (Dahl *et al.* 1993; Busse *et al.* 1998).

In addition to underlying asthma, other co-morbidities may contribute to asthma being difficult to treat including: bronchiectasis, COPD, vocal cord dysfunction, gastro-oesophageal reflux, allergen exposure and occupational exposures (Heaney & Robinson 2005). However management of these conditions does not improve quality of life in treatment-resistant asthmatics (Coughlan *et al.* 2001; Heaney *et al.* 2003; Heaney & Robinson 2005). General factors which may predict therapy resistant asthma have been identified including: specific inflammatory changes in airways, structural changes in the lung, genetic polymorphisms or environmental factors such as exposure to pollution (Chung *et al.* 1999). Two populations of steroid insensitive asthma have been identified; firstly chaotic steroid insensitive asthma with a large variability in lung function, and secondly, non chaotic steroid insensitive asthma, with less variability of lung function, a later diagnosis of asthma and requirement for oral GCs later in life (Chan *et al.* 1998). Having a low FEV_1 did not predict response to steroid as some patients which failed to respond to oral GC had near normal FEV_1 scores (Chan *et al.* 1998). The presence of eosinophils and active T cells in patients with asthma despite treatment with anti-inflammatory therapy may be a clue to

therapy resistance (Chung *et al.* 1999). But non eosinophilic asthma has also been shown to be unresponsive to steroid treatment (Pavord *et al.* 1999).

One of the reasons steroids may be so effective in asthma is that they illicit their anti-inflammatory effects through multiple mechanisms and pathways. There are therefore several possible ways in which steroid insensitivity could occur, and this is likely to differ between patients. Plasma clearance of cortisol was shown to be faster in steroid-resistant asthmatics that un-selected asthmatics, which may be one explanation for lack of steroid efficacy (Schwartz *et al.* 1968). Insensitivity of T cell proliferation to steroid treatment may also be involved in the lack of clinical response to steroid treatment (Corrigan *et al.* 1991*a*): dexamethasone was found to inhibit T cell proliferation from patients with steroid sensitive but not steroid resistant asthma. (Corrigan *et al.* 1991*b*).

The cytokine profile as well as alterations in the binding abilities or levels of GR may determine whether a patient with asthma responds to steroid treatment. It is suggested that steroid resistance in asthma may be due to dysregulation of Th1/Th2 cytokines (IL-4, IL-5 and IFN- γ) and that IL-2 and IL-4 may contribute to steroid insensitivity (Leung *et al.* 1995) by mediating a reduction in GR binding ability. Several studies support this theory. Reduced GR binding affinity in T cells compared to other cells, and compared to T cells from normal individuals is observed in steroid resistant asthmatics (Sher *et al.* 1994). This could be reversed by incubation of T cells with medium, or prolonged by incubation with IL-2 and IL-4 (Sher *et al.* 1994). This implies that something in the lung environment of steroid resistant asthmatics promotes reduced GR binding affinity and that this may well be mediated by the IL-2 and IL-4 (Sher *et al.* 1994). Levels of IL-2 and IL-4^{+} cells and IL-2 and IL-4 expression in T cells is observed in BAL from steroid resistant asthmatics compared to steroid responsive asthmatics (Kam *et al.* 1993). Further to this the combination of IL-2 and IL-4 also reduced GR binding affinity and increased GR number in PBMCs isolated from normal donors, and this effect was associated with a reduced efficacy of steroids in T cell proliferation (Kam *et al.* 1993). Finally steroid treatment in steroid insensitive asthmatics fails to illicit the reduction in BAL cells expressing IL-4 and IL-5 which occurs in normal individuals (Leung *et al.* 1995). IL-2 and IL-4-induced reduction in GR binding affinity may be through p38 MAPK and can be inhibited by a p38 MAPK inhibitor (Irusen *et al.* 2002). In conjunction with this IFN-γ treatment blocked the effects of IL-2 and Il-4 on GR binding (Kam *et al.* 1993) and can restore dexamethasone mediated nuclear translocation of the GR in T cells through inhibition of p38 MAPK (Goleva *et al.* 2009) and interestingly in normal individuals steroid treatment increases IFN-γ $^{+}$ cells in the BALF, while it reduces levels of these cells in steroid resistant asthmatics (Leung *et al.* 1995). Insensitivity of T cell

proliferation to steroid treatment may also be involved in the lack of clinical response to steroid treatment (Corrigan *et al.* 1991*a*).

It has been suggested that cytokines can increase AP-1 activity and that increased AP-1 interaction with GR (Jonat *et al.* 1990; Schüle *et al.* 1990; Yang-Yen *et al.* 1990) may cause the GR binding defect (Kam *et al.* 1993) and the associated reduction in steroid efficacy described above. AP-1 interaction with GR is likely to occur by reducing the number of GRs available to bind to DNA (Corrigan *et al.* 1991*a*, *b*; Leung *et al.* 1995; Adcock *et al.* 1995*a*, *b*). Dexamethasone caused GR-GRE binding PBMCs from steroid sensitive asthmatics and normal subjects, but not in steroid resistant asthmatics; this was attributed to reduced availability of GRs for binding rather than a change in binding affinity (Adcock *et al.* 1995*a*). A reduction in GR-AP1 interaction and an increase in AP-1 DNA binding were then noted in steroid resistant patients. This implicates either a reduced binding of GR to GRE or an increase in AP-1 DNA binding preventing GR:DNA binding in the glucocorticoid insensitive asthmatics (Adcock *et al.* 1995*b*). However there is evidence to contradict this hypothesis. The level of GR mRNA or the sites of its localisation was not different between non asthmatic and asthmatic donor lungs Adcock *et al.* (1996*b*) and Corrigan *et al.* (1991*a*) found no difference between the numbers or binding affinities of GRs in steroid resistant asthmatics.

Furthering this hypothesis the ratio of GR-α to GR-β may be involved. GR-α is the classical GR, whilst GR-β is an alternatively spliced form of the glucocorticoid receptor. This is thought to be transcriptionally inactive, and does not bind to GC ligands (Oakley *et al.* 1996). GR-β may act as an inhibitor of GR-α by competing for GRE binding, or through heterodimerisation with GR-α (Bamberger *et al.* 1995; Oakley *et al.* 1996; de Castro *et al.* 1996) upon activation with glucocorticoids (Strickland *et al.* 2001). Upon activation with GR, a heterodimer of GRβ with ligand bound GRα translocates to the nucleus where GR-β can act as a dominant negative regulator of the glucocorticoid receptor (de Castro *et al.* 1996). An increased level of GR-β has been noted for example in PBMCs from steroid-insensitive asthmatics (Leung *et al.* 1997; Hamid *et al.* 1999). Transfection of GRβ into cultured cells results in reduction of binding capacity of GRα which as mentioned has been observed in steroid resistant asthmatics (Leung *et al.* 1997). Therefore increased level of GR-β may be responsible for the reduced steroid responsiveness in some asthmatics.

As I described previously one of the mechanisms by which glucocorticoids reduce inflammatory gene expression is through recruitment of HDACs (Ito *et al.* 2006; Tsaprouni *et al.* 2007), and subsequent modification of the histone complex and chromatin structure of DNA. Impaired HDAC2 activity has been suggested as a mechanism for a reduced effect of

steroids on NF-κB mediated inflammation (Ito *et al.* 2006). This is also backed up in a steroid-resistant, neutrophilic model of asthma exacerbations where increased HAT and reduced HDAC activity were observed. Dexamethasone treatment reduced eosinophilia and T cell driven inflammation but not neutrophilia or AHR. Dexamethasone also blocked the increased HAT activity but not the reduction in HDAC activity; oxidative stress was implicated in the reduced HDAC activity due to the increase in lipid peroxidation markers which was not inhibited by steroid treatment (Ito *et al.* 2008).

1.4. Cigarette smoke and pollution in asthma

Several clinical papers suggest that smoking worsens symptoms of asthma (Siroux *et al.* 2000; Apostol *et al.* 2002; Thomson *et al.* 2004, 2013; Eisner & Iribarren 2007; Jang *et al.* 2009; O'Byrne *et al.* 2009). Smoking has also been shown to adversely affect the treatment impact of steroids in asthmatics (Chalmers *et al.* 2002; Chaudhuri *et al.* 2003) and to worsen disease outcome (Lazarus *et al.* 2007). While education around the dangers of cigarette smoking is good in the developed world, levels of smoking are still high, and worryingly, smoking prevalence in developing countries continues to increase (Abdullah & Husten 2004). What is most interesting is that despite asthma often being a severe and debilitating illness the levels of smoking in asthmatics patients are still high (Rabe *et al.* 2004) and are comparable with levels in healthy individuals (Vozoris & Stanbrook 2011). Some estimates suggest smoking asthmatics in developed countries represent approximately ¼ of asthmatics (Thomson *et al.* 2004; Cerveri *et al.* 2012), with more being previous smokers; one estimate suggests that as many as half of all adult asthma patients may be active, or previous smokers (Thomson *et al.* 2004). Furthermore childhood asthma does not lead to a reduction in take up of smoking (Siroux *et al.* 2000). Pollution and passive exposure to cigarette smoke are also likely to increase asthma severity, and these exposures may strongly promote the development of asthma.

1.4.1. Pollution, ETS and risk of asthma development in children

High levels of exposure to traffic pollution in early life has been shown to increase risk of asthma development, and asthma exacerbations (Zmirou *et al.* 2004; McConnell *et al.* 2006; Salam *et al.* 2008; Wallace *et al.* 2011; Patel *et al.* 2011). Exposure to pollutants such as carbon monoxide, carbon dioxide, nitric oxide, nitrogen dioxide, PM10, and sources of pollution early in life is associated with increased asthma diagnosis, with traffic pollution sources being most highly associated (Clark *et al.* 2010). Parents cite air pollution as both an initiator of asthma and a factor driving exacerbations (Stevens *et al.* 2004). Others have suggested that pollution alone does not cause asthma, but may increase levels of asthma in

individuals who are susceptible and live near sources of heavy traffic pollution (Gowers *et al.* 2012). Air pollution due to traffic and industrial sources is increasing in areas including Asia where it is linked with an increase in incidence of asthma (Chung *et al.* 2011).

Passive exposure to cigarette smoke (CS) is also likely to be a substantial problem in asthmatic children. One study in Chicago suggested that up to 65% of children were exposed to carer or household tobacco which substantially increased child salivary cotinine levels (a marker of CS exposure) compared to non-exposed children (Kumar *et al.* 2008). High cotinine levels in children is associated with increased asthma exacerbations, reduced FEV_1 and FEV_1/FVC ratio, wheezing and asthma incidence (Chilmonczyk *et al.* 1993; Mannino *et al.* 2001). Childhood exposure to ETS predominantly from paternal or maternal smoking may also increase the risk of asthma development (Skorge *et al.* 2005). A meta-analysis suggested that pre- and postnatal smoke exposure resulted in a 30-70% increased risk of wheeze, and a 21-85% increased risk of asthma (Burke *et al.* 2012). It was also found that maternal and paternal smoking are detrimental to offsprings' respiratory health even decades into adulthood (Accordini *et al.* 2012).

In addition maternal smoking or passive exposure during pregnancy is strongly linked with abnormal lung development in utero and increased levels of asthma in offspring (Gilliland *et al.* 2000, 2001; Zlotkowska & Zejda 2005; Leung *et al.* 2010); in utero CS exposure was also associated with reduced lung function in healthy children (Leung *et al.* 2010). Even in low risk infants with no history of atopy in either parent, maternal smoking during pregnancy can increase cord serum IgE and risk of atopy in infants (Magnusson 1986). Cotinine is found in new born infants' blood at higher levels than in maternal blood suggesting that pollutants can concentrate in the foetal bloodstream. This is possibly due to reduced ability of the foetus to clear pollutants from the system (Perera *et al.* 2004). (Magnusson 1986) hypothesised that the increased risk of sensitisation was due to synergy between the increased foetal IgE and subsequent damage to the airway mucosa enabling passage of allergens across the epithelial barrier. Even in utero exposure to air and traffic pollution was associated with increased risk of asthma (Zhou *et al.* 2013); important factors included CO, PM10, black carbon and exposure to specific industrial point sources (Clark *et al.* 2010).

1.4.2. Active smoking and risk of asthma development

There is also an association of active smoking with increased chance of development of asthma in adults (Björnsson *et al.* 1994; Lúdvíksdóttir *et al.* 1996; Wieringa *et al.* 1997; Kogevinas *et al.* 1998; Torén & Hermansson 1999; Plaschke *et al.* 2000; Piipari *et al.* 2004) Smoking for as little as three years increases risk of developing asthma (Flodin *et al.* 1995)

and this effect is dose-related depending on both intensity and duration of smoking (Polosa *et al.* 2008). Risk of developing asthma in adults was higher in both smokers and ex-smokers than never-smokers (Piipari *et al.* 2004), thus even after smoking cessation existing pathologies may continue to promote asthma generation. CS exposure to the lung has been shown to induce increased levels of TSLP mediated by oxidative stress. CS may promote allergic Th2 sensitisation by inducing production of the Th2-promoting cytokines TSLP (Nakamura *et al.* 2008).

1.4.3. Smoking, pollution and increased asthma severity

Several papers have shown active smoking to be associated with increased asthma severity: for example, active smoking was associated with increased severity scores and symptoms, increased frequency of asthma attacks, worse asthma related quality of life, and greater risk of hospitalisation (Siroux *et al.* 2000; Eisner & Iribarren 2007). Smoking was associated with an increase in medical service use (Shavit *et al.* 2007) and smoking asthmatics were also more likely to require antibiotics during hospital admissions with acute asthmatic episodes (Patel *et al.* 2009). Smoking has also been listed as a factor which predicts death from asthma (Ulrik & Frederiksen 1995). An increased risk of severe asthma was observed in current and ex-smokers, both of which were more likely to have uncontrolled disease, and effect of smoking was dose-related (Polosa *et al.* 2011). Again, smokers had worse levels of asthma control and increased unscheduled medical care. They required more OCS, and had a higher level of psychological factors (Thomson *et al.* 2013). Worryingly, even in children aged 13-14 active smoking was associated with an increase in wheeze (Austin *et al.* 2005). A more rapid decline in lung function was also found in smokers than non-smokers (Lange *et al.* 1998); smoking asthmatics had evidence of fixed airflow obstruction and emphysema than non-smoking asthmatics. Furthermore more smokers with asthma had airflow limitation by age 45 than non-smokers with asthma (Harmsen *et al.* 2010). This implies the beginnings of COPD-like symptoms in these patients (Jang *et al.* 2009; Harmsen *et al.* 2010). Smoking also affected the growth of FEV_1 which occurs during adolescence (Harmsen *et al.* 2010) thus the effects of smoking on lung function decline in asthma may well be initiated early in life. Both chronic airflow obstruction and smoking were significant risk factors for asthma-related deaths (Ulrik & Frederiksen 1995). Therefore there is substantial evidence that smoking leads to worsened asthma and also may cause permanent airflow obstruction in asthmatics.

CS is the primary cause of COPD which is another chronic lung disease associated with pulmonary inflammation and remodelling, however in COPD the primary feature is irreversible airflow limitation, progressive loss of lung function chronic bronchitis and emphysema (caused by dramatic tissue destruction in the lungs). The pathological processes

driving the disease are quite different from those in asthma. The important inflammatory cells in asthma include $CD4^+$ Th2 lymphocytes and eosinophils while in COPD they are macrophages, neutrophils and $CD8^+$ lymphocytes (Tamimi *et al.* 2012). COPD is also characterised by a lack of response of inflammation to steroid treatment (Keatings *et al.* 1997; Culpitt *et al.* 1999, 2003). While ICS improve exacerbation rate and health status in COPD patients, they do not alter disease progression i.e. decline in lung function (Vestbo *et al.* 1999; Pauwels *et al.* 1999; Burge *et al.* 2003; Spencer *et al.* 2004). Some cases of steroid resistant or severe asthma may therefore actually be due to overlapping features of co-morbid COPD, especially in smokers (Thomson *et al.* 2004; Tamimi *et al.* 2012). However in many cases (especially in younger patients) severe asthma in smokers is likely to be a distinct phenotype.

Not only is active smoking thought to worsen asthma but passive tobacco smoke exposure (ETS) and other types of pollution may also have a detrimental effect on asthma condition. Passive CS exposure was associated with increased medical service use in children aged 13-14, (Austin *et al.* 2005) and is also associated with reduced FEV_1 and FEV_1/FVC ratio and an increase in asthma exacerbations in children (Chilmonczyk *et al.* 1993). In adults with asthma, exposure to ETS is associated with worsened asthma on several counts: increased severity, reduced quality of life, reduction in medical outcome, and increased emergency medical visits and hospitalizations (Eisner *et al.* 1998, 2005).

ETS cessation improved asthma severity measures and reduced both emergency visits and hospitalisations (Eisner *et al.* 1998). Indeed a recent publication showed a dramatic reduction in the levels of childhood asthma-related hospital admissions since the introduction of the smoking ban in public places in England in 2007 (Millett *et al.* 2013). A similar finding was also found in a Scottish study (Mackay *et al.* 2010). Experimental CS exposure in asthmatics induced AHR which persisted for up to 14 days after challenge (Menon *et al.* 1992) and a proportion of asthmatic patients (irrespective of atopy) showed a substantial reduction in lung function upon challenge with ETS (Stankus *et al.* 1988). Similarly some of the worsened disease features in smoking asthmatics were restored in ex-smokers suggesting benefit can be achieved from smoking cessation (Broekema *et al.* 2009).

There are thought to be many differences in the pathology in the lungs of asthmatic patients who smoke compared to non-smokers which may account for the increase in disease severity. One such feature is the different airway inflammatory cell profile observed in smoking asthmatics. Neutrophils and macrophages, and cytokines such as IL-1β and IL-8 are increased in the BAL fluid of smokers and the effects of smoking are dose dependent

(Kuschner *et al.* 1996). Cigarette smoke can cause release of neutrophil and macrophage chemo-attractants from airway epithelial cells (Masubuchi *et al.* 1998). Similarly to the severe asthma phenotype which I described earlier smoking asthmatics have lower levels of airway eosinophils (Broekema *et al.* 2009; Thomson *et al.* 2013) and higher levels of sputum neutrophils than non-smoking asthmatics (Boulet *et al.* 2006). Asthmatic smokers also have more macrophages in BAL than non-smoking asthmatics (Kane *et al.* 2009). As key mediators of COPD, neutrophils and macrophages in the lungs may be responsible for worsened disease in smoking asthmatics. Neutrophils in induced sputum were associated with decline in lung function in smokers (Stănescu *et al.* 1996). In addition neutrophils and eosinophils were associated with a low pre-bronchodilator FEV_1, whilst neutrophils alone were associated with a low post-bronchodilator FEV_1; this implicates the neutrophil in chronic airflow obstruction (Shaw *et al.* 2007). Furthermore in smoking asthmatics there was a relationship between smoking history, lung function changes and airway inflammation; sputum IL-8 correlated with smoking history and airway neutrophilia, and FEV_1 was related to sputum IL-8 and neutrophilia (Chalmers *et al.* 2001). Diesel exhaust particle exposure in healthy individuals can also cause neutrophilic airway inflammation (Nightingale *et al.* 2000) so the immune processes driven by pollution may parallel those caused by active or passive smoking. In a study investigating the effects of pollution on immediate asthma symptoms, a greater reduction in FEV_1, FVC and increased neutrophilic biomarkers were observed in asthmatics who walked in a busy London street, compared to those walking in Hyde Park. This correlated with increased exposure to diesel traffic related pollution street including fine particles, ultrafine particles carbon and NO_2 (McCreanor *et al.* 2007). These effects were enhanced in moderate asthmatics compared to mild asthmatics (McCreanor *et al.* 2007).

In addition to an altered cellular inflammatory profile and reduced lung function, smoking asthmatics also have more goblet cells and mucus production, and increased epithelial thickness than non-smoking asthmatics (Broekema *et al.* 2009). The effect of smoking on IgE levels in asthmatics is unclear; some studies have shown active smoking to be associated with an increase in total IgE which was related to level of smoking, and sensitisation to HDM was more prevalent in smokers (Jarvis *et al.* 1999; Accordini *et al.* 2012). However reduced allergen-specific IgE has been observed in severe asthmatics, smokers and ex-smokers than never-smokers (Thomson *et al.* 2013).

CS exposure in man and in murine studies has been shown to have dose-dependent inflammatory effects in the lung (Kuschner *et al.* 1996; Clatworthy *et al.* 2009; Eltom *et al.* 2011). Smoking asthmatics may therefore have different disease phenotypes, which is likely to be dependent on smoking intensity. Indeed there is a suggestion that in smoking

asthmatics the inflammatory profile and whether this is most contributed to by smoking or the underlying asthmatic condition is dependent on the intensity and duration of smoking history (Polosa & Thomson 2013). Furthermore the level of smoking or smoke exposure was equivalent across non-severe and severe asthmatics (Gaga *et al.* 2005) which implies that not all smoking asthmatics develop severe disease. Intrinsic differences in the patient may determine the response to cigarette smoke or pollution. For example, single nucleotide polymorphisms (SNPs) have been discovered which confer a genetic risk of early onset asthma, including one for which the risk of asthma is exacerbated by exposure to ETS (Bouzigon *et al.* 2008)**.**

1.4.4. How CS exposure may modulate disease processes in asthma

The majority of studies looking at the effect of smoking in asthma investigate asthma symptoms, rather than looking at how smoking may mechanistically impact on asthma. However there is some data on this topic. After a single exposure to CS in mice, pro-collagen and growth factor production were increased after 2 hours and had subsided by 24 hours, however levels were sustained after multiple exposures (Churg *et al.* 2006). Acute exposure to CS may not be particularly damaging to ones' health, however it is chronic exposure which is responsible for the substantial lung damage that occurs in COPD and worsened asthma in smokers.

Acute CS exposure causes inflammation in the lungs. Studies in humans and animals have shown that acute CS results in increased levels of neutrophils and macrophages and increased markers of oxidative stress in the lungs (van der Vaart *et al.* 2004). T cells and macrophages are found in the airway wall of smokers including young smokers with lack of disease, while neutrophils are found in the airway lumen of smokers with COPD (Niewoehner *et al.* 1974; Saetta *et al.* 1993; Keatings *et al.* 1996). High levels of neutrophils in the lungs of smokers may be due to increases in neutrophil retention in the lungs (MacNee *et al.* 1989). CS exposure also changes cytokine regulation in the lung (Brown *et al.* 1989; Soliman & Twigg 1992; Yamaguchi *et al.* 1993; Dubar *et al.* 1993; McCrea *et al.* 1994; Sauty *et al.* 1994). Multiple inflammatory mediators are released from airway epithelial cells and immune cells such as TNF-α, MIP-1α, MCP-1, IL-8, and IL-1β in response to acute CS exposure (van der Vaart *et al.* 2004). Increased levels of IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α are observed in the exhaled breath of smokers compared to non-smokers (Gessner *et al.* 2005). As airway inflammation in asthma is likely to drive many of the pathological disease processes it may be that the increased disease severity in smoking asthmatics occurs as a result of the combination of inflammation resulting from CS in addition to the underlying asthmatic inflammation.

Many papers have also suggested that CS exposure or smoking can cause or promote remodelling of the airways; resulting in many pathological features associated with asthma. Cigarette smoke can induce small airway remodelling through increasing production of pro-collagen and growth factors such as TGF-β (Churg *et al.* 2006; Guo *et al.* 2008; Kim *et al.* 2011). Smoking also increases elastic fibres, collagen and myofibroblast matrix in the bronchial tree (Carroll *et al.* 2000) and 4 months of smoke exposure in mice induced features of remodelling such as collagen deposition and airway thickening measured by α-SMA levels (Melgert *et al.* 2007). CS has also been shown to increase airway remodelling in a murine OVA-driven asthma model (Min *et al.* 2007) and a guinea pig fibrosis model (Cisneros-Lira *et al.* 2003). In a HDM-driven model CS co-exposure also increased collagen levels compared to HDM-challenged mice (Botelho *et al.* 2011).

Cigarette smoke has been shown to damage the respiratory epithelium, increasing epithelial permeability and reducing epithelial barrier function (Jones *et al.* 1980; Burns *et al.* 1989; Dye & Adler 1994) which may increase the permeability of the airways to allergens. This may promote sensitisation to allergens such as HDM and increase the effect of HDM exposure in sensitised individuals. Indeed CS has been shown to potentiate HDM-induced increase in epithelial permeability and transit of allergens across the epithelial cell layer (Rusznak *et al.* 1999). This may be through modulation of kinase pathways or by cytoskeletal alterations and redistribution of tight junction proteins (Olivera *et al.* 2007, 2010).

Much of the damage to the lungs mediated by cigarette smoke is likely to be through generation of free radicals which have been implicated in many chronic diseases associated with cigarette smoke, reviewed in (Church & Pryor 1985). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are unstable particles which can initiate oxidation through their unpaired electrons (Rahman *et al.* 2006). Cigarette smoke contains over 4700 chemicals including 10^{14} reactive species (Church & Pryor 1985). The gas phase of cigarette smoke is thought to have 10^{15} free radicals per puff including reactive oxygen species, nitric oxide, nitrogen dioxide, epoxides, peroxides and peroxynitrite, while the tar phase contains 10^{18} free radicals per gram such as H_2O_2, hydroxyl ions, semiquinone and phenol (Yoshida & Tuder 2007). Free radicals may also come from endogenous sources such as inflammatory cells in the lungs (Yoshida & Tuder 2007). Free radicals such as RNS and ROS are generated endogenously by inflammatory cells to protect the body from invading microorganisms and chemicals; inflammatory cells are activated by CS which may potentiate the damaging effect of cigarette smoke. Pathologically high levels of ROS and RNS damage organs such as the lung through oxidation effects on DNA and proteins, and through production of secondary metabolic products (Rahman *et al.* 2006). ROS and RNS

have many biological effects which can include: ECM remodelling, increased mucus production, activation of alveolar repair responses, inactivation of anti-proteases (or protease inhibitors) and modulation of immune responses (Rahman *et al.* 2006). These effects are likely to be mediated through activation of TFs such as NF-κB and AP-1 and the resultant increased expression of proinflammatory mediators (Rahman *et al.* 2006).

One of the most widely studied pathological effects of CS is its effect on proteases and antiproteases; in the lungs there is a balance between proteases and antiprotease activity (Taylor 2010). Proteases are required to protect the lung, but their activity is strictly controlled by endogenous protease inhibitors (Taylor 2010). An imbalance in this system is likely to contribute to the destruction of the lung observed in COPD and CS-driven oxidant stress is a likely candidate for driving this imbalance. Levels of neutrophils and macrophages are increased in the lungs of smokers (van der Vaart *et al.* 2004): neutrophils are a primary source of oxidants and elastases while macrophages are an important source of oxidants and proteases of the extracellular matrix such as matrix metalloproteinases (MMPs) (Yoshida & Tuder 2007). BALF fluid from smokers also have increased levels of neutrophil and macrophage derived elastases (Janoff *et al.* 1983) which are key drivers of emphysema (Damiano *et al.* 1986; Hautamaki *et al.* 1997). In addition it was discovered that a deficiency in α1-antitrypsin or α1-antiprotease (AAT) could cause emphysema (Stockley *et al.* 2009). AAT is an inhibitor of neutrophil elastase, and in deficient individuals the ability to counteract the activity of neutrophil-derived elastases is lost (Stockley *et al.* 2009). These findings support the hypothesis of the protease-protease inhibitor imbalance (Gadek *et al.* 1981) in the pulmonary destruction observed in pulmonary disease. Free radicals and oxidants in CS or those released by inflammatory cells recruited to the airways after CS exposure can cause inactivation of α1-antiproteinase (Evans & Pryor 1994.). Even a partial deficiency in AAT (heterozygous genotype) can cause increased decline in lung function (Sandford *et al.* 2001) and different phenotypes of the AAT gene or deficiency have been implicated in asthma and AHR. In these AAT deficient individuals smoking increased asthma symptoms (Townley *et al.* 1990; Eden *et al.* 1997, 2003; Sigsgaard *et al.* 2000; Piitulainen & Sveger 2002). Therefore a protease imbalance in smoking asthmatics may well contribute to increased disease severity and decline in lung function.

In addition α1-antiprotease is known to inhibit cleavage of CD23 by HDM resulting in a reduction in HDM-induced IgE production (Sherr *et al.* 1989; Flores-Romo *et al.* 1993; Yu *et al.* 1994). Inhibition of α1-antiprotease by CS may therefore be one explanation for the increased sensitisation to allergens observed in smokers (Hewitt *et al.* 1995).

Matrix metalloproteinases (MMPs) are a collection of enzymes with proteolytic activity such as collagenase and elastase. These cleave extracellular matrix components such as collagen, proteoglycan, laminin, fibronectin and elastin (O'Connor & FitzGerald 1994) and are important in tissue remodelling and repair (Yoshida & Tuder 2007). Counteracting the effect of MMPs in the lungs are TIMPs (which are inhibitors of MMPs). TIMPs and MMPS have been proposed to be involved in the response of airway epithelium to oxidative stress (Freishtat *et al.* 2009). Levels of MMPS and elastinolytic and collagenolytic activity are increased in the BALF and BAL macrophages of emphysema patients (Finlay *et al.* 1997*a*, *b*; Yoshida & Tuder 2007). While these processes have been predominantly implicated in COPD there is also evidence that oxidative stress, MMPs and TIMPS may play a role in asthma. The ratio of MMP-9/TIMP-1 is likely to be important in asthma, as a low MMP-9/TIMP-1 ratio predicts low FEV_1, increased collagen deposition and pulmonary extracellular remodelling (Vignola *et al.* 1998; Mautino *et al.* 1999; Chiappara *et al.* 2001; Atkinson & Senior 2003; Araujo *et al.* 2008; Watson *et al.* 2010). CS and oxidative stress can induce inflammation which increases TIMP-1 (Gomez *et al.* 1997; Atkinson & Senior 2003). Furthermore CS exposure increased levels of TIMP-1 in asthmatic and normal airway epithelial cells but decreased MMP-9 only in asthmatic airways (Watson *et al.* 2010). This meant the MMP-9/TIMP-1 ratio was reduced by CS in asthmatic but not normal cells and that asthmatic airways therefore respond to CS by inducing a pro-remodelling environment. Airway epithelial cells from asthmatics were more susceptible to the oxidant effects of H_2O_2 than cells from normal individuals (Bucchieri *et al.* 2002). In addition when pollution related exposure to oxidants was increased, this was positively associated with shortness of breath and bronchodilator use in asthmatics (Hiltermann *et al.* 1998).

Whilst the classical role of MMPs is in remodeling and repair of the extracellular maxtric there is some evidence that MMPs such as MMP 12 may also be involved in the generation of inflammation, and specifically that driven by CS. Mice deficient in macrophage metalloelastase (or MMP 12) do not develop CS-induced neutrophilia (Churg *et al.* 2002; Leclerc *et al.* 2006) and MMP 12 appears to drive CS-induced neutrophilia by activating macrophages to release TNF-α (Churg *et al.* 2003).

1.4.5. CS and steroid insensitivity

As I have previously mentioned, smoking is associated with poor asthma control; asthma control was related to the number of cigarettes smoked per day (Laforest *et al.* 2006; Chaudhuri *et al.* 2006; Leuppi *et al.* 2006; Clatworthy *et al.* 2009). Several clinical studies have shown that the therapeutic response to steroid treatment in asthmatics is reduced by

smoking. Smokers showed reduced improvement of FEV_1 after ICS compared to non-smokers (Pedersen *et al.* 2007); ICS improved lung function, sputum eosinophils and eosinophil markers; morning PEF, FEV_1 and AHR in non-smokers but not in smokers (Pedersen *et al.* 1996; Chalmers *et al.* 2002). In other studies ICS improved sputum eosinophils and eosinophil markers in smokers and non-smokers, but only improved FEV_1 in non-smokers (Lazarus *et al.* 2007). Smoking also worsens the short term improvement of FEV_1 with ICS in individuals with chronic airflow obstruction in both asthma and COPD (Kerstjens *et al.* 1993). This effect is not just restricted to ICS; the response to oral corticosteroids is also impaired by smoking. OCS improved FEV_1, am PEF, and asthma control in never smokers but not in smokers (Chaudhuri *et al.* 2003). OCS showed improved efficacy in ex-smokers however, which implies some level of restoration of response to steroid treatment following smoking cessation (Chaudhuri *et al.* 2003). Low dose ICS elicited less of an improvement in PEF in smokers than non-smokers but this was overcome by a higher dose of steroid (Tomlinson *et al.* 2005) suggesting that steroid resistance is relative, and that improved treatment efficacy can be achieved in smoking asthmatics with higher doses of ICS. In contrast others have shown that addition of a LABA to treatment with ICS elicits a better treatment effect in smoking asthmatics than doubling the dose of ICS (Clearie *et al.* 2012). The combination of theophylline and ICS also induced a better treatment response than ICS alone in smoking asthmatics (Spears *et al.* 2009).

The effect of ICS on lung function decline in smoking asthmatics is controversial. On one hand, ICS have been shown to slow decline in lung function only in non-smoking asthmatics (Dijkstra *et al.* 2006), however in other studies ICS did attenuate decline in lung function in smoking asthmatics (Lange *et al.* 2006) despite smokers have worse decline in lung function than non-smokers (O'Byrne *et al.* 2009). Therefore although efficacy of ICS may be impaired in smokers, their use may still be warranted to help prevent decline in lung function.

1.4.5.1. Mechanisms of CS-induced steroid resistance

The inflammatory phenotype in smoking asthmatics is more neutrophilic than that which is observed in non-smoking asthmatics (Boulet *et al.* 2006; St-Laurent *et al.* 2008; Meghji *et al.* 2011) and CS is widely accepted to induce an increase in airway neutrophils (Hunninghake & Crystal 1983; Kuschner *et al.* 1996; Roth *et al.* 1998; Amin *et al.* 2000). This cell type is insensitive to steroid treatment in smokers (Cox *et al.* 1999; Culpitt *et al.* 1999) and has been associated with a decline in lung function (Stănescu *et al.* 1996). Furthermore the production of IL-8 (a neutrophil chemokine) from alveolar macrophages in smoking asthmatics was resistant to glucocorticoids and this may explain the persistent airway neutrophilia in smoking asthmatics (Kane *et al.* 2009). As the pathogenesis of asthma

in smokers may at least in part be driven by neutrophilia this may explain the lack of clinical efficacy of steroids in smoking asthmatics.

1.4.5.1.1. HDACS

A highly cited mechanism for CS-induced steroid resistance involves HDACs, and was originally proposed for COPD but may also be important in steroid resistance in asthmatics. As described earlier one of the mechanisms by which glucocorticoids reduce inflammatory gene expression is through recruitment of HDACs (Ito *et al.* 2000, 2006; Kagoshima *et al.* 2001; Tsaprouni *et al.* 2007), and subsequent modification of the histone complex and chromatin structure of DNA. CS exposure, possibly through oxidative stress, has been shown to downregulate HDAC2 activity (Marwick *et al.* 2004; Ito *et al.* 2008; Adenuga *et al.* 2009) and impaired HDAC2 recruitment and activity has been suggested as a mechanism for a reduced effect of steroids on NF-κB mediated inflammation (Ito *et al.* 2006). CS may therefore negate one of the mechanisms by which glucocorticoids mediate their anti-inflammatory effects.

The methylxanthine theophylline is thought to restore steroid sensitivity (Cosio *et al.* 2009; To *et al.* 2010; Sun *et al.* 2012). It has been suggested as a treatment approach in COPD patients (Barnes 2003, 2010*a*) and has shown efficacy in some studies (Cosio *et al.* 2009; Ford *et al.* 2010). This hypothesis and treatment approach may also be of relevance in the asthma field. Theophylline has shown to inhibit allergen-induced symptoms in asthmatics (Crescioli *et al.* 1991) and has shown benefit as an add-on therapy (Spears *et al.* 2009). Although theophylline is thought to have many targets its effect on steroid sensitivity is thought to be through PI3K-δ inhibition (To *et al.* 2010), which lead to the proposal of using PI3K-δ inhibition to restore glucocorticoid responsiveness (Marwick *et al.* 2009, 2010).

1.4.5.1.2. GRα: GRβ ratio

As I explained above, another hypothesis regarding steroid resistance in asthmatics is related to the ratio of GRα: GRβ. Reduced GRα: GRβ ration has been observed in PBMCs from smoking asthmatics (Livingston *et al.* 2004), therefore this hypothesis may be highly relevant to smoking asthmatics. Smoking is widely accepted to increase levels of neutrophils in the lungs of smokers (Chalmers *et al.* 2001) and these are likely to contribute to the pathology in the lungs of smoking asthmatics. Although both neutrophils and PBMCs express GRα and GRβ, PMBCs express more GRα while neutrophils express more GRβ and more GRα:GRβ heterodimers (Strickland *et al.* 2001). Steroid treatment was associated with a reduced GRα:GRβ ratio in neutrophils (Strickland *et al.* 2001). Cigarette smoke results in increased production of multiple cytokines including IL-8 (Chalmers *et al.* 2001) and IL-8

treatment was also shown to increase GRβ levels and reduce GRα levels in neutrophils (Strickland *et al.* 2001). Therefore CS may contribute to a reduced GRα: GRβ ratio in smoking asthmatics (Strickland *et al.* 2001) and this may explain the lack of steroid responsiveness in these patients.

1.5. Models of asthma

As I have described asthma is a multifactorial disease and there is no overarching definition that covers all its guises, thus it is a difficult disease to model. Many different models have been established in various species, including the guinea pig the Brown Norway rat, and the mouse, which with its highly characterised immune system and the ever expanding choice of genetically modified strains is increasingly becoming the species of choice. As this thesis concerns modelling asthma in mice, I will concentrate on work published on murine asthma models.

1.5.1. Strain dependence in murine asthma models

Balb/c mice have in the past been the preferred strain for asthma models however development of genetically modified mice on the C57Bl/6 background – lacking either cell types or mediators of interest – has meant use of C57Bl/6 mice in these models has increased. Several studies have compared immunological responses to allergen between Balb/c and C57Bl/6 mice (Zhang *et al.* 1997; Wilder *et al.* 1999; Morokata *et al.* 1999, 2000; Takeda *et al.* 2001; Hayashi *et al.* 2001; Gueders *et al.* 2009). Dogma often suggests that C57Bl/6 mice are poor IgE and Th2 responders compared to Balb/c mice, in line with publications by Zhang et al. (1997) and Takeda et al. (2001). But, others have actually shown C57Bl/6 mice to produce higher levels of allergen-specific IgE than Balb/c mice (Wilder *et al.* 1999; Morokata *et al.* 1999, 2000). Reduced allergen-induced bronchial lesions associated with suppressed Th2 response, reduced eosinophils and lymphocytes have also been observed in C57Bl/6 mice compared to Balb/c (Hayashi *et al.* 2001) however others have shown OVA-challenge to elicit a more robust airway eosinophilia in C57BL/6 mice. Thus although there may be immunological differences between the two strains, allergen-driven models in both strains have been shown to induce robust asthma-relevant pathologies. Our group currently favours C57BL/6 mice due to the abundance of genetically modified mice strains raised in this background. These can be used to investigate the role of mediators and cell types in the responses observed in our various murine disease models.

1.5.2. Allergen challenge models

Much of the early understanding of disease processes in asthma has resulted from allergen challenge experiments in asthmatic patients, as reviewed: (Gauvreau & Evans 2007; Cockcroft *et al.* 2007). This procedure has also been adopted for pre-clinical models of asthma where a surrogate or etiological allergen is used to induce asthma-like phenotypes. Few animals spontaneously develop allergic disease however, so the models need to engineer allergic sensitisation. In addition, the normal response to an inhaled insult is tolerance, thus asthma models must also overcome this general tendency towards tolerance. The classical model which achieves this is the ovalbumin or OVA model.

1.5.2.1. OVA model

Ovalbumin (OVA), is a protein allergen isolated from (hen) egg white, and is generally immunologically inert in the absence of prior sensitisation. The OVA model typically involves two phases: firstly a 'sensitisation' phase, followed by a 'challenge' phase to induce an allergic response. Typically intraperitoneal sensitisation with OVA plus an adjuvant (described below) induces production of OVA-specific immunoglobulin responses, while a subsequent challenge induces AHR and eosinophilic pulmonary inflammation (Zhang *et al.* 1997). The general model protocol requires at least two systemic doses to induce sensitisation; systemic sensitisation with OVA (plus adjuvant) has been shown to induce a robust increase in total and OVA-specific IgE (Beck & Spiegelberg 1989; Brusselle *et al.* 1994) in both C57Bl/6 and Balb/c mice. On-going Th2 responses may prevent IgE tolerance developing to aerosolised OVA, instead promoting Th2 priming to the allergen (Hurst *et al.* 2001). This explains the widespread use of Alum, a Th2 polarising adjuvant (Comoy *et al.* 1998) in OVA models. Following systemic sensitisation, topical OVA challenge in various models has been shown to result in multiple features of asthma including lung function changes such as acute bronchospasm, LAR and non-specific AHR; allergic airway inflammation, including influx of airway eosinophils and lymphocytes, and airway remodelling (reviewed in (Stevenson & Belvisi 2008)). Although dogma suggests that a systemic adjuvant is an absolute requirement for OVA-driven asthma models, adjuvant-free models have been described (Renz *et al.* 1992; Hessel *et al.* 1995; Blyth *et al.* 1996; De Bie *et al.* 1996; Besnard *et al.* 2011), predominantly in Balb/c mice. This evidence has however been largely ignored in the field, and most groups still use adjuvants in their OVA models despite multiple reports citing the requirement of adjuvant as a downside of the OVA model (Renz *et al.* 1992; Stevenson & Belvisi 2008).

It is generally accepted that OVA models require systemic sensitisation; however because atopy in asthma is directed towards the lungs, there is an increased belief that systemic sensitisation is less clinically relevant than other topical routes. In asthma in the

clinic, allergens are likely to be encountered in the lungs or the skin, this means that the APCs populations stimulated by systemic sensitisation may induce a different immune response to that observed in the clinic (Gregory *et al.* 2009). Entirely topical OVA models have been reported in Balb/c mice (Larsen *et al.* 1992; Renz *et al.* 1992, 1993; Saloga *et al.* 1993; Hamelmann *et al.* 1997*b*) which require neither exogenous adjuvant, nor systemic delivery however others have shown that while topical OVA initially induced IgE production this response abrogated with time (Holt *et al.* 1981). Another group compared different routes for sensitisation to OVA in Balb/c mice and found that intranasal application of OVA lead to higher levels of OVA-specific IgE than intraperitoneal application (Nelde *et al.* 2001). However the intranasal approach has not been adopted in the field.

1.5.2.1.1. Tolerance to chronic OVA models

The development of tolerance in chronic OVA models has been repeatedly reported in both C57Bl/6 and Balb/c mice (Swirski *et al.* 2002; Van Hove *et al.* 2007) and is another practical disadvantage to using OVA-driven models. Typically BAL macrophages and lymphocytes; BAL and lung tissue eosinophils; total and OVA specific IgE, and AHR to MCh can be observed after acute OVA challenge (e.g. 2 weeks) in mice sensitised with OVA plus adjuvant, however this is abolished after chronic challenge (6-8 weeks) (Yiamouyiannis *et al.* 1999). Another group showed that prolonged OVA challenge resulted in a suppression of AHR; airway eosinophilia and markers of a Th2 type response; and OVA-specific IgE, whilst remodelling markers (goblet cell hyperplasia and airway fibrosis) were maintained (Sakai *et al.* 2001). Tolerance occurred despite persistent plasma immunoglobulin levels, suggesting development of local tolerance (Yiamouyiannis *et al.* 1999; Swirski *et al.* 2002; Schramm *et al.* 2004; Van Hove *et al.* 2007). Interestingly although adjuvants may promote sensitisation they may not prevent the development of tolerance in chronic models as Alum was utilised in several of the above studies. Tolerance may depended on continuous OVA challenge, as discontinuous allergen challenge failed to induce tolerance (Yiamouyiannis *et al.* 1999; Schramm *et al.* 2004). Tolerance has been attributed to suppressor cells directed against OVA-specific IgE which tolerise mice to subsequent systemic exposure (Holt *et al.* 1981). Tolerance to inhaled allergen may be mediated through pulmonary DCs and IL-10 production (Akbari *et al.* 2001). Antigen-specific γδ T cells have also been implicated in tolerance to OVA, possibly mediating suppression of OVA-specific $CD4^+$ Th2 cell proliferation and reduction of OVA-specific IgE (McMenamin *et al.* 1994). Regulatory $CD4^+TGF\text{-}\beta^+Foxp3^+T$ cells and plasmacytoid DCs have been implicated in the generation of tolerance in the absence of sensitisation (Ostroukhova *et al.* 2004; de Heer *et al.* 2004).

1.5.2.1.2. Remodelling in OVA models

OVA models have been criticised for failing to induce chronic remodelling events characteristic of clinical asthma (McMillan & Lloyd 2004; Fulkerson *et al.* 2005; Ahn *et al.* 2007; Wegmann 2008) however several chronic OVA models which result in remodelling have been described (Temelkovski *et al.* 1998; McMillan & Lloyd 2004; Kumar *et al.* 2004; Fulkerson *et al.* 2005; Yang *et al.* 2005; Wegmann 2008; Fernandez-Rodriguez *et al.* 2008); these models appear to overcome the tolerant effects described above. Notably (McMillan & Lloyd 2004) characterised a prolonged OVA model to try to circumvent the issues associated with previous acute models. In this model mice were systemically sensitised with OVA and Alum and then received numerous aerosolised challenges out to 55 days post first sensitisation. This protocol resulted in airway remodelling characterised by collagen deposition, smooth muscle and GC hyperplasia, which was persistent even after cessation of OVA challenge (McMillan & Lloyd 2004).

Our group currently utilises a model in C57Bl/6 mice based around a Balb/c model which they have published on previously (Birrell *et al.* 2003), in addition a similar model is used in house to generate the LAR in the same strain (Raemdonck *et al.* 2012). In this thesis I will utilise both of these models to investigate the effect of CS co-exposure.

Although the OVA model has provided great progress in the understanding of asthma, there are criticisms of its use. Firstly OVA is not particularly clinically relevant because it is not a clinical cause of asthma. Secondly, as I have described above in most cases systemic sensitisation and the use of an adjuvant is required to generate allergic sensitisation in the models. It is suggested that this does not replicate the sensitisation to airborne allergens which occurs in human asthmatics and I will discuss this in more detail below. Because of this an alternative model utilising a more clinically relevant allergen was sought; thus the HDM model was developed.

1.5.2.2. HDM model

Although house dust mite is a clinically relevant allergen, shown to induce positive skin prick test responses in approximately 80% of asthmatic patients (Smith *et al.* 1969), it has only been in the last 20 years or so that the trend in the field has been to switch to the use of HDM in preclinical asthma models rather than the more traditional OVA models.

HDM extract is most commonly used, containing a mixture of multiple allergenic proteins. Der p 1 and 2 have been shown to be the major allergens contained in HDM

extracts, and 50-70% of Der p directed IgE allergens taken from sera from HDM allergic patients has been shown to be directed towards Der p 1 (Chapman & Platts-Mills 1980).

Protocols used for HDM driven models are typically different to those used for OVA, with multiple topical instillations over several weeks being favoured over systemic sensitisation followed by separate challenge phases. The advantages of these HDM driven models for the investigation of asthma are: an adjuvant is not required to induce a Th2 type response, there is no need for systemic sensitisation prior to challenge, and that eosinophilic inflammation can be maintained with chronic exposure (Johnson *et al.* 2004). Chronic instillation of HDM (usually 5 times per week for multiple weeks) has been shown to cause chronic inflammation and accompanied structural remodelling (Johnson *et al.* 2004; Southam *et al.* 2007), and Th2 driven pathology (including IL-4, IL-5 and IL-13 release) (Gregory *et al.* 2009). Th2 cell and eosinophil recruitment into the lung, and development of increased airway resistance are also reported (Gregory *et al.* 2009). Furthermore increased levels of total IgE (Johnson *et al.* 2004; Cates *et al.* 2004) and HDM specific IgG1 (Cates *et al.* 2004) have been detected in plasma in HDM models after multiple HDM challenges. Another study showed purely intranasal HDM dosing to cause AHR, and influx of eosinophils, $CD4^+$ and $CD8^+$ T cells, Th17 cells, γδ T cells and Tregs into the lungs/airways (Gregory *et al.* 2009).

Airway inflammation in a 5 week intranasal HDM model in Balb/c mice was sensitive to treatment with both topical and systemic steroids (Ulrich *et al.* 2008) showing the inflammation to be relevant to that observed in clinical asthma however few further studies have documented the response of HDM models to asthma-relevant treatments.

Although entirely topical HDM models are favoured, some have been described in the literature which utilise systemic sensitisations. A HDM model was described early on utilising systemic sensitisation to HDM plus Alum followed by intranasal HDM challenge. This model resulted in HDM-specific IgE and airway eosinophilia (Clarke *et al.* 1999) but this has not really been followed up. Another group utilised systemic (i.p) sensitisation to recombinant Der p 1 plus Alum to induce increases in serum total IgE and Der p 1 specific IgE, IgG1 and IgG2a and 2b (Kikuchi *et al.* 2006). This group also showed that the proteolytic activity of Der p 1 was crucial for sensitisation in this model (induction of HDM-specific IgE, IgG1 and IgG2a and 2b (Kikuchi *et al.* 2006). Another model has been reported where i.p, sensitisation with HDM without adjuvant on days 0 and 7, followed by a single topical HDM challenge on day 14 induced airway eosinophilia and production of Th2 cytokines which was greater in C57Bl/6 mice than Balb/c mice. However in this model AHR to MCh was only detected in Balb/c mice (Kelada *et al.* 2011). A further group utilised systemic sensitisation to HDM in C57Bl/6 mice using a single i.p. administration of purified

Der p 1 followed by aerosolised HDM extract on days 14-20. Sensitisation alone induced increased HDM-specific IgE, while the combination of sensitisation and challenge resulted in airway eosinophilia and AHR but again this model has not been adopted in the field.

1.5.2.2.1. Processes driving allergic sensitisation to HDM

In general, the following properties of an allergen are thought to be important in the activation and migration of APCs such as DCs and the subsequent generation of an immune response: production of and/or detection of a danger signal through activating a PRR such as TLR; allergen proteolytic activity, or indirect activation of DCs via activation of structural cells such as epithelial cells.

1.5.2.2.1.1. Proteases and epithelial barrier disruption

As to how HDM allergens promote allergenicity, most of the evidence surrounds the function of group 1 allergens (Der p 1 and Der f 1) as serine and cysteine proteases (Chua *et al.* 1988; Ino *et al.* 1989; Ando *et al.* 1991; Stewart *et al.* 1991; Dilworth *et al.* 1991; Hewitt *et al.* 1997). This protease activity may promote the allergenicity of HDM by inducing epithelial barrier dysfunction and increasing epithelial permeability; causing disruption to epithelial intercellular tight junctions by cleavage of the tight junction protein, occludin or by inducing E cadherin delocalisation (Herbert *et al.* 1995; Wan *et al.* 1999*a*, 2000; Heijink *et al.* 2010; Post *et al.* 2012). The epithelium usually acts as a barrier for inhaled pathogens, so this epithelial disruption may promote the passage of allergens across the epithelium providing a mechanism as to how allergens cross the epithelial barrier and induce allergy. Asthmatic bronchial epithelial cells are permeable to allergens, enabling allergens to access and activate the DCs below epithelial surface in asthmatics (Mori *et al.* 1995) culminating in activation of adaptive immune processes and ultimately in allergy.

1.5.2.2.1.2. Protease activity and cell surface markers

In addition to cleaving epithelial tight junctions, the proteases found in allergens such as HDM may cleave cell surface markers. Through cleavage of CD23 on B cells, HDM may cause increased production of IgE; CD23 on B cells is involved in the negative regulation of IgE production (Sherr *et al.* 1989; Flores-Romo *et al.* 1993; Yu *et al.* 1994; Schulz *et al.* 1995, 1997; Hewitt *et al.* 1995). Der p 1 administration in mice results in total and Der p 1-specific IgE production, which is dependent on the protease activity (Gough *et al.* 1999), therefore the proteolytic activity of HDM may indeed promote allergenicity through IgE production (Shakib *et al.* 1998). Der p 1 also cleaves CD25 (IL-2 receptor) from human peripheral blood T cells, and as the IL-2R is important in generation of Th1 cells, this may mean Der p 1 in HDM can promote the Th2 environment of asthmatics (Schulz *et al.* 1998). Der p 1 may promote a Th2 environment by altering the balance between IL-4 and

IFN- γ production (Comoy *et al.* 1998; Ghaemmaghami *et al.* 2002) or by promoting loss of Th1 activity through cleavage of C-type lectins on DCs (Furmonaviciene *et al.* 2007). This was found to be through cleavage of CD40 on DCs resulting in reduced production of IL-12.

1.5.2.2.1.3. Proteases and cytokine release from epithelial cells

The next theory involves the role of protease activity in house dust mite in promoting cytokine release from epithelial cells via PAR-2 receptor activation; the cytokine environment is known to promote and polarise adaptive immune responses. PAR-2 stimulation promoted DC uptake of antigen (OVA) and DC migration; Th2 sensitisation, airway inflammation and AHR in response to allergen challenge (Ebeling *et al.* 2007). PAR-2 activation by HDM allergens causes release of cytokines such as GM-CSF, IL-6, IL-8 and eotaxin from epithelial cell types which may promote and polarise the adaptive immune system (King *et al.* 1998; Sun *et al.* 2001; Asokananthan *et al.* 2002; Kauffman *et al.* 2006). However others have shown HDM allergens to induce cytokine release from airway epithelial cells via protease independent mechanisms, such as Der p 1-induced IL-8 release (Kauffman *et al.* 2006; Adam *et al.* 2006). The role of proteases in sensitisation to allergens are not restricted to HDM however; the protease activity in Aspergillus allergens has been shown to be required for AHR, BAL eosinophilia and allergen-specific Th2 responses in response to inhaled Aspergillus in mice (Kheradmand *et al.* 2002).

1.5.2.2.1.4. Direct effect on DCs, basophils, mast cells and T cells

In addition to the effects on epithelial cells HDM allergens have also been shown to directly affect other cell types including dendritic cells, basophils and mast cells and T cells causing release of asthma relevant mediators such as IL-5, IL-5 and IL-13 (Hammad *et al.* 2001; Phillips *et al.* 2003). Der p 1 treatment of DCs from asthmatic patients resulted in an increased capacity of DCs to induce T cell proliferation through expression of CD86 (Hammad *et al.* 2001) and release of Th2 chemokines CCL17 (TARC) and CCL22 (MDC) which implies a role for HDM activated DCs in promoting a Th2 response (Hammad *et al.* 2003). Mast cells can also be directly activated by HDM (Der f extract) and were important for Der f mediated allergic asthma in mice. This was thought to be via activation of monocytes and promotion of Th2 responses, Th2 cell differentiation and differentiation (Yu & Chen 2003). Together all of these effects promote antigen presentation and lead to the generation of a Th2, IgE production and eosinophilia which are typical of asthmatics (Phillips *et al.* 2003).

1.5.2.2.1.5. TLR activation

One signalling pathway which has received much attention is that of the Toll-like receptors. Der p 2, a major allergenic component of HDM has been shown to possess structural and functional homology with MD-2 (the LPS binding component of TLR4). Thus Der p 2 may have auto-adjuvant properties by facilitating activation of TLR4 (Trompette *et al.* 2009). The TLR pathway has been implicated in HDM-induced eosinophilia, Th2 responses, AHR and the recruitment and maturation of mDCs in the lung (Phipps *et al.* 2009) and also in the migration of DCs and IL-4 competent basophils to the draining mediastinal lymph nodes (Hammad *et al.* 2010). Airway response to HDM may be mediated through activation of TLR4 on epithelial cells, resulting in the release of TSLP, GM-CSF, IL-25 and IL-33 which go in to interact with DCs to promote Th2 immunity and asthma-like responses (Hammad *et al.* 2009).

1.5.2.2.1.6. Other mediators and pathways

Other factors implicated in the effects of HDM include GM-CSF, cysteinyl-leukotrienes, Dectin-1 and -2 and spleen tyrosine kinase (Syk) (Cates *et al.* 2004; Barrett *et al.* 2009; Nathan *et al.* 2009). For example GM-CSF may drive Th2 sensitisation to intranasal HDM (Cates *et al.* 2004). HDM extracts can release cysteinyl-leukotrienes from dendritic cells via activation of Dectin-2 receptors and subsequent activation of Syk (Barrett *et al.* 2009). Considering Cysteinyl leukotrienes are potent bronchoconstrictors, inflammatory mediators and may control Th2 inflammation (Dahlén *et al.* 1980; Weiss *et al.* 1982; Laitinen *et al.* 1993; Kim *et al.* 2006) they are a strong contender for mediating some of the effects of HDM in asthma and asthma models. A further pathway involves the induction of release of CCL20 (MIP-3α) – a chemokine for immature DCs—from human from epithelial cells. This is stimulated by β-glucan moieties on HDM through activation of Dectin-1 and Syk (Nathan *et al.* 2009)

Mediators such as TSLP, GMCSF, IL-25 and IL-33 are innate Th2 promoting cytokines which have been implicated in activation of DCs in asthma and asthma models (Willart *et al.* 2012). TSLP (produced from the airway epithelium) has been implicated in airway inflammation, airway remodelling and AHR in a HDM model (Chen *et al.* 2013). IL-25 has been implicated in driving TSLP and IL-33 production in the lung and as such may drive remodelling, inflammation and AHR in a house dust mite model (Gregory *et al.* 2013). In contrast others have shown that IL-33 but not TSLP or IL-25 was important for mite induced allergic asthma and Th2 responses through induction of DC OX40L (Chu *et al.* 2013). A model has been proposed whereby TLR stimulation of epithelial cells resulted in IL-1α release. This caused autocrine release of GM-CSF and IL-33 which can activate and

attract DCs. In contrast a role for TSLP was only observed at high HDM doses (Willart *et al.* 2012).

1.5.2.2.2. Acute HDM

The murine topical HDM model system has been shown to induce several desirable features of an allergic asthma model; however data published by our group (De Alba *et al.* 2010) suggests that inflammation with a similar profile to that observed in asthma can be induced directly by a single, topical HDM dose, without any prior exposure. This draws into question the allergic nature of the response in these chronic models. It is possible that some of the phenotypes observed are built up as a result of multiple acute inflammatory insults and although the responses are similar to that which is described in asthma, the responses may not be allergically mediated (De Alba *et al.* 2010; Birrell *et al.* 2010).

One aim of this project is therefore to develop a model of HDM-induced allergic asthma where sensitisation with HDM is able to induce a clear increase in IgE, prior to inducing an allergic response through HDM challenge. As part of this model development I will compare the efficacy of sensitisation to HDM through different dosing routes. Dogma in the field is that asthmatics become sensitised to HDM and other aeroallergens through the airways and that topical sensitisation is therefore preferable (Renz *et al.* 1992). However there is evidence to refute this claim. There are several other ways in which patients may become sensitised and ultimately develop asthma. These include in-utero sensitisation, or as a result of early atopic dermatitis and the atopic march which I will now describe.

1.5.3. Prenatal sensitisation

Prenatal exposure to allergens such as HDM, cats and dogs is associated with increased cord blood IgE and increased systemic IgE 3 days after birth (Schönberger *et al.* 2005; Peters *et al.* 2009). Maternal total IgE is also associated with cord blood total IgE (Peters *et al.* 2012). Maternal exposure to Der p 1 affected the immune profile of cord blood cells and was associated with increased development of atopic dermatitis in offspring within 1 year of life (Hagendorens *et al.* 2004). Mononuclear cells from cord blood have been shown to proliferate in response to common indoor allergens such as cockroach, Der p and Der f and this occurred even in the absence of antigen-induced cell proliferation in maternal blood (Miller *et al.* 2001). Furthermore Der p 1 has been found in amniotic fluid and cord blood, thus transamniotic or transplacental allergen exposure may be a mechanism by which in utero sensitisation to allergens such as Derp1 may occur (Holloway *et al.* 2000). In addition Der p specific IgE has been detected in cord blood, and in those infants with atopic mothers this is associated with an increased development of allergic disease at 3 years of age

(Nambu *et al.* 2003). Altered (Th2) cytokine profile in the blood of pregnant women has been postulated as a mechanism as to how maternal allergy may increase risk of atopy in infants (Breckler *et al.* 2010). Ineffective conversion of the predominant Th2 immune profile in early life due to reduced Th1 capacity may also be involved in the development of atopy (Prescott *et al.* 1998*a*). These studies suggest that even in the absence of inhaled allergen exposure infants may be predisposed to sensitisation or have the beginnings of atopy and Th2 skewed immune responses at birth.

1.5.4. Atopic march

Another theory surrounding allergic sensitisation in asthma surrounds the atopic march. This is a phenomenon noted in certain atopic individuals whereby atopic dermatitis (AD) is developed early on, followed by allergic rhinitis and subsequently atopic asthma (the atopic triad). In these cases it is highly likely that sensitisation may be systemic, rather than through airway exposure (Spergel 2010). The atopic march has been documented in several clinical studies where young children with atopic dermatitis have increased risk of subsequently developing allergic rhinitis and or asthma (Gustafsson *et al.* 2000; Ricci *et al.* 2006; van der Hulst *et al.* 2007; Kapoor *et al.* 2008). Evidence of the atopic march has also been observed in several animal models (Spergel *et al.* 1998; Lee & Flavell 2004; He *et al.* 2007; Jiang *et al.* 2012). TSLP, expressed in high levels in the skin of children with atopic dermatitis (Lee *et al.* 2010) and is important for activation of DCs and induces allergic inflammation via the interaction of epithelial cells and dendritic cells (Liu 2006). TSLP may also drive the atopic march from AD to asthma (Demehri *et al.* 2009; Leyva-Castillo *et al.* 2013). A model has been proposed whereby mutations in fillagrin – a component important for formation of skin barriers – lead to penetration of allergens through the epidermis. This leads to increased TSLP expression and interaction with APCS such as DCs culminating in Th2 sensitisation, and Th2/Th17 driven airway sensitisation (Palmer *et al.* 2006; Spergel 2010).

Considering both the evidence concerning in utero sensitisation and that concerning the atopic march, it may well be that airway sensitisation may not in fact be involved in allergic asthma in all cases. Thus as is yet to be established, the trend to opt for solely intranasal models of asthma may not be appropriate. Until such a time as this is fully established, it is important to utilise models which generate the most disease appropriate endpoints, rather than focusing on the sensitisation methods perceived to be the most relevant.

1.5.5. Modelling allergen-induced bronchoconstriction: EAR and LAR

Thus far the models I have described above concentrate on features of asthma such as allergen-specific immunoglobulin production, AHR, pulmonary inflammation and remodelling. Allergen-induced bronchoconstriction, both the early and the late response, are important features of allergic asthma which are largely overlooked in murine asthma models. This may be reflected in the difficulty in obtaining these endpoints in mice. However both early and late bronchoconstriction have been reported in murine allergen models (Cieslewicz *et al.* 1999; de Bie *et al.* 2000; Crosby *et al.* 2002; Nabe *et al.* 2005). In one model both an early and a late response (Cieslewicz *et al.* 1999) is generated in C57Bl/6 mice after systemic sensitisation to OVA plus Alum followed by aerosolised OVA challenge. This resulted in an EAR 5-30 minutes after challenge, followed by an LAR which peaked at 6 hours after challenge and was associated with AHR and airway eosinophilia. The EAR was dependent on B and T cells and IgG but not IgE or mast cells, so the pathogenesis may contrast with that observed in asthmatics (Crosby *et al.* 2002), whereas the LAR was driven by IL-5 and eosinophilia (Cieslewicz *et al.* 1999). Another group also published a model in Balb/c mice which generated the EAR and LAR, this time following systemic sensitisation with OVA plus Alum followed by 4 intratracheal doses of OVA (Nabe *et al.* 2005). In contrast despite recording an EAR in Balb/c mice, (de Bie *et al.* 2000) failed to observe an LAR in an adjuvant-free OVA model, even when AHR and pulmonary eosinophilia was maximal. To my knowledge, none of the recently developed HDM-driven murine models of asthma have been shown to generate either an early or a late response. This is an important point as it casts further doubt over the allergic nature of the HDM models; however it is possible that nobody has yet looked for these features as most of the publications have aimed to further understand the mechanisms driving inflammation and AHR in these models.

1.5.6. Adjuvants

Adjuvants have been widely used for almost a century in vaccinations to aid the development of immunity, and are now widely used in preclinical models of allergic disease to aid sensitisation. Common adjuvants contain aluminium salts, such as aluminium hydroxide or a mixture of aluminium hydroxide and magnesium hydroxide; these are often referred to as Alum. Adjuvants promote the adaptive immune system, including APC uptake of allergen, and differentiation and proliferation of T cells (Mannhalter *et al.* 1985; Grun & Maurer 1989) which in turn promotes B cell production of immunoglobulins. Adjuvants such as Alum generally promote Th2 type responses typified by IgE production and eosinophilia; this explains their historical use in models of allergic asthma. Despite being used in human vaccines so widely their mechanism of action is poorly understood, however some mechanisms have been suggested which I will describe below. Different adjuvants are

likely to have different mechanisms of action (Grun & Maurer 1989). Alum is thought to induce a Th2 response whereas other adjuvants such as CFA are less polarising (Comoy *et al.* 1998). Here I will concentrate on Alum, as this is the adjuvant used in-house in the pre-clinical asthma models.

The depot theory is the oldest theory surrounding the mechanism of action of adjuvants. According to this theory adjuvants form a depot from which allergens are slowly released to enable uptake by APCs and subsequent allergen presentation. To support this there is a suggestion that the effect of adjuvants can be transferred between mice by transplanting Alum precipitates from one mouse to another (Kool *et al.* 2012); DCs and allergen-specific T cells can be found around these alum precipitates (Kool *et al.* 2012). However others have shown that while adjuvants may form depots *in vivo*, depot formation may not be important for the immunogenic effects of adjuvants. Signals for antigen presentation by B cells and DCs predominantly occur between 6 and 24 hours after adjuvant-allergen injection and removal of the adjuvant-alum injection site and the adjuvant depot 2 hours after administration failed to affect allergen-specific T and B cell responses. This suggests that prolonged release of allergen is not important (Hutchison *et al.* 2012). Another group also corroborated this lack of involvement using mice deficient in fibrin, an essential component of adjuvant nodules; normal T cell and antibody responses to immunization with allergen plus adjuvant occurred in these mice (Munks *et al.* 2010).

Adjuvants may act at the level of antigen presentation (Mannhalter *et al.* 1985) and DCs are likely to be vital for the allergenic effects of Alum (Mannhalter *et al.* 1985; Kool *et al.* 2008*a*). Adjuvants can promote monocyte differentiation into dendritic cell phenotypes including increased expression of: MHC-II, ICAM-1; CD40, CD83 and CD86, co-stimulatory molecules on APCs which trigger T cell activation; CD58, a cell adhesion molecule on APCs; which together will promote antigen presentation (Ulanova *et al.* 2001; Seubert *et al.* 2008). Alum also promotes IL-4 expression which may drive the increase in MHC-II expression (Ulanova *et al.* 2001). From this data a mechanism of action was proposed whereby Alum activates T cells to produce IL-4; this in turn promotes increased MHC-II expression on monocytes and thus promotes their APC capabilities (Ulanova *et al.* 2001).

Another theory surrounding the inflammatory effects of adjuvants has been the most widely investigated. There is strong evidence from both *in vitro* and *in vivo* studies that Alum can induce innate inflammatory responses and that this inflammation may promote recruitment of APCs to take up the allergen. Several studies have now shown that Alum can induce release of inflammatory mediators such as IL-1β and IL-18, and chemokines such as

IL-5 (Mannhalter *et al.* 1985; Martinon *et al.* 2006; Li *et al.* 2008; Franchi & Núñez 2008; McKee *et al.* 2009; Kuroda *et al.* 2011) and local inflammatory responses consisting of neutrophils, eosinophils, monocytes and DCs recruited to the site of Alum injection within 2 hours (McKee *et al.* 2009). IL-1β is an Alum responsive cytokines which is likely to be involved in the recruitment of inflammatory cells to the injection site and has been highlighted for a role in allergenicity (Huber *et al.* 1998); Alum-induced T cell proliferation was dependent on the release of IL-1 and IL-4 production (Grun & Maurer 1989). How alum activates innate inflammatory responses and whether this is involved in the resultant adaptive response is controversial, but the prevailing theory is based on the immunological response to organic molecules such as LPS which are known to possess adjuvant activity (Eisenbarth *et al.* 2002). Their effects are likely to be mediated through receptors which detect PAMPS (pathogen associated molecular patterns) and DAMPS (damage associated molecular patterns) – TLR and Nod-like (NLR) receptors respectively – and subsequent inflammasome-mediated release of inflammatory cytokines. It may be that adjuvants such as Alum may utilise similar pathways to promote immunity. Alum has been shown to cause production of mature IL-1β, IL-18 and IL-33 from human macrophages and IL-1β from DCs via activation of the inflammasome (caspase-1, NLRP3 and ASC) (Li *et al.* 2008; Hornung *et al.* 2008). Furthermore Alum-induced IL-1β release and recruitment of inflammatory leukocytes into the peritoneum, along with DC-induced Ag-specific T cell expansion and antigen-specific antibody production are driven by the NLRP3 inflammasome (Eisenbarth *et al.* 2008; Li *et al.* 2008; Kool *et al.* 2008*b*). Thus there is substantial evidence for the role of the inflammasome and mediators such as IL-1 in the adjuvant effects of Alum. In addition the NLRP3 inflammasome and IL-1β may also be involved in the generation of Th2 immunity in an adjuvant free OVA model (Besnard *et al.* 2011). Uptake of an adjuvant by DCs causes NLRP3 activation, and IL-1β secretion. However, an additional TLR agonist was required for IL-1β to be produced *in vitro*, whereas *in vivo* this was not required (Sharp *et al.* 2009). This interestingly implies that *in vivo*, Alum induces production of endogenous factors which activate the inflammasome (Sharp *et al.* 2009). Aluminium salt-induced activation of the inflammasome involved phagocytosis of the salt crystals causing lysosomal damage; this may mimic cell damage and act as a danger signal (Hornung *et al.* 2008). Uric acid (UA) is an endogenous danger signal which is released from dying cells, and these necrotic cells are found at Alum injection sites (Goto & Akama 1982, 1984; Goto *et al.* 1997). When released from dying cells UA can activate DCs and promote T cell responses to antigen presentation (Shi *et al.* 2003) and has been shown to activate the NLRP3 inflammasome resulting in IL-1β and IL-18 release from macrophages (Martinon *et al.* 2006). *In vivo*, Alum injection induced UA release in the peritoneum, activation and migration of DCs, antigen presentation and T cell proliferation; these events were abolished

by uricase treatment (Kool *et al.* 2008*a*). Uric acid is not just found in the peritoneum; it can also be detected in the airways following allergen challenge in asthmatics and mice (Kool *et al.* 2011). The UA-inflammasome-IL-1β axis is now being described as a probable mechanism of action of adjuvants (Kool *et al.* 2011), but may also be important in Th2 responses in the absence of adjuvant, possibly playing a role in asthma and murine models of asthma (Kool *et al.* 2011).

Despite the above work, there is also evidence to contradict this mechanism of action; other studies have suggested that NLRP3 and ASC are important for Alum-induced IL-1β production, but not the adjuvant properties of Alum (Franchi & Núñez 2008). In addition to a lack of role for the NLRP3 inflammasome others showed that the adjuvant activity of Alum (Th2 and antibody responses) was not dependent on mast cells; macrophages or eosinophils; or IL-1β (McKee *et al.* 2009). Furthermore others showed that adjuvant effect was not through the NLRP3 inflammasome or IL-1/Myd88 but rather through spleen tyrosine kinase and PI3Kδ signalling which activated DCs (Kool *et al.* 2011). In support of this the combination of Alum and OVA was shown to induce the production of extracellular ATP – another DAMP which can promote Th2 immunity (Idzko *et al.* 2007); however this was shown not to contribute to Alum-induced Th2 immunity (Kool *et al.* 2011). Thus it may be that NLRP3 mediated innate immune responses may be dissociated from the mechanism which promotes adaptive immunity. Other inflammasome independent mechanisms have been suggested: Alum may stick to dendritic cells and – by altering the DC membrane lipid composition through lipid sorting – promote high affinity binding of DCs to $CD4^+$ T cells via ICAM-1 and LFA-1 (Flach *et al.* 2011). PGE_2 production, which is inflammasome independent, may also control the adjuvant induced antibody response (Kuroda *et al.* 2011).

While there has been a flurry of publications in the last few years aiming to elucidate the mechanism behind the adjuvant activity of Alum, the results are far from conclusive.

1.5.7. The effect of CS in murine allergen models

A few groups have investigated the effect of combining allergen-driven models with CS exposures; the majority of the work has been conducted with the OVA model. A collection of approximately 30 papers have been published on this topic in the last 20 years with only 2 utilising the more clinically relevant allergen – HDM. Despite this work the field does not appear to have reached a general consensus on the effect of CS in these models.

Even within a single research group CS has been shown to have quite different effects. The disparate results observed in the various publications are likely to result from the variations in the protocols used. I will now discuss the existing data in murine OVA models. Several papers have investigated the effect of *in utero* CS exposure on asthma development; however in this thesis I am interested in the effect of CS on the response to allergen exposure. These *in utero* papers are therefore beyond the scope of this thesis and will not be discussed.

1.5.7.1. CS and OVA co-exposure

The first of these studies was published in 1997 by Seymour et al. In this study they found that second hand smoke resulted in increases in serum total IgE and OVA-specific IgG1; blood and BALF eosinophil levels; and IL-4 and IL-10 compared to levels observed in mice exposed to OVA alone (Seymour *et al.* 1997). In this study they proposed the increase in IL-4 and IL-10 as a mechanism for the enhancing effects of CS in the OVA model. Subsequently Rumold et al. (2001) determined whether second hand smoke could induce sensitisation in mice exposed to OVA (in the absence of an additional adjuvant). ETS plus OVA resulted in increased serum OVA-specific IgE and IgG1, which did not occur after OVA alone. OVA recall challenge *in vivo* resulted in increased BAL eosinophils, IL-5, GM-CSF and IL-2 only in OVA sensitised mice which were exposed to ETS. This shows clearly that ETS can facilitate sensitisation to an allergen which on its own was innocuous. Furthering this idea, an attempt was made to determine whether ETS could overcome the normal tolerance to inhaled allergen. The three strains compared – Balb/c, C57Bl/6 and A/J – showed different sensitivities for immunoglobulin production in response to OVA. However ETS was unable to enhance antibody production, airway inflammation or AHR (Bowles *et al.* 2005) in any of the strains. This study failed to show any adjuvant properties of CS on solely inhaled allergen.

In OVA-sensitised Balb/c mice mainstream CS co-exposure caused a small increase in inflammation and the appearance of AHR compared to OVA challenge alone (Moerloose *et al.* 2005). In this study OVA alone resulted in increased BAL eosinophils whereas CS alone resulted in increased BAL neutrophils; when the two challenges where combined there was a further increase in eosinophilia and a large increase in the levels of BAL macrophages. OVA challenge alone did not result in AHR, however AHR to i.v. carbachol was observed in OVA challenged mice exposed to CS. There was also a non-significant trend towards increased OVA-specific IgE after CS and OVA co-exposure compared to OVA alone. A year later the same group reported CS to facilitate allergic sensitisation in an OVA model involving repeated aerosolised OVA challenge without any prior sensitisation or adjuvant (Moerloose *et al.* 2006). OVA alone induced a small increase in OVA-specific IgE, but no

inflammatory response was observed. Where CS was combined with OVA, significantly elevated levels of OVA-specific IgE, BAL eosinophils, lymphocytes and DCs; lung tissue DCs, $CD4^+$ and $CD8^+$ T lymphocytes; and airway goblet cells were observed. This response was also associated with an increase in IL-5. The normal homeostatic tolerance mechanisms that prevent a robust response to aerosolised OVA alone appeared to be disrupted by CS co-exposure. This group has also published that repeated aerosolised OVA challenge will result in tolerance after 8 weeks. In this model of OVA tolerance, CS co-exposure delayed the development of tolerance to OVA (Van Hove *et al.* 2008). Interestingly the neutrophilia induced by 8 weeks of CS was also abrogated in the OVA-tolerised mice which implied that the process of tolerance also dampened the airway response to other inflammatory stimuli. (Van Hove *et al.* 2008).

Another group sought to further understand the inconsistent effects of CS in models and clinical studies (Robbins *et al.* 2005; Trimble *et al.* 2009). Cigarette smoke was reported to cause a 'heightened state of allergen-specific sensitisation, but dampened local immune inflammatory responses in the lung' (Robbins *et al.* 2005). They showed mainstream CS to attenuate airway inflammation in a Balb/c, GM-CSF-adjuvanted aerosolised OVA model (Robbins *et al.* 2005). BAL eosinophils and neutrophils, and lung tissue eosinophils were attenuated in MTS and OVA co-exposed mice compared to OVA alone. There was also a trend for reduction of DCs, activated $CD4^+$ T lymphocytes and Th2 associated lymphocytes in MTS and OVA co-exposed mice (Robbins *et al.* 2005). AHR was reduced in MTS and OVA co-exposed mice, which in this case was likely to be associated with the reduction in inflammatory cells such as eosinophils (Robbins *et al.* 2005). In contrast there was a trend for BAL levels of IL-5, Il-13, eotaxin and IgG2a to be reduced but no effect was observed for IgE or IgG1. Overall the group hypothesised that MTS increased systemic sensitisation through increased production of cytokines by splenocytes (Robbins *et al.* 2005). The same group then proposed that the contradictory data on the effect of CS in OVA models could be explained by cigarette smoke having adjuvant as well as anti-inflammatory properties (Trimble *et al.* 2009). To investigate this hypothesis they combined MTS with their aerosolised OVA model (minus adjuvant). CS and OVA resulted in an increase in BAL eosinophilia and goblet cell hyperplasia after 2 and 7 weeks respectively and neither of these changes were observed in mice challenged with OVA alone. Increased IgE, IgG1 and IgG2a were also reported after CS plus OVA, along with an increase in DCs and activated T cells (Trimble *et al.* 2009). Together this implies that CS conferred adjuvant properties to enable the mice to respond to OVA. However if the cigarette smoke exposure was continued after cessation of OVA challenge and then OVA recall challenge was performed, there was a decrease in OVA-induced eosinophilia compared to the group which was sham exposed

during this period (Trimble *et al.* 2009) – this highlighted the anti-inflammatory effects of CS.

Others have concentrated on the role of ETS on allergic asthma (Min *et al.* 2007): this is of particular relevance when considering TS as an environmental pollutant. ETS enhanced several features of the OVA model including: allergen-induced airway remodelling (smooth muscle thickening, α-smooth muscle actin levels, peribronchial fibrosis, collagen deposition, TGF-β+ cells); BAL and lung tissue eosinophilia; and AHR to MCh, in Balb/c mice (Min *et al.* 2007).

Melgert *et al.* (2004) reported 'short term' CS to attenuate OVA induced airway inflammation in C57Bl/6 mice. CS blocked the OVA-induced increases in BAL eosinophilia, macrophages in lung tissue and AHR but had no effect on immunoglobulin levels. Interestingly none of the endpoints measured in this model were worsened by CS exposure. Subsequently the group then investigated whether a longer term 4 months CS exposure would show any 'negative' effects on the OVA model (Melgert *et al.* 2007). But in this publication again CS did not impact on any of the OVA-induced phenotypes measured in this model.

The way in which a study is set up in terms of the temporal relationship between the allergen and the CS delivery will affect the results obtained in each case. Furthermore the level of CS will also impact on the effect of CS. This was highlighted by Thatcher et al. (2008). In this study a high dose of CS resulted in suppressed OVA responses (BAL eosinophilia, IL-4 and 5, and immunoglobulin responses) whereas a low dose did not. In this case the 'level' of CS was altered in terms of TSP, but the same could be suggested for number or frequency of CS exposures.

Despite clinical evidence that cigarette smoke has a negative impact on treatment efficacy in asthma patients, few studies have assessed the impact of steroid treatment in the CS and OVA co-exposure models described above. Only one study to date – as far as I am aware – has looked at the effect of CS exposure on the responsiveness of allergen-induced endpoints to steroid treatment (Song *et al.* 2009). Here steroids were shown to elicit a significantly significant reduction in multiple OVA-induced endpoints following co-exposure with ETS including: BAL eosinophilia, mucus cell levels, peribronchial fibrosis, smooth muscle thickening and α-smooth muscle acing staining; and AHR (Song *et al.* 2009). Therefore it is suggested that ETS did not impact on the ability of steroids to reduce the asthma-relevant endpoints in this model (Song *et al.* 2009). Unfortunately however, in this publication the steroid treated/OVA challenged/air exposed controls were not included so it is difficult to interpret the effect of cigarette smoke on the response of the OVA-induced endpoints to steroid treatment. Although steroid treatment was shown to be effective against most endpoints in this paper in ETS co-exposed mice, it is impossible to tell whether the

efficacy of steroids against the responses induced by OVA alone would have been greater. This model utilises a 'low level' ETS exposure, which also raises the question as to whether a higher level MTS exposure would have rendered any of these endpoints insensitive to steroid treatment.

1.5.7.2. CS and HDM co-exposure

To date only two publications have investigated the effect of CS exposure on a HDM-driven murine model of asthma. Firstly Balb/c mice were challenged with HDM intranasally for 5 weeks followed by concomitant exposure to CS and HDM. Here CS reduced levels of BAL eosinophilia, B cells, mucus expression, serum HDM-specific IgE and reduced expression of V-CAM and eotaxin-1 expression compared to HDM alone. Therefore it was suggested that the reduction in eosinophils was due to reduced eosinophil trafficking into the lung, as blood eosinophil levels were not affected (Botelho *et al.* 2011). CS also increased features of airway remodelling such as collagen expression compared to HDM alone (Botelho *et al.* 2011).

In a second study (Lanckacker *et al.* 2012) showed that CS exposures for 3 weeks concomitantly with HDM in Balb/c mice enhanced HDM-driven responses including: airway eosinophilia, goblet cell metaplasia and AHR; cytokine release including IL-4, IL-5, IL-13 and IL-10; and levels of serum HDM specific IgG1. In a different protocol where CS was given during sensitisation, CS was shown to facilitate sensitisation by increasing DC-mediated transplant of HDM to lymph nodes, and promoting a local Th2 response (Lanckacker *et al.* 2012). Finally, 2 weeks of HDM and CS co-exposure following 5 initial weeks of HDM challenges resulted in reduced BAL eosinophil levels; reduced B cells and serum IgE; and reduced mucus expressed; however little effect on AHR to MCh was observed. V-CAM1 and eotaxin-1 expression were reduced by CS exposure which was muted as the possible mechanism for the observed reduction in airway eosinophils. Conversely an increase in collagen expression was observed suggesting an enhancing effect of CS on airway remodelling (Lanckacker *et al.* 2012). This study clearly demonstrates how different temporal relationships between the CS and the allergen exposure can result in dramatically different outcomes.

1.6. Thesis aims

To summarise, severe asthma is in important subset of asthma which contributes a large proportion of the medical disease burden; patients with severe disease often have poorly controlled symptoms and poor quality of life. Active cigarette smoking, passive

smoke exposure, or exposure to air pollution may contribute to worsened asthma severity and a reduced response to asthma treatment.

The aim of this thesis is to develop a murine CS and allergen co-exposure model in which the effects of CS in asthma can be investigated. The group already utilises a murine OVA-driven asthma model which will be combined with the group's existing CS exposure model (Eltom *et al.* 2011; Rastrick *et al.* 2013). A HDM-driven murine asthma model will be developed to parallel the existing OVA model, which will also be combined with CS. I will investigate the effect of CS co-exposure on airway inflammation, AHR and the LAR in these models and also the effect of CS on the response of these endpoints to steroid treatment. The hypothesis of this thesis is that CS co-exposure will confer a change in the phenotype of the murine allergen-driven asthma models, and their response to steroid treatment.

Chapter 2. General methods

This chapter provides an overview of the general methodologies used throughout this thesis. More detailed experimental protocols are described in the relevant chapters.

2.1. Animal studies

All work described in this thesis was carried out in male C57Bl/6 mice (18-20g) because the group has extensive colonies of genetic knockout mice raised in this strain – these may be used in the future to evaluate mechanisms involved in the models described in this thesis. Mice were housed for at least 5 days prior to conducting experiments, or bred in-house. Throughout housing and experimental periods, food and water were supplied *ad libitum* and all studies were conducted under the Animals Scientific Procedures Act 1986 UK Home Office guidelines.

2.1.1. Sample harvest

2.1.1.1. Overdose

Mice were euthanized with an overdose (200mg.kg^{-1}) of intraperitoneal sodium pentobarbitone and samples were harvested once a level of terminal anaesthesia had been achieved.

2.1.1.2. Plasma

Heparinised blood samples were obtained by cardiac puncture performed with a syringe containing heparin.

2.1.1.3. Bronchoalveolar lavage

The trachea was exposed by blunt dissection, and cannulated. Bronchoalveolar lavage (BAL) was performed by instilling the lungs with 0.3ml of Roswell Park Memorial Institute 1640 medium + GlutaMAX-1 (RPMI). The RPMI was left in the lungs for 30 seconds, and then removed. This was performed 3 times, and the three samples were then pooled for each animal.

2.1.2. Sample processing

2.1.2.1. Plasma processing

Heparinised blood samples were centrifuged at 2500 rpm for 10 minutes at 4°C. The supernatant (plasma) was then aspirated and stored at -20°C for subsequent analysis.

2.1.2.2. BAL fluid processing

In order to evaluate the level of airway cellular inflammation induced by the various model treatments described in this thesis, the BAL fluid was prepared for total and

differential cell counts. For differential cell counts, microscopy slides were prepared as follows. Briefly, 100µl of BAL sample was spun onto a microscopy slide using a cytospin (Shandon, Runcorn, UK) at 700 rpm with low acceleration for 5 minutes. The slides were then stained and fixed with modified Wright-Giemsa stain using an automated slide stainer (Hema-tek 200, Ames Co, Elkhart, USA). Stained slides were then cover-slipped by hand using DPX.

2.1.2.3. Total cell counts

An automated cell counter (Sysmex UK Ltd, Milton Keynes, UK) was used to obtain total cell counts from samples. Prior to counting samples, the cell counter was calibrated with a reference blood sample containing a known number of white and red blood cells. Briefly, 200µl of neat BAL fluid was mixed with 10ml of Sysmex diluent and then treated with Quicklyser to lyse any red blood cells which may be found in the sample. The total concentration of white blood cells in the sample was then determined using the Sysmex counter.

2.1.2.4. Differential cell counts

Differential cell counts were performed on slides using light microscopy (40x magnification). For each sample 200 cells were counted from at least 2 separate, representative regions of the slide; the percentage of eosinophils, lymphocytes, macrophages and neutrophils within the total population of cells in the sample was determined. The previously determined total counts were then used to determine the total concentration of each cell type in the sample. Standard morphological criteria were used to identify the different cell types as explained below. Figure 2.1 shows illustrations of the morphology of the different cell types.

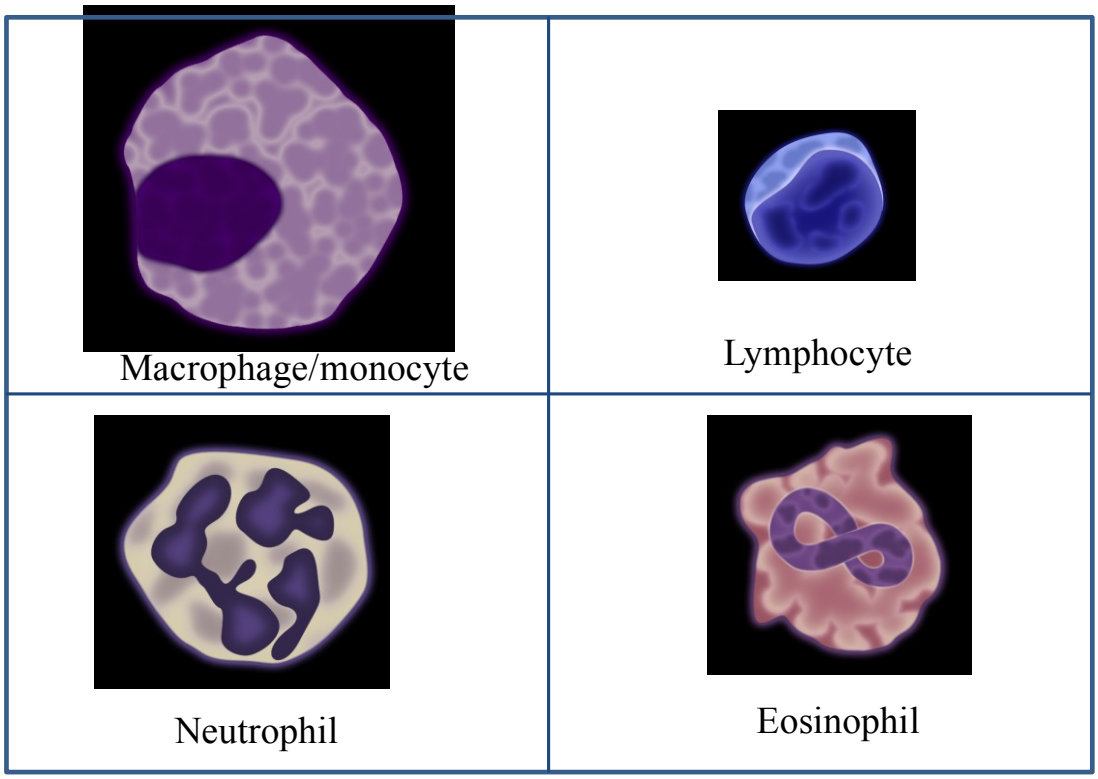


Figure 2.1: Illustrations of the morphology of the different cell types counted in the BAL fluid

2.1.2.4.1. Eosinophils

Eosinophils possess a bi- or multi-lobed nucleus, which often takes on a donut shape or figure-of-8 appearance, and is stained dark blue. They have a granular cytoplasm which stains pink, enabling them to be easily distinguished from other cell types.

2.1.2.4.2. Neutrophils

Neutrophils contain a multi-lobed nucleus, which stains dark blue. Like eosinophils, the cytoplasm of neutrophils is also granular; however the staining of the cytoplasm is very pale.

2.1.2.4.3. Lymphocytes

Lymphocytes are the smallest cell type found in the BAL fluid, and are characterised by having little or no cytoplasm and a single, very darkly stained nucleus.

2.1.2.4.4. Monocytes

Finally, macrophages and monocytes (counted as one cell type) are the largest cell type. They have a nucleus which stains dark blue, and a large region of cytoplasm which is stained a light blue. This gives them their characteristic 'fried-egg'-like appearance.

2.1.3. Saline

Where saline is used for *in vivo* dosing or exposures this refers to endotoxin-free saline (0.9% w/v; Fresenius Kabi, Warrington, UK)

2.1.4. Cigarette smoke exposures

A whole body cigarette smoke exposure system has previously been developed and characterised in house (Eltom *et al.* 2011). This system comprises of a time-set pinch valve (C Lee Machining, Horsham, UK), 136L exposure chambers (Teague Enterprises, CA, USA), an extraction unit (Grainger Industrial Supply, USA) and a total suspended particulate (TSP) sampling unit (Teague Enterprises, CA, USA). A separate exposure system was used for room air and for cigarette smoke exposures to avoid contamination.

Animals were placed in metal cages, inside the exposure chambers, and exposed to room air or cigarette smoke (3R4F cigarettes, Tobacco Health Research Institute, University of Kentucky, Lexington, KY, USA) for 50 minutes, followed by a 10 minute venting period. A negative pressure was generated by the extraction unit (flow-rate set at 1500 ml/min) which drew cigarette smoke into the exposure chamber for 2 seconds followed by 4 seconds of room air, as controlled by the pinch-valve (500ml/min of CS). These settings were previously determined in dose response studies (Eltom *et al.* 2011). A fan was placed at the bottom of the chamber to ensure that the smoke was uniformly distributed throughout the chamber. TSP levels within the chamber were regularly assessed throughout a study (30 minutes into an exposure, 1min sampling period) to validate the consistency of the smoke concentration within the chambers. During the venting period, the flow through the system was increased to maximal flow to clear the smoke from the exposure chamber. After which the mice were removed from the exposure chambers and returned to their cages. Figure 2.2 shows a diagrammatic representation of the CS exposure system.

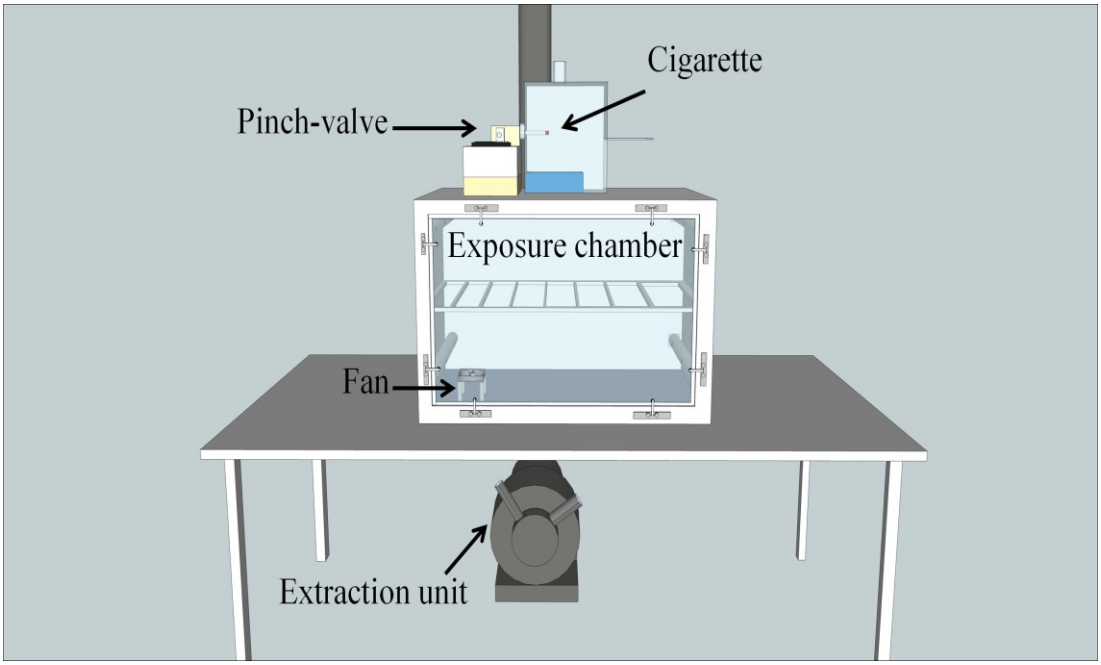


Figure 2.2: Diagramatic representation of the CS exposure system

2.1.5. Oral dosing

Conscious mice were dosed orally with an oral dosing gavage (dose volume 10 $ml.kg^{-1}$).

2.1.6. Allergen models

Ideally, studies should contain 4 control groups: saline-sensitised/saline-challenged, saline-sensitised/allergen-challenged, allergen-sensitised/saline-challenged and allergen-sensitised/allergen-challenged. This is especially important with the HDM model to delineate whether responses are due to an allergic effect in sensitised mice, or due to an innate response to allergen occurring independently of prior sensitisation. This was however not practical for all studies. In these cases, allergen-sensitised/allergen-challenged mice were compared with allergen-sensitised/saline-challenged mice as the control. In studies where CS and either of the allergen-driven asthma models were combined, the term OVA- or HDM-challenged, or CS-exposed mice refers to mice which received the allergen (OVA/HDM) combined with air exposure as a control, or the CS exposure combined with saline challenge respectively.

2.1.6.1. Alum

Throughout this thesis Alum will be used as an adjuvant during sensitisation of mice to either OVA or HDM. Alum refers to 20 $mg.ml^{-1}$ aluminium hydroxide and 20 $mg.ml^{-1}$

magnesium. Where the term "Alum" is used in methods or protocols, including as a vehicle for OVA or HDM, this will refer to Alum diluted 1:1 in saline. Where OVA or HDM is prepared in Alum, this is first made up in endotoxin-free saline at double the required concentration, and then the corresponding volume of Alum is added. Once prepared, both vehicle and OVA- or HDM-Alum solutions will be placed on a magnetic stirrer for at least 1 hour prior to sensitisation.

2.1.6.2. Standard OVA model

An OVA-induced allergic asthma model has previously been developed in-house. In addition a similar, modified model is used for generating the OVA-induced LAR (Raemdonck *et al.* 2012). For clarity, throughout this thesis these models will be referred to as the "standard OVA model" and the "OVA-induced LAR model". These models will be discussed in more detail in the relevant chapters, but the general model protocols are described below.

Standard OVA model

Sensitisation	10µg OVA per mouse in 100µl Alum i.p.	Days 0 and 14
Challenge	50µg OVA per mouse in 50µl of endotoxin free saline i.n.	Days 24, 25, 26

OVA-induced LAR model

Sensitisation	50µg OVA per mouse in 500µl Alum i.p.	Days 0 and 14
Challenge	25µl of 2% OVA in endotoxin-free saline per mouse i.t.	Days 24, 25, 26

2.1.6.3. Intranasal and intratracheal dosing under anaesthesia

Mice were placed in Perspex exposure chambers attached to an anaesthetic machine (Bowring Medical Engineering Ltd, Witney, UK) and exposed to 4% isofluorane in oxygen. For intranasal dosing: once lightly anaesthetised, mice were dosed by dropping small amounts of the dosing solution with a Gilson pipette onto both nostrils until passively inhaled by the mouse (50µl total dose volume). Mice were then monitored until fully recovered from the anaesthesia.

For intratracheal dosing: once completely anaesthetised, mice were dosed into the trachea using a dosing gavage (25µl dose volume). Mice were then monitored until fully recovered from the anaesthesia.

2.1.7. Lung function

2.1.7.1. Measuring Lung function in mouse models

In asthma one of the principal symptoms is shortness of breath. Narrowing of the airways causes an increase in lung resistance and a reduction in airflow, resulting in this characteristic shortness of breath. When modelling asthma it is therefore very important to measure changes in lung function in addition to the more routine endpoints such as airway or lung tissue inflammation.

Measuring lung resistance and compliance is a highly accurate and specific method to assess lung function in animals. This technique however is invasive, requiring anaesthesia, tracheal intubation and artificial ventilation. Thus using this technique lung function is assessed under non-physiological conditions. The technique is also terminal so may preclude further endpoints being assessed in the same animals. In addition our group has previously shown that anaesthesia abolishes the allergen-driven LAR (Raemdonck *et al.* 2012) and as such resistance and compliance is not always a viable technique.

An alternative technique uses whole body plethysmography (WBP) to measure Penh (described below). The advantages of this technique are that it is non-invasive and so can be performed on conscious animals, and that the animals are unrestrained and thus less anxious which allows for more accurate measurements of breathing patterns. Some publications have shown Penh to correlate with airway resistance (Hamelmann *et al.* 1997*c*). However this technique is associated with controversy, with several publications raising concerns over its validity as a technique for measuring respiratory parameters (Adler *et al.* 2004; Bates *et al.* 2004; Sly *et al.* 2005; Lundblad *et al.* 2007). The main arguments against the use of Penh are based on the possible contribution of conditioning and the nasal airways to the respiratory signal. The following section will explain, in brief, the theory behind the derivation of Penh.

2.1.7.2. WBP and Penh

There are two types of plethysmographs, a sealed, pressure whole body plethysmograph (PWBP) and a flow whole body plethysmograph (FWBP) which contains a

pneumotacograph. In a plethysmograph, the total air volume is comprised of the air within the chamber, and the air within in the animal. In PWBP, the waveform is derived from the net change in **air volume** due to air exchange from the chamber to body during respiration. This type of plethysmograph therefore measures the difference between the respired nasal volume (volume drawn into the animal from the chamber) and the thoracic displacement volume. The waveform is affected by two processes, firstly, resistance acting on the respired air, and secondly conditioning: the heating and humidification of respired air as it moves from chamber conditions to body conditions. During inspiration the increase in chest volume is greater than the air removed from the chamber. This results in a **net volume change**, which creates a pressure waveform. The conditioning component dominates the waveform in PWBP. To try to circumvent this, FWBP is used, where the **rate of net volume change** is measured instead or rather the difference between the rate of change of thoracic displacement, and the nasal flow; FWBP will be used for all studies in this thesis. Nasal flow always lags behind thoracic flow: as the thorax expands, there is a delay before air is drawn into the lungs due to airway resistance. This difference is greater in a state of constriction. The difference between the two flows makes up the FWBP waveform. In contrast to the PWBP, in FWBP conditioning is proportional to the animals flow. During a breath cycle, there are regions where flow is zero, for example in the transition between inspiration and expiration (known as zero flow crossings). Here, the conditioning component of the waveform is minimal, and the resistive component dominates. Penh is calculated from the FWBP waveform (concentrating on the zero flow crossings). Penh is "a non-dimensional parameter based on a characteristic change in the expiratory wave shape of the unrestrained plethysmography box signal" (Lomask 2006). In this thesis Penh is used as an arbitrary measure of airway constriction and where possible, measurements using this technique are backed up using resistance and compliance or studies in the isolated trachea.

2.1.7.3. Non-invasive lung function (whole body plethysmography)

Mice were placed in whole body plethysmography (WBP) chambers (Buxco Electronics, Troy, New York, USA). Pressure changes due to the animal breathing were continuously computed by a Buxco XA-analyser (Troy, New York, USA) and enhanced pause (Penh) was derived from the resulting waveform.

2.1.7.4. Late asthmatic response

Mice were placed in the WBP chambers immediately after intratracheal challenge with saline or OVA and monitored for up to 15 hours. Mice were challenged in the evening and the LAR was recorded overnight. Average Penh values were calculated for each 10 minutes of recording.

2.1.7.5. Airway reactivity studies

Mice were placed in WBP chambers and a baseline Penh value was recorded for 5 minutes. Subsequently mice were exposed to aerosolised saline and increasing doses of spasmogen (as detailed in the specific methods in each chapter) generated by an ultrasonic nebuliser (Buxco, Troy, New York, USA). Each spasmogen dose (50µl per chamber) was nebulised over a 15minute period for 5-HT or AMP, or 10 minute period for ACh and MCh. Penh area under the curve was calculated for the 10 or 15 minute nebulisation period for each dose.

2.1.7.6. Resistance and compliance

Mice were anaesthetised with 200µl urethane (i.p. at $2g.kg^{-1}$). Once under a level of surgical anaesthesia, the trachea was exposed, and mice were connected via a tracheal cannula to a ventilator (Ugo Basil, Comerio, Varese, Italy) set at 190 breaths per minute, with the tidal volume adjusted to 0.15ml. Whole body plethysmography was then used to measure airflow. A water-filled cannula was inserted into the oesophagus to measure transpulmonary pressure; resistance ($cmH_2O/ml/s$) and compliance (ml/cmH_2O) were then continuously computed by a Buxco XA-analyser (Troy, New York, USA). Mice were exposed to aerosolised spasmogen by a Buxco nebuliser connected in-line with the ventilator. Airway response to increasing concentrations of spasmogen (20µl per mouse, 5 minutes per dose) was then assessed as detailed in the relevant chapters.

2.1.8. Tissue bath methods

In addition to measuring lung function parameters in the whole animal, I also wanted to study the effect of the models on the contractility of the isolated airways.

Mice were euthanized with an overdose of sodium pentobarbitone (200 mg/kg, i.p.) and the trachea carefully dissected, minimising damage to the airway smooth muscle. The trachea was placed in Krebs-Henseleit solution (KHS, composition in mM: NaCL 118, KCL 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5, glucose 5.6, pH7.4, bubbled with

95% 0_2/5% CO_2). Where stated tracheas were prepared in Krebs solution contain 10μM indomethacin; indomethacin is commonly used in organ bath experiments at 10μM as a non-selective COX inhibitor to inhibit the production of endogenous prostanoids (Patel *et al.* 1995). The trachea was cut into two pieces around which two loops of silk thread were tied (top and bottom). These threads were used to set-up the tracheal rings in organ baths (Linton Instrumentation, Palgrave, Norfolk, UK) containing 10ml KHS, maintained at 37°C and gassed with 95% 0_2/5% CO_2. The bottom thread was attached to a fixed steel hook within the organ bath, and the top thread attached to a Grass FT-03 force-displacement transducer (Grass Instruments, Quincy, MA, USA). Isometric tension was then measured by these transducers, connected to a data acquisition system (Biopac Systems MP100 workstation) operated on a Windows PC using AcqKnowledge software (Biopac Systems, CA, USA). Resting tension of the tissues was set to 800mg.

2.1.8.1. Isolated smooth muscle contraction

Tracheal rings were left to equilibrate in the organ baths for 1 hour (refreshing the KHS to wash the tissues every 20 minutes). During this time the tension was adjusted to maintain the tissues at 800mg tension. The maximal contractile response of each individual tissue was then assessed by administering 1mM acetylcholine (ACh) to each bath. After the responses had plateaued, tissues were washed by emptying the baths and then adding fresh KHS solution. Tissues were repeatedly washed until the tissues had plateaued back to baseline tension; where necessary, tension was reset to 800mg. This process was repeated 3 times and the final ACh response was taken as the maximal tissue response.

As described in more detail in the relevant chapters, tissues were treated with vehicle, ligand or antagonist followed by a cumulative dose responses to spasmogen (ACh, 5-HT or MCh).

2.1.9. Analysis of immunoglobulin levels in plasma samples

To evaluate the effectiveness of the sensitisation protocols used in this thesis, the level of plasma IgE was used as a marker of allergic sensitisation. IgE levels were measured by enzyme-linked immuno-sorbent assay (ELISA) which is a technique universally used to detect and quantify levels of a specific protein of interest within a biological sample.

Analysis of IgE levels for this thesis was carried out while on placement at GSK (Stevenage, UK).

2.1.9.1. Total IgE

IgE assays were performed using 96-well Maxisorb plates (Nunc, Thermo Fisher Scientific, MA, USA). Unless otherwise stated, reagents were prepared and diluted in assay diluent (4% bovine serum albumin (BSA) and 0.05% Tween20 in PBS). Wash buffer contained 2.5ml Tween 20 500ml of 10x dPBS made up to 5L in dH_20.

Firstly plates were coated with 50µl of 2µg.ml^{-1} rat anti-mouse IgE made up in PBS, and left overnight at room temperature. The following day plates were washed with wash buffer 3 times and tapped dry after each wash. To block the non-specific binding, 100µl of 4% BSA in PBS was added to each well. This blocking step was left for 1.5 hours after which the blocking buffer was flicked out and the plates tapped dry. Assay samples (in duplicate) and standards (in triplicate) were then added (50µl per well). To prepare the standards, purified mouse IgE was diluted to 100ng.ml^{-1} in assay dileunt. This was then serial diluted 1:2 to make the standards (100-1.5625ng.ml^{-1}). Samples were diluted 1:2000 or 1:1000 as required. Plates were incubated with standards and samples overnight, at room temperature. On the third day, plates were again washed 3 times and tapped dry at the end of each wash. Biotinylated anti-IgE was then added to each well, 50µl at 2µg.ml^{-1}. This was then left for 1 hour at room temperature on a plate shaker after which the plates were again washed 3 times and tapped dry. Subsequently, 50µl of streptavidin-HRP (diluted 1:4000 in assay diluent) was added to each well and left at room temperature for 30 minutes. After this step the plates were washed again and tapped dry. Then, 100µl TMB (3,3',5,5'-Tetramethylbenzidine) substrate was added to each well and left in the dark for 5 minutes at room temperature, or until sufficient colour change had been observed. The reaction was stopped by adding 100µl of 0.25M sulphuric acid to each well. Plates were then read at 450nm using a spectrophotometer (Biotek PowerWave XS Plate Reader, Potton, UK).

2.1.9.2. HDM specific IgE

Unlike for total mouse IgE, there is no commercially available HDM-specific IgE to use as a standard. Without a standard curve, an ELISA only provides qualitative analysis of samples. Therefore for this assay, a reference curve was prepared from pooled plasma taken from HDM-sensitised and -challenged mice (generated at GSK by Sorif Uddin), which had previously been shown to have high HDM-specific IgE levels. This allowed semi-quantitative analysis of the plasma samples described in this thesis.

Unless otherwise stated, reagents were prepared and diluted in assay diluent (4% BSA and 0.05% Tween20 in PBS). Wash buffer contained 2.5ml Tween 20 500ml of 10x dPBS made up to 5L in dH_20.

Nunc Maxisorb 96-well Elisa plates were coated with 50µl of 5µg.ml^{-1} HDM in PBS and left overnight at room temperature. The following day plates were washed with wash buffer 3 times and tapped dry after each wash. To block the non-specific binding, 100µl of 4% BSA in PBS was added to each well. This blocking step was left for 1.5 hours after which the blocking buffer was flicked out and the plates tapped dry. For the reference curve, the pooled HDM-specific plasma was diluted 1:2000 in assay diluent containing normal mouse plasma (diluted 1:500). This was the top reference sample (1 unit) and was then serial diluted 1:2 in assay diluent to prepare the remainder of the reference samples (down to 0.015625 units,). The diluted normal mouse plasma made up the blank standard. Reference samples (in triplicate) and samples (in duplicate, diluted 1:500 in assay diluents) were then added to the plates (50µl per well). Plates were incubated with standards and samples overnight, at room temperature. On the third day, plates were again washed 3 times and tapped dry at the end of each wash. Biotinylated anti-IgE was then added to each well, 50µl at 2µg.ml^{-1}. This was then left for 1 hour at room temperature on a plate shaker after which the plates were again washed 3 times and tapped dry. Subsequently, 50µl of streptavidin-HRP (diluted 1:4000 in assay diluents) was added to each well and left at room temperature for 30 minutes. After this step the plates were washed again and tapped dry. Then, 100µl TMB (3,3',5,5'-Tetramethylbenzidine) substrate was added to each well and left in the dark for 5 minutes at room temperature, or until sufficient colour change had been observed. The reaction was stopped by adding 100µl of 0.25M sulphuric acid to each well. Plates were then read at 450nm using a spectrophotometer (Biotek PowerWave XS Plate Reader, Potton, UK). Data will be presented as units.ml^{-1}compared to the results in the reference plasma.

2.1.9.3. OVA-specific IgE

Levels of OVA-specific IgE were measured in plasma samples using a mouse ovalbumin-specific IgE ELISA assay kit (AbD Serotec, MorphoSys, Oxford, UK) according to the manufacturer's instructions.

2.1.10. Statistical analysis

Unless otherwise stated, data will be expressed as mean ± SEM (standard error of the mean) of n observations. The statistical significance of data will be assessed using a Mann-Whitney U-test for non-parametric data, with each group being compared to its relevant time-matched or vehicle-treated controls. To compare multiple groups, the Kruskal-Wallis test, followed by Dunn's Multiple Comparison post-test for non-parametric data will

be used. A p value less than 0.05 will be classed as significant. Statistical analysis was performed using Graphpad Prism software.

2.2. Materials

Abbott laboratories, Maidenhead, Berkshire, UK: Isoflurane (Isoflo)

Amersham Biosciences (now GE Healthcare, Buckinghamshire, UK): Streptavidin HRP

BD Biosciences, CA, USA: BD OptEIA mouse IgE ELISA set, TMB substrate solution

BD Pharmingen, CA, USA: biotinylated rat anti-mouse IgE, purified rat anti-mouse IgE, purified mouse IgE.

BOC Industrial Gases, Guilford, UK: Medical oxygen

Cayman Chemical, Michigan, USA: PGE_2

CP Pharmaceuticals Ltd, Wrexham, UK: Heparin

Fresenius Kabi, Warrington, UK: Endotoxin free saline

Gibco-Invitrogen, Paisley, UK: DPBS

Greer Labs, Lenoir, USA: House dust mite (dermatophagoides pteronyssinus) lot number 124632

Harlan, Bicester, UK: C57BL/6 mice

Invitrogen, Paisley, UK: RPMI 1640 medium + GlutaMAX-1, FBS

National Veterinary Services Ltd, Stoke-on-Trent, UK: Sodium pentobarbitone

Pierce Biotechnology Inc, Illinois, USA: Imject Alum ($AlOH_3/MGOH_4$)

Sigma-Aldrich Co Ltd, Poole, UK: Modified Wright Giemsa stain, acetylcholine, 5-HT, adenosine monophosphate (AMP), methacholine, indomethacin, DMSO, methylcellulose, Tween80, Tween20, ovalbumin, Bovine serum albumin, urethane

Sysmex UK Ltd, Milton Keynes, UK: Quicklyser

Tobacco Health Research Institute, University of Kentucky, Lexington, KY, USA: 3R4F research cigarettes.

VWR International Ltd, Lutterworth, UK: Glucose, NaCL, KCL, $NaHCO_3$, NaH_2PO_4, $CaCl_2$, $MgSO_4$, DPX, Sulphuric acid,

Chapter 3. OVA model optimisation

3.1. Rationale

Previous work in OVA models in-house has been conducted in Balb/c mice (Birrell *et al.* 2003, 2008*b*) but due to the availability of genetically modified strains of mice on the C57Bl/6 background the group switched to using this strain for the majority of their murine disease models. The group had previously set-up an OVA-driven model in C57Bl/6 mice but it was important to perform a detailed characterisation of the OVA model and its endpoints in order to interpret the subsequent effect of CS co-exposure. The endpoints optimised in the OVA model in this chapter will also be used as a guide for development of a HDM-driven model (Chapter 5).

When utilising an allergen-driven asthma model it is important that the model displays as many of the key features of asthma as possible. These include but are not restricted to allergic airway inflammation, non-specific airway hyperresponsiveness and an early and/or late response to allergen challenge. These are the features of the model which I will evaluate in this chapter.

3.1.1. Cellular inflammation

To measure cellular inflammation in the lungs in murine disease models the most commonly utilised method is bronchoalveolar lavage (BAL) which collects inflammatory cells from the airways. Our group has utilised this technique in several publications (Birrell *et al.* 2003; De Alba *et al.* 2010; Eltom *et al.* 2011) therefore this technique will be utilised in the present chapter.

3.1.2. AHR

There are several ways to measure AHR in an animal model of asthma. Firstly, conscious whole body plethysmography which is more akin to lung function measurements in man, where airway sensory nerves and reflex signalling remains intact. Secondly invasive lung function such as resistance and compliance provides more classical resistance measurements. And lastly, studying isolated airways in organ baths allows the measurement of changes in airway function at the level of the tissue itself.

It is important to utilise a range of spasmogens to ensure that any changes observed are not just specific to a given spasmogen. Most of the published data describing smooth muscle responses in asthma concern human or guinea pig ASM; however the effect of mediators may differ in other species. For example histamine and cysteinyl leukotrienes are potent bronchoconstrictors and asthmatic mediators in man and guinea pigs, mediating the

response of isolated trachea to allergen (Adams & Lichtenstein 1979), whereas cysteinyl leukotrienes, 5-HT and products of the COX pathway have been shown to mediate allergen-induced bronchospasm in the rat (Dahlbäck *et al.* 1984; Hele *et al.* 2001). In human airways the role of 5-HT as a contractile agent is more controversial. Different groups have shown 5-HT to have little or no direct contractile effect, but others have shown 5-HT to cause substantial bronchoconstriction in up to 65% of subjects (Cushley *et al.* 1986). 5-HT has also been shown to play a facilitatory role in cholinergic contraction by acting on pre-junctional 5-HT_3 and 5-HT_4 receptors (Takahashi *et al.* 1995; Dupont *et al.* 1999). However 5-HT is unlikely to mediate allergen induced airway responses in man. Histamine and cysteinyl leukotrienes are thought to play a less important role in allergen-induced bronchoconstriction in the mouse, despite being released from murine mast cells (Weigand *et al.* 2009). The primary mediator of allergic bronchoconstriction in the mouse is 5-HT (Eum *et al.* 1999; Weigand *et al.* 2009), although others have suggested a role of both 5-HT and histamine in murine OVA-induced AHR (De Bie *et al.* 1998).

I therefore selected the following spasmogens to use for the conscious lung function assessment: ACh, 5-HT, MCh and AMP. ACh is the neurotransmitter responsible for autonomic control of airway tone causing direct contraction of airway smooth muscle via M3 receptors; 5-HT and MCh are commonly used to induce bronchospasm *in vivo* in murine studies. AMP is used in the clinic to identify or diagnose asthmatics based on their having an enhanced response to this agent (Avital *et al.* 1995; Berkman *et al.* 2005) and to measure airway reactivity in asthmatics in clinical studies (Kanniess *et al.* 2001; Singh *et al.* 2008); it has also occasionally been used as a spasmogen in murine studies (Mustafa *et al.* 2007).

In disease states there is an up-regulation of several mediators which may modulate airway tone and thus may be important in the AHR observed in the OVA model. Of these the prostanoids, such as PGE_2, are widely reported to modulate airway tone. Several groups have shown inhibition of the COX pathway – which is responsible for generation of prostanoids – to modulate airway responses, AHR and allergen-induced airway contractions (Watts & Cohen 1993; Peebles *et al.* 2002; Swedin *et al.* 2010*b*; Larsson-Callerfelt *et al.* 2013). In addition PGE_2 was protective against allergen-induced bronchospasm and AHR in asthmatics, and against MCh challenge in both conscious mice and anaesthetised mice (Gauvreau *et al.* 1999*a*; Sheller *et al.* 2000; Hartney *et al.* 2006). Indomethacin, a non-selective COX inhibitor, is commonly used at 10μM in organ bath experiments to inhibit production of endogenous prostanoids (Patel *et al.* 1995), therefore indomethacin was included in the isolated tracheal studies to investigate the role of endogenous prostanoids in the AHR observed in the OVA model.

3.1.3. LAR

The group has recently published a paper demonstrating an OVA-induced LAR in C57Bl/6 mice (Raemdonck *et al.* 2012). The LAR is a key symptom-based feature of allergic asthma but is rarely studied in murine OVA models. This endpoint will be utilised to support inflammation and AHR data in this thesis.

3.2. Methods

3.2.1. General OVA sensitisation and challenge protocol

Mice were sensitised on days 0 and 14 with vehicle (Alum in saline, 100µl per mouse i.p.) or Alum plus ovalbumin (10 µg per mouse, i.p). On days 24-26 mice were challenged once daily with either vehicle (50µl of endotoxin-free saline) or ovalbumin (50µg in 50µl) intranasally. Figure 3.1 shows a diagrammatic representation of the general OVA model protocol. Mice were given an overdose of sodium pentobarbitone (200mg.kg^{-1}, i.p.) at various time points after final saline or OVA challenge as described below. BAL was performed, and the BAL fluid analysed for total and differential cell numbers.

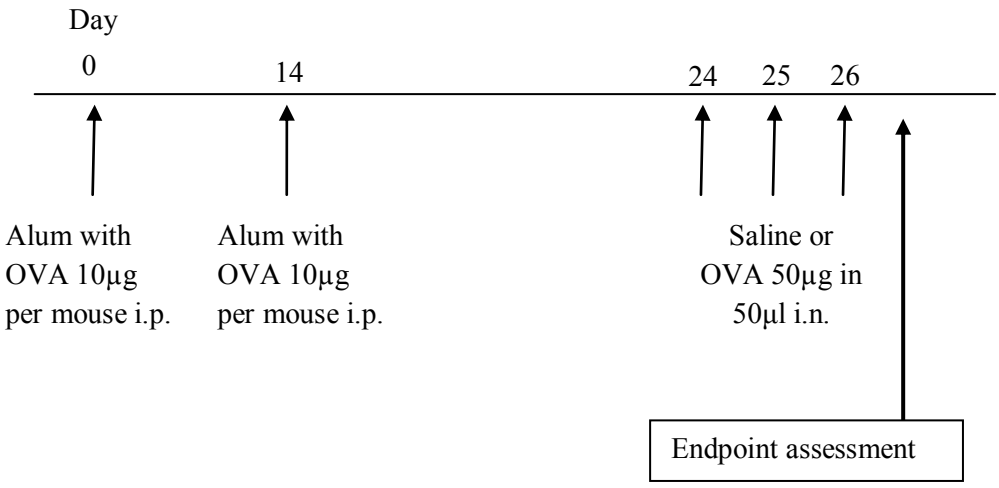


Figure 3.1: General schematic protocol for OVA model

3.2.2. Time course analysis of OVA-induced inflammation

Mice were sensitised with Alum and OVA, and challenged with either saline or OVA as detailed above. To determine the temporal airway inflammatory response to OVA, mice were culled 2 and 6 hours, and 1, 2, 3, 4, 7, 10, 14 17, 21, 24, 28, 24 and 28 days after final OVA challenge, and cellular inflammation was assessed in the BAL fluid.

3.2.3. Optimisation of airway hyperresponsiveness measurement in the OVA model

Allergic asthma is known to cause lung function changes in asthma patients including non-specific airway hyperresponsiveness. Once a time point for evaluating inflammatory changes in this model had been selected the presence of airway hyperresponsiveness at this time point was investigated.

3.2.4. Conscious whole body plethysmography

Mice were sensitised with vehicle (Alum in saline) or Alum with OVA and challenged with either saline or OVA as detailed above. Mice were placed in whole body plethysmography boxes 3 days after final challenge and were exposed to saline followed by increasing doses of the relevant spasmogen. Airway response to inhaled 5-HT (1-30mg.ml^{-1}), methacholine (1, 3 mg.ml^{-1}), adenosine monophosphate (AMP, 3-30mg.ml^{-1}) or acetylcholine (1-10mg.ml^{-1}) was evaluated.

3.2.5. Resistance and compliance

Having shown an increased response to 5-HT in OVA-sensitised and -challenged mice compared to OVA-sensitised, saline-challenged mice, I then investigated whether this enhancement was also associated with an increased response to spasmogen when measured by classical resistance and compliance.

Mice were sensitised with Alum with OVA, then challenged with saline or OVA and finally prepared for resistance compliance 3 days after final saline or OVA challenge. Mice were exposed to saline followed by increasing doses of 5-HT (0.1-3mg.kg^{-1}).

3.2.6. Isolated tracheal contraction

I was then interested to know whether the functional changes observed *in vivo* were replicated at the tissue level.

Mice were sensitised with Alum plus OVA and subsequently challenged with saline or OVA. Tracheal rings were obtained from these mice 3 days after final challenge and prepared in organ baths. Firstly the rings were incubated with vehicle ($NaHCO_3$) or indomethacin (10μM) for 30 minutes. The KHS in the organ baths was then refreshed twice to wash any existing COX products from the tissues and surrounding KHS. Indomethacin

was then re-added to the organ baths and the tension was re-set to 800mg. Dose responses to ACh (1nM-10mM), MCh (1nM-10mM) and 5-HT (1nM-10μM) were then performed.

3.2.7. OVA-induced late asthmatic response

A key symptom of allergic asthma is the late asthmatic response to allergen challenge, however this is not observed after any of the three allergen challenges in the standard allergic OVA model. The LAR has previously been demonstrated in a murine, OVA-driven model of asthma (Nabe *et al.* 2005); this protocol has been adapted in-house and has been published on by our group (Raemdonck *et al.* 2012).

Mice were sensitised with Alum plus OVA on days 0 and 14 and were then intratracheally challenged with saline or OVA. Mice were then immediately placed in whole body plethysmography chambers and Penh was recorded overnight for up to 15 hours.

3.3. Results

3.3.1. Time course analysis of OVA-induced inflammation

To understand the temporal profile of the cellular inflammatory response to OVA challenge a time-course was performed in OVA-sensitised mice challenged with either saline or OVA. Ovalbumin challenge in OVA-sensitised mice resulted in increases in levels of BAL eosinophils, lymphocytes, macrophages and neutrophils (Figure 3.2). The different cell types appear to be recruited to the lungs at different rates, for example elevated levels of neutrophils were observed by 2 hours after final challenge compared to time-matched saline-challenged control. Levels of BAL fluid neutrophils had fallen dramatically by 48 hours after challenge, whereas levels of eosinophils, macrophages and lymphocytes were still substantially elevated compared to saline-challenged controls 7 days after final challenge. Indeed levels of eosinophils were significantly elevated compared to saline-challenged time-matched controls 17 days after final challenge, while lymphocyte levels were still elevated 24 days after final challenge; the model exhibits a persistent cellular inflammatory response in the lungs.

Using this time course data, 3 days after final OVA challenge was selected as the optimum time point for measuring inflammation in this model. At this time point the neutrophilia in this model had predominantly resolved, and a robust eosinophilia and lymphocyte infiltration was established. In the literature the most common time points in acute allergen-driven models for measuring inflammation range between 24 and 72 hours. From the present time course data, other time points such as 7 days after challenge – where the peak of eosinophilia occurred – could be argued to be better time points, but time points beyond 72 hours are rarely used. Selecting 72 hours provides a robust eosinophilia, and also enables comparison with other published models and data.

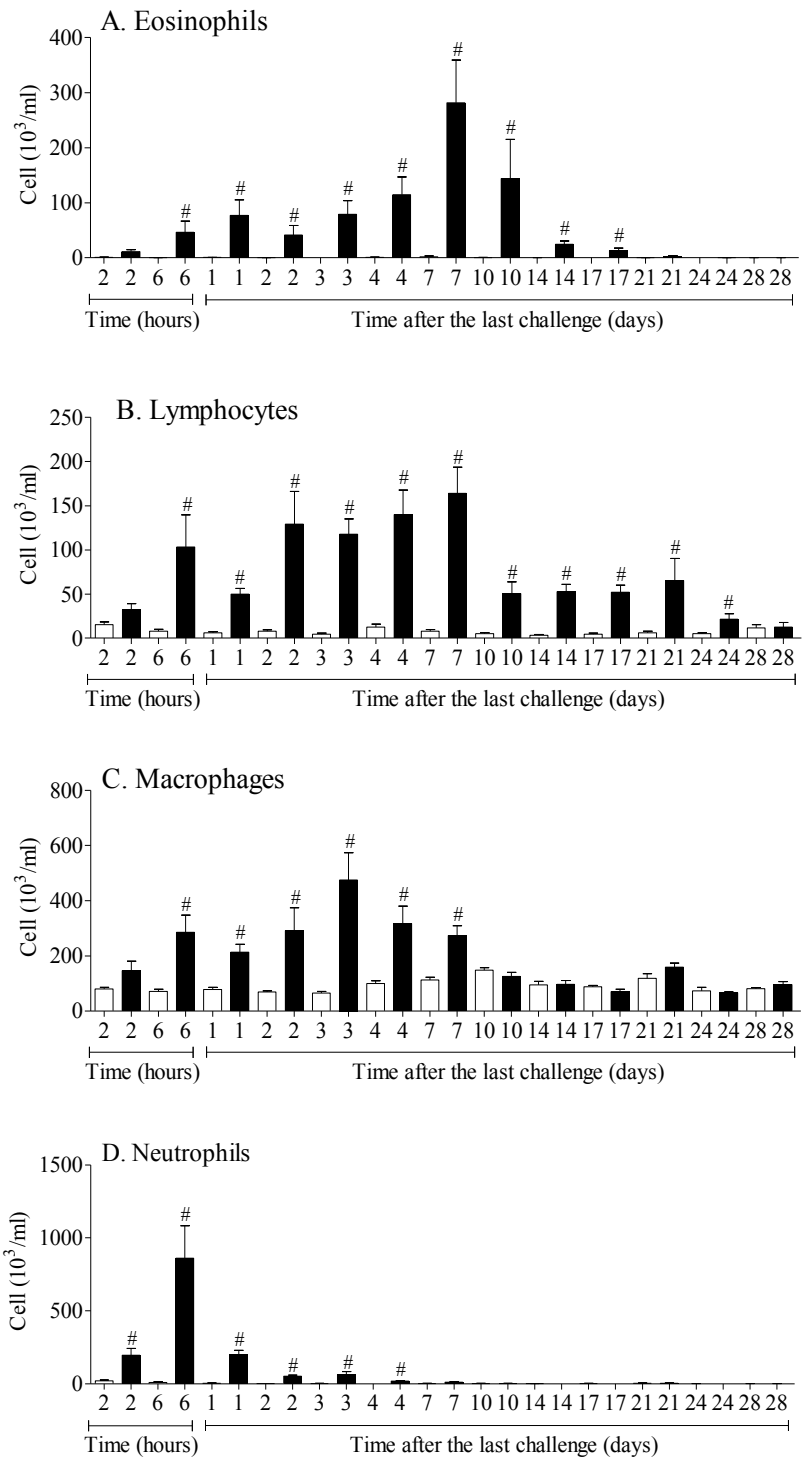


Figure 3.2: The cellular inflammatory response in BAL fluid to OVA sensitisation and challenge over time.

Male C57Bl/6 mice were sensitised with Alum plus OVA and challenged with saline (open bars) or OVA (black bars). Inflammation in the BAL fluid was assessed at various time points after final challenge. Data expressed as mean cell number.ml^{-1} for n= 8 per group. #=$p<0.05$ OVA challenged mice vs. saline challenged time-matched controls, Mann-Whitney U-test. Where no symbol is shown on the figure this indicates a non-significant difference.

3.3.2. Optimisation of airway hyperresponsiveness in the OVA model

3.3.2.1. Conscious lung function

In addition to measuring cellular inflammation in asthma models, it is also important to use functional disease measures, including airway hyperresponsiveness. I investigated *in vivo* contractile responses to a range of spasmogens to determine firstly whether OVA challenge in this model resulted in AHR, and secondly to determine which spasmogen generated the most robust window of AHR for use in subsequent studies.

In saline-sensitised/saline-challenged mice inhaled 5-HT caused a mild dose-dependent airflow obstruction, as indicated by the increase in Penh. The response to 5-HT observed in this group was also comparable to the response in saline-sensitised/OVA-challenged and OVA-sensitised/saline challenged mice. In OVA-sensitised and challenged mice the response to 5-HT was significantly enhanced compared to OVA-sensitised/saline challenged mice at 3, 10 and 30mg.ml^{-1} 5-HT (Figure 3.3 A, $p<0.005$, 0.05, 0.05 respectively, Mann-Whitney U-test). At 10mg.ml^{-1} the increase in Penh from the responses to saline was two-fold higher in the OVA/OVA group than in the OVA/saline group.

Having shown allergic AHR to 5-HT in OVA-sensitised/OVA-challenged mice compared to OVA-sensitised/saline challenged mice – the standard control used in this model – I then concentrated on these two groups to look at responses to the remainder of the spasmogens.

Regarding the MCh dose response, OVA-sensitised and -challenged mice appeared to have an increased response to 3mg.ml^{-1} inhaled MCh compared to the OVA-sensitised/saline-challenged controls however this failed to reach statistical significance (Figure 3.3 B). Again a similar profile was seen with inhaled AMP, where exposure to 30mg.ml^{-1} AMP appeared to induce a greater response in OVA-sensitised and challenged mice compared to controls (Figure 3.3 C); however this enhanced response was not significantly different to responses seen in the control mice (OVA-sensitised/saline-challenged). Conversely, when ACh was used as the spasmogen, OVA-sensitised and challenged mice showed consistently elevated responses across all of the doses (Figure 3.3D); the responses were significantly higher than responses in the control mice at 1 and 10mg.ml^{-1} ACh ($p<0.005$, Mann-Whitney U-test).

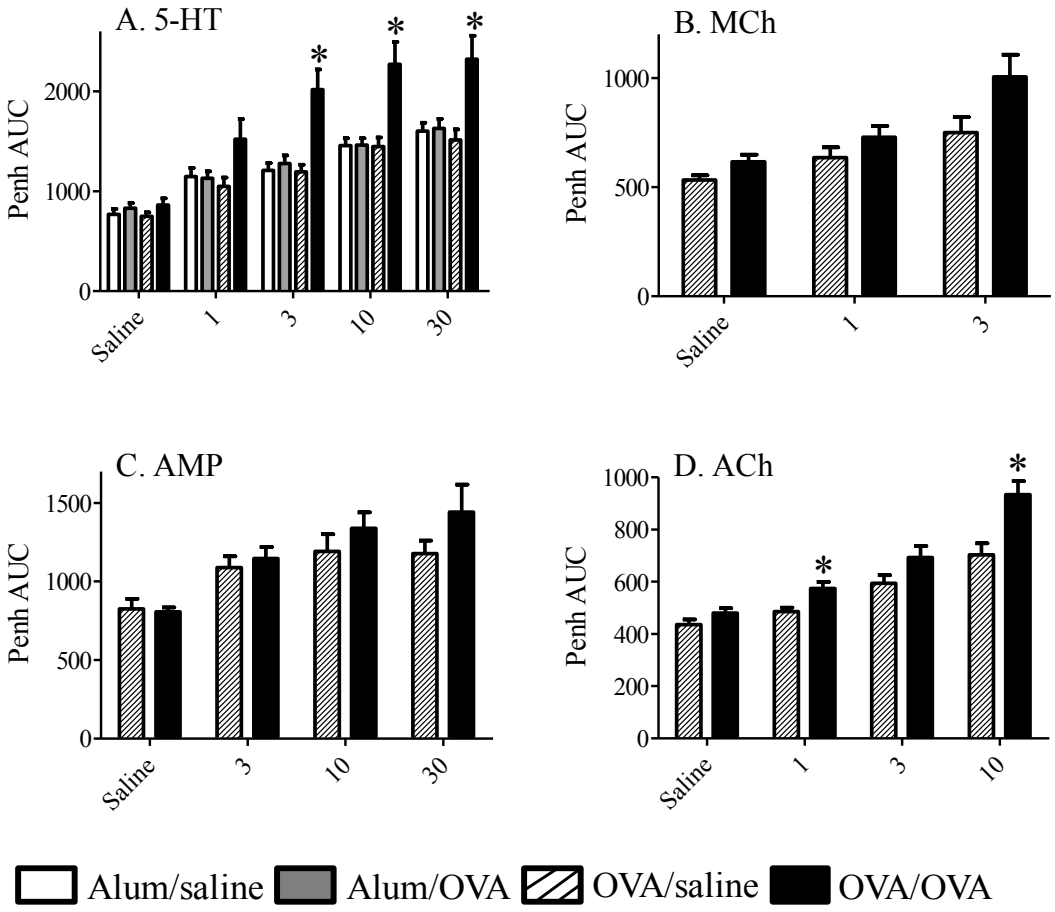


Figure 3.3: Effect of OVA sensitisation and challenge on responses to inhaled spasmogen: conscious whole body plethysmography.

Male C57Bl/6 mice were sensitised with vehicle (Alum) or OVA and challenged with saline or OVA. 3 days after final challenge mice were placed in whole body plethysmography chambers and exposed to saline and increasing concentrations of (A) 5-HT (1-30mg.ml^{-1}), (B) MCh (1-3mg.ml^{-1}), (C) AMP (3-30mg.ml^{-1}), and (D) ACh (1-10mg.ml^{-1}) to assess airway reactivity, measured as Penh. Open bars – Alum sensitised/saline challenged, grey bars – Alum sensitised/OVA challenged, striped bars – OVA sensitised/saline challenged, black bars – OVA sensitised/OVA challenged. Data expressed as mean Penh area under the curve + SEM for n=8-12 per group. *= P<0.05 Mann-Whitney U-test, OVA/OVA compared to relevant OVA/saline group. Where no symbol is shown on the figure this indicates a non-significant difference.

3.3.2.2. Airway resistance

It is important to back up any data obtained using conscious whole body plethysmography with classical airway resistance studies. Therefore I used resistance compliance to determine if the AHR observed in this model could also be detected using this method. Previously, I showed that 5-HT was the spasmogen which generated the most robust AHR using whole body plethysmography. I therefore decided to concentrate on this spasmogen for the resistance compliance study. In addition, the data in Figure 3.3 clearly show that the observed AHR is an allergic response. Therefore to reduce the number of mice

used for the resistance and compliance studies, I decided to concentrate on the OVA-sensitised mice, using saline challenged mice as the control.

Figure 3.4 shows resistance levels in OVA-challenged mice compared to controls, corrected for baseline resistance values recorded prior to nebulisation of saline. OVA-sensitised and challenged mice showed a clear increased response to inhaled 5-HT compared to the control mice, which was observed across all doses of 5-HT; the response to 5-HT was significantly higher in OVA-challenged mice compared to saline-challenged mice at 0.1, 0.3 and 3mg.ml-1 5-HT ($p<0.05$, 0.01, 0.05 respectively, Mann-Whitney U-test). This corroborates with the data obtained using conscious lung function and implies that the changes observed in the Penh study are due to a genuine increase in airflow obstruction in response to spasmogen. This gives increased confidence in the data described in this thesis which has been obtained using Penh.

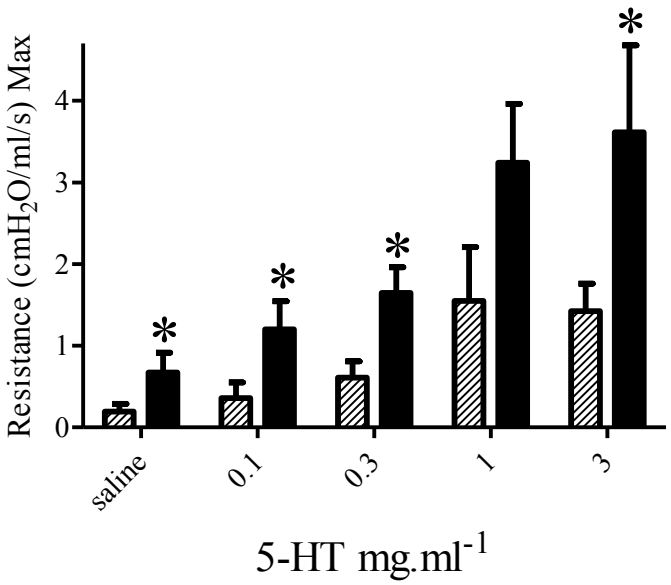


Figure 3.4: Effect of OVA sensitisation and challenge on airway responsiveness to 5-HT: anesthetised resistance

Male C57Bl/6 mice were sensitised with Alum and OVA and challenged with saline (striped bars) or OVA (closed bars). Approximately 3 days after final challenge mice were prepared for invasive lung function. Airway response to nebulised saline followed by increasing doses of 5-HT (0.1-3mg.ml^{-1}) was assessed. Data expressed as mean peak resistance corrected for baseline values, plus or minus SEM for n=6-8 per group. *= $P<0.05$ Mann-Whitney U-test, OVA/OVA compared to relevant OVA/saline group. Where no symbol is shown on the figure this indicates a non-significant difference.

3.3.2.3. Effect of OVA sensitisation and challenge on the contractile responses of isolated murine trachea

I was interested to determine whether the AHR observed *in vivo* would be associated with a change in reactivity at the tissue level.

Tissues from OVA-sensitised and -challenged mice showed substantially increased contraction to 5-HT across the whole dose response compared to tissues from OVA-sensitised/saline-challenged mice (Figure 3.5 E, F); a shift in the sensitivity to 5-HT was observed in addition to an increase in maximum response. This was observed both in the absence and the presence of indomethacin. Responses to MCh were also enhanced in the tissues from the OVA-challenged mice compared to control tissues (Figure 3.5 C, D). Again this occurred both in the absence and presence of indomethacin, however the enhanced response to MCh observed in OVA exposed tissues appeared to be different to that of 5-HT. The tissues from the OVA-challenged mice exhibited an increased maximal response to MCh, but at the lower doses of MCh the response in the asthmatic mice were equivalent to those seen in control mice. With ACh as the spasmogen, results replicated those seen with MCh (Figure 3.5 A, B), however the OVA-induced enhanced response to ACh was less pronounced than the enhancements seen to MCh or to 5-HT.

These data show that the AHR observed *in vivo* is still present when the airway tissue is removed from the animal, implying that a change in responsiveness has occurred at the level of the tissue itself. An enhanced response to 5-HT, MCh and ACh was observed which, in addition to the WBP data, provides further evidence that the OVA model demonstrates non-specific AHR. This endpoint could provide a valuable means to further investigate the mechanism behind allergen-induced AHR, and will also provide another functional endpoint with which to investigate the effects of CS exposure in this model.

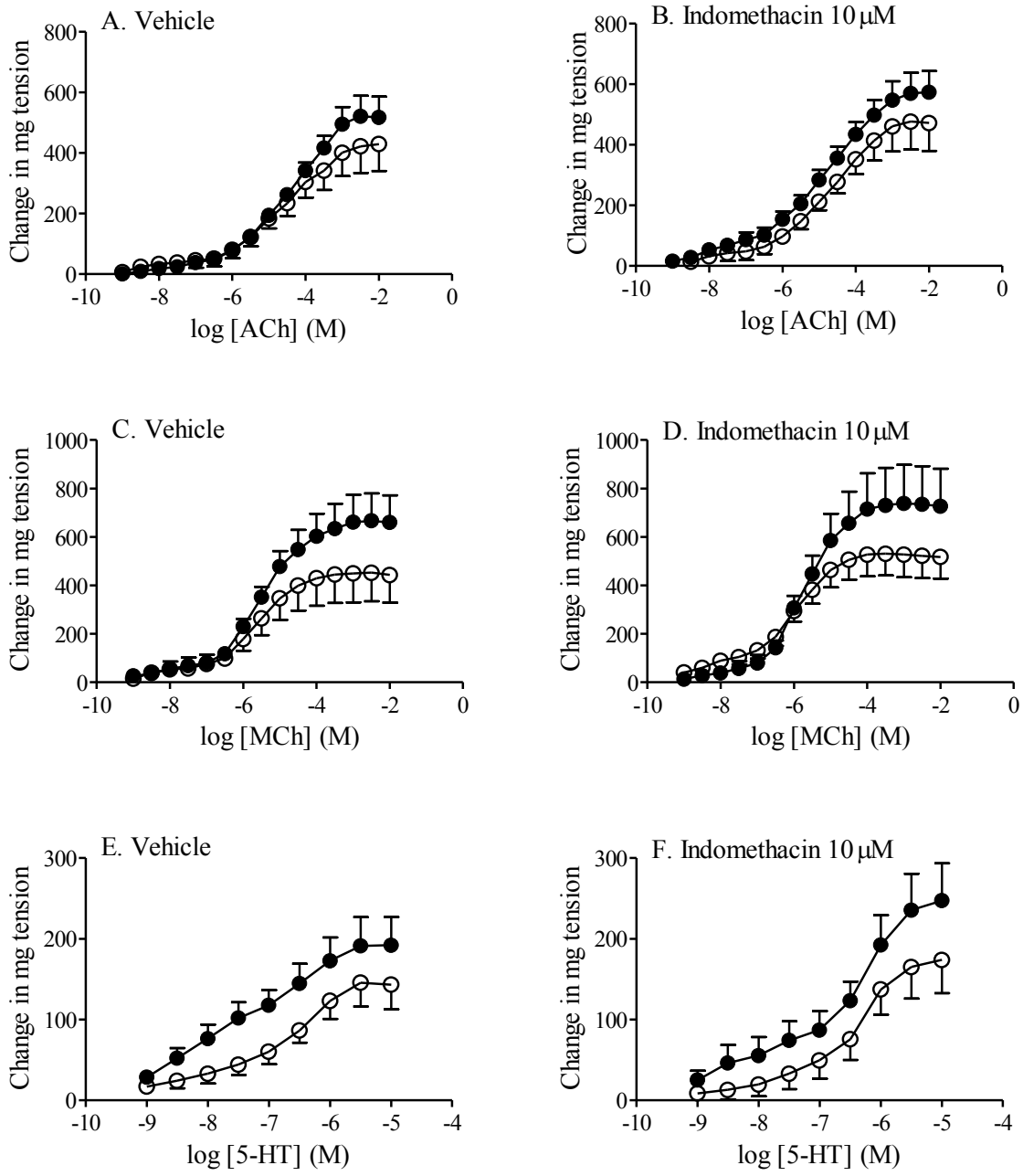


Figure 3.5: Effect of OVA sensitisation and challenge on isolated ASM responses. Male C57Bl/6 mice were sensitised with Alum and OVA, and challenged with saline (open circles) or OVA (closed circles). Tracheal rings were prepared and maintained with vehicle (0.01% $NaHCO_3$) (A, C, E) or indomethacin (10µM) (B, D, F). Cumulative dose response curves were performed to ACh (A, B), MCh (C, D), and 5-HT (E, F). Data show change in tension (mg) expressed as mean plus or minus SEM for n= 6-8 per group. Statistical analysis was not performed on this data.

3.3.3. OVA-induced late asthmatic response

As described, an important feature of allergic asthma is the LAR; however this response is not observed after any of the three allergen challenges given in the standard allergic model. An adjusted protocol is therefore used to generate the LAR which is based on the protocol developed by (Nabe *et al.* 2005), and our group has previously published on this model and its mechanisms (Raemdonck *et al.* 2012). In this model a late response occurs in OVA-sensitised and –challenged mice indicated by the increase in Penh compared to the stable respiratory pattern seen in OVA-sensitised/saline-challenged mice (Figure 3.6). No response to OVA challenge is observed in saline-sensitised mice – OVA is an innocuous allergen unless delivered in the context of prior sensitisation - therefore these controls were not included here.

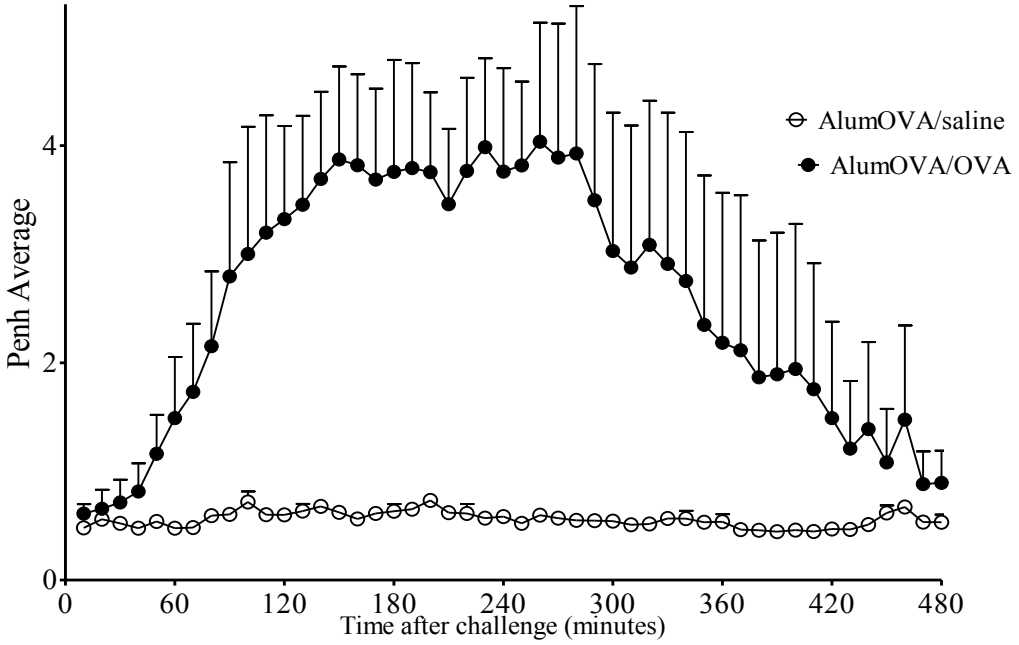


Figure 3.6: A typical late asthmatic response observed in sensitised C57Bl/6 mice after a single i.t. OVA challenge.

Male C57Bl/6 mice were sensitised with Alum or OVA and challenged intratracheally with 2% OVA. Immediately after challenge mice were placed in whole body plethysmography chambers, and the late response recorded as changes in Penh. Open circles –Alum/OVA sensitised and OVA challenged. Closed circles – Alum/OVA sensitised and OVA challenged. Data expressed as mean Penh average + SEM for n=4 per group. Statistical analysis was not performed on this data

3.4. Discussion

Firstly, a time course was performed to understand at what time points an elevated level of inflammatory cells could be detected in the ovalbumin-driven model. An initial increase in the levels of airway neutrophils in OVA challenged mice was followed by sustained airway cellular inflammation consisting of macrophages, lymphocytes and eosinophils. There are some limitations to perfoming cellular analysis on the BAL fluid alone, as this only collects inflammatory cells present in the airways and not those which have infiltrated the lung tissue itself. Formalin fixed lung tissue samples were obtained from the OVA model which could permit a more comprehensive analysis of the inflammatory phenotype in the model in the future.

With the development of genetically modified mice which are most commonly bred on the C57Bl/6 strain, the use of C57 mice in asthma models has increased. The majority of OVA models in C57Bl/6 mice utilise 1-2 systemic sensitisations to OVA plus Alum followed by 1-3 aerosolised challenges; similar protocols to the one used in this chapter. (Brusselle *et al.* 1994, 1995) published a model where OVA sensitisation was associated with an increase in total and OVA-specific IgE. The model also demonstrated robust allergic airway inflammation (comprised predominantly of eosinophils), and AHR to carbachol and 5-HT (anaesthetised resistance measurements) 24 hours after aerosolised OVA challenge. (Hamelmann *et al.* 2000) also showed a robust BAL eosinophilia, OVA-specific IgE and IgG1 and AHR to MCh (resistance) in C57 mice, measuring endpoints 48 hours after final challenge. Another group measured airway inflammation 3 days after challenge, and the inflammatory response was made up of monocytes, neutrophils, eosinophils and lymphocytes; OVA-specific IgE was also detected in the plasma (Stämpfli *et al.* 1998). These papers are just a selection of several detailing allergic OVA-driven asthma models in the C57Bl/6 mouse; I am therefore by no means the first to demonstrate allergic airway inflammation, or AHR in this strain.

Balb/c mice have in the past been the strain of choice for asthma models, and several studies have compared responses in OVA models between this strain and C57Bl/6s (Zhang *et al.* 1997; Wilder *et al.* 1999; Morokata *et al.* 1999, 2000; Takeda *et al.* 2001; Hayashi *et al.* 2001; Gueders *et al.* 2009); again the majority of studies utilise systemic OVA-sensitisation in conjunction with Alum, followed by varying numbers of aerosolised OVA challenge. Dogma often suggests that C57Bl/6 mice are poor IgE and Th2 responders compared to Balb/c mice in line with publications by (Zhang *et al.* 1997) and (Takeda *et al.* 2001). But, others have actually shown C57Bl/6 mice to produce higher levels of OVA-

specific IgE than Balb/c mice (Wilder *et al.* 1999; Morokata *et al.* 1999, 2000). OVA-challenge produced a robust and comparable eosinophilia in both strains, along with increased levels of Th2 cytokines (Il-4, IL-5, IL-13) compared to control mice in both strains, but cytokine levels were lower in C57 mice (Zhang *et al.* 1997). Some have shown OVA-challenge to elicit a more robust airway eosinophilia in C57BL/6 mice (Wilder *et al.* 1999; Morokata *et al.* 1999, 2000; Takeda *et al.* 2001; Gueders *et al.* 2009) although another study showed reduced airway eosinophils and lymphocytes in a C57Bl/6 OVA model compared to Balb/c mice (Hayashi *et al.* 2001). Another study, this time utilising intranasal challenge with OVA in systemically sensitised mice showed that Balb/c and C57Bl/6 mice generate comparable Th1/Th2 responses and eosinophilia, but the eosinophil distribution around the lung was different; in Balb/c mice eosinophils were found localised to airways and vessels, whereas in C57Bl/6 mice the eosinophils were more evenly distributed throughout the lung (Lu *et al.* 2010). Balb/c mice may exhibit greater AHR as OVA-induced AHR to i.v. methacholine (Resistance) was observed in Balb/c mice but not in C57Bl/6 mice with an analogous protocol (Wilder *et al.* 1999). Indeed others have shown AHR to nebulised MCh and 5-HT (resistance and Flexivent) to be greater in Balb/c than C57Bl/6 mice (Takeda *et al.* 2001; Gueders *et al.* 2009).

Although there is some dispute as to whether Balb/c mice are the more appropriate strain for use in murine asthma models, a model developed in C57Bl/6 mice with the potential for utilising genetically modified strains will be invaluable to understanding the mechanisms driving the model. I have clearly shown in the present chapter that using the in-house OVA model, OVA sensitisation and challenge in C57Bl/6 mice results in a robust allergic airway inflammation that is sustained for several days after allergen challenge. Interestingly the majority of papers listed above use aerosolised OVA challenge whereas the present model utilises intranasal challenge. Intranasal challenge has been shown to be more effective at inducing AHR and airway inflammation than aerosolised challenge (Swedin *et al.* 2010*a*) and intranasal challenge has the advantage of using less allergen; this will be important when developing the parallel HDM model, where the cost implications are greater.

In the present chapter, 3 days after final saline or OVA challenge was selected for the measurement of cellular inflammation in this model based on the establishment of a robust allergic cellular response comprised of eosinophils, macrophages and lymphocytes. I then opted to measure AHR in the model at the same time-point. This decision was based on the widely muted hypothesis that AHR is driven by airway inflammation, and thus a time point when a robust allergic inflammation had been established was selected. In addition, measuring AHR immediately prior to sample harvest means that only one set of animals is

required for the two endpoints; an extra set of animals would be required for all subsequent studies if the AHR measurements were to be performed at a later time point than the sample harvest. This would have implications surrounding the 3Rs.

Using this time-point – 3 days after final challenge – airway hyperresponsiveness to 5-HT was detected in the asthmatic mice compared to controls using conscious, non-invasive lung function; there was also a trend towards an increased responsiveness to other spasmogens. The fact that the Sal/OVA group or the OVA/Sal group had indistinguishable responses from the Sal/Sal group means that the increased response observed in the OVA/OVA group was indeed an allergic response, i.e. did not occur in OVA challenged mice which had not been previously sensitised to OVA. It may be that a further heightened response to 5-HT, or indeed a more robust AHR to other the spasmogens could have been detected if measured at other time points after challenge. Indeed several publications have dissociated inflammation and AHR (Hessel *et al.* 1995; Tournoy *et al.* 2000; Birrell *et al.* 2003; Swedin *et al.* 2009). However the measurement of AHR or pulmonary mechanics at 3 days after final challenge in allergen-driven models is supported by numerous publications (Lee *et al.* 2004*b*; Busse *et al.* 2009; Kelada *et al.* 2011; Possa *et al.* 2012). Having detected a robust AHR at this time point there was no rationale for measuring AHR at further time points.

It appears that in this model there is a trend for the asthmatic mice to develop a heightened response to multiple inhaled spasmogens after allergen challenge; however this difference is most reproducible when using 5-HT as the spasmogen. The window of AHR to 5-HT is robust and could be confidently used to assess the effect of pharmacological interventions, or in the case of this thesis, the effect of cigarette smoke on AHR.

To limit the number of animals used, 5-HT was selected to confirm the observation of AHR using classical airway resistance measurements; a robust AHR to 5-HT was also observed here. In the field there is substantial distrust of conscious lung function (Penh) as a method to record airway reactivity (Adler *et al.* 2004; Bates *et al.* 2004; Lundblad *et al.* 2007), however I have corroborated the AHR to 5-HT obtained in the Penh studies using resistance compliance. I can now be confident to continue measuring AHR in this model using Penh as a representative measure of airflow obstruction changes. Previous studies have also shown results obtained using unrestrained conscious plethysmography to correlate with results obtained using invasive lung function methods (Hamelmann *et al.* 1997*c*).

AHR has long been reported as a cardinal feature of asthma, but the mechanisms driving it are not clear. Changes in airway responsiveness could be due to many factors including intrinsic changes to the smooth muscle structure or function: smooth muscle proliferation, increased smooth muscle contractility, or mediators released by the tissue which consequently regulate contractile responses. Or, a higher level effect on reflex regulation of smooth muscle tone may be involved. I therefore paralleled the *in vivo* AHR studies with studies using isolated trachea. Here I measured airway responsiveness to 5-HT, the spasmogen used for the *in vivo* studies; MCh, a spasmogen commonly used for *in vivo* and *in vitro* studies; and ACh, the endogenous muscarinic agonist which is used routinely in the group to elicit contraction of isolated airways. AHR to all three spasmogens was observed in trachea obtained from OVA-challenged mice. The fact that allergen-induced AHR was detectable in isolated airways is of interest in itself although not a novel finding (Moir *et al.* 2003; McVicker *et al.* 2007; Birrell *et al.* 2008*b*). This implies that a change in the structure or function of the airway smooth muscle itself occurred in the OVA model. The presence of AHR in the isolated trachea and in anaesthetised resistance compliance studies also implies that the AHR was not due to an effect on reflex control of airway tone as these processes would not be present in either of these two experimental preparations.

True AHR in asthma is described as being 'non-specific'; asthmatic airways have increased reactivity to multiple stimuli. The *in vitro* AHR studies in this chapter show that the AHR observed in this model is indeed non-specific as the airways of the asthmatic mice showed increased responses to multiple stimuli: ACh, MCh and 5-HT. The fact that AHR to all three spasmogens was detected in the organ bath studies is also interesting as this is in contrast with the *in vivo* data where a robust AHR was only detected to 5-HT. In the isolated trachea an increased maximal response to 5-HT was observed as well as a shift in sensitivity; the tissue from OVA-challenged mice responded to 5-HT at lower doses than the tissue from saline-challenged mice. Conversely OVA-challenged mice only exhibited an increased maximal response to ACh and MCh. During *in vivo* dose responses breathing difficulties and respiratory distress preclude the use of very high spasmogen doses. This may explain the difference in the Penh data and the data generated in the isolated trachea; it may have been possible to observe an enhanced response to ACh or MCh *in vivo* if higher spasmogen doses were used. In addition there may be neural mechanisms to limit *in vivo* bronchospasm for example pre-junctional M_2 autoreceptors. These serve as a feedback mechanism to limit cholinergic bronchospasm, although notably this response is reported to be absent in asthmatics (Minette *et al.* 1989).

The discrepancy between AHR data using the different spasmogens and different measurement techniques has been reported previously. Others have shown AHR to 5-HT but

not methacholine when using compliance to measure AHR in C57Bl/6 mice (Takeda *et al.* 2001). In a murine OVA model, bronchial hyperresponsiveness to ACh, but not tracheal hyperresponsiveness was observed (Chiba *et al.* 2004), which shows the importance of measuring AHR through multiple methods and considering multiple airway levels. A recently developed technique – PCLS (precision-cut lung slices) –can now be used to measure contractile responses in small airways and investigate lung diseases and therapeutics (Liberati *et al.* 2010; Sanderson 2011). Several groups have started utilising the PCLS technique to investigate small airway responses and airway hyperresponsiveness in human tissue and in animal models (Wohlsen *et al.* 2003; Chew *et al.* 2008; Henjakovic *et al.* 2008; Banerjee *et al.* 2012). Studying the lower airways in the OVA model would be a worthwhile experiment to further understand the *in vivo* AHR observed in this model. Notably one mouse study failed to demonstrate OVA-induced AHR to ACh on PCLS despite observing *in vivo* AHR to MCh and airway remodelling (Chew *et al.* 2008), implying the lower/smaller airways may not be important in the AHR observed in this model.

To further understand the AHR observed in the present OVA model, tissue responses in OVA-sensitised and challenged mice were compared in the absence and presence of indomethacin to highlight a possible role of endogenous prostanoids in this endpoint. Interestingly, although the presence of indomethacin tended to enhance contraction in all tissues (saline and OVA treated) it did not affect the AHR observed to any of the spasmogens. AHR to all three stimuli – 5-HT, MCh, and ACh – was observed both in the absence and presence of indomethacin. This implies that altered levels of prostanoids or other COX-derived mediators were unlikely to be involved in the observed *ex vivo* AHR. In other cases inhibition of the COX pathway has generated varied results on airway responses and AHR. In rat PCLS COX inhibition was shown to attenuate OVA-induced contraction of PCLS, and highlighted a role for PGE_2 in allergen-induced contraction in the rat (Larsson-Callerfelt *et al.* 2013). Alternatively, others have shown inhibition of the COX pathway to induce AHR and enhance bronchospasm (Watts & Cohen 1993; Peebles *et al.* 2002) and to increase AHR in a murine asthma model (Swedin *et al.* 2010*b*). Exogenous PGE_2 was protective against allergen-induced bronchospasm and AHR in asthmatics (Gauvreau *et al.* 1999*a*) and against methacholine challenge in conscious mice (Sheller *et al.* 2000). Endogenous PGE_2 was also protective against MCh-induced bronchospasm in anaesthetised mice (Hartney *et al.* 2006). The increased *in vivo* AHR in a murine asthma model with indomethacin (Swedin *et al.* 2010*b*) is at odds with the data presented in this thesis. However the effect of the COX inhibitors was not tested in saline-challenged mice in the study by (Swedin *et al.* 2010*b*); COX inhibition was only shown to enhance the response in

OVA-challenged mice. In the present study indomethacin appeared to increase airway contraction in both saline-challenged and OVA-challenged mice meaning the AHR compared to controls was not modulated. The *in vitro* data in this chapter suggests that the COX inhibitors would also have increased the *in vivo* response in saline-challenged mice in the Swedin study and therefore conferred a general effect on airway responses rather than on OVA-induced enhanced response. However in an earlier study COX inhibitors failed to affect *in vivo* responses in saline-challenged mice (Swedin *et al.* 2009) so the discrepancy between the present data and the data published by Swedin et al may be due to differences between *in vivo* bronchospasm and contractile responses of the isolated airway.

Airway smooth muscle proliferation has been shown to be increased in asthmatic patients (Johnson *et al.* 2001), resulting in increased contractile ability of the airways. This could be the mechanism for the AHR observed in the present OVA model. Airway smooth muscle proliferation has been previously demonstrated by our group in a murine OVA-driven model (Birrell *et al.* 2008*b*) by staining histological samples for α-smooth muscle actin (α-SMA) (a common smooth muscle marker). Similar studies could be performed in samples from the OVA model to determine whether any changes in airway smooth muscle mass are observed.

The final endpoint I have described in this chapter is the OVA-induced LAR, which was generated using a different protocol than the one used to generate allergic inflammation and AHR. The absence of LAR in murine allergic asthma models is not unusual; others have shown a lack of LAR in murine models in which AHR and eosinophilia are observed (de Bie *et al.* 2000; Zosky *et al.* 2008).

In human asthmatics, the LAR typically occurs 4-8 hours after allergen challenge (Booij-Noord *et al.* 1971). The LAR described here occurs at an earlier time point than this, beginning approximately 1 hour after allergen challenge and reaching a peak at around 2.5 hours after challenge. It is also noted that no EAR is observed in this model; these factors may call into question whether the LAR observed here is a true LAR. The lack of EAR in the present model compared to the Nabe study – on which the present model is based – could be explained by the difference in mouse strain used in the two studies; (Nabe *et al.* 2005) used Balb/c mice. In the Nabe paper the early response in Balb/c mice occurred within 10 minutes of OVA challenge followed by an LAR which occurred around 2 hours after challenge (Nabe *et al.* 2005). The time scale of the LAR described in this chapter is more reminiscent of the LAR. In addition our group has previously published that steroid treatment inhibits the LAR in both a rat model, and the present murine LAR model (Raemdonck *et al.* 2012), which would not be consistent with an early response.

Another caveat to the LAR in this model is that it has only been demonstrated using conscious, non-invasive plethysmography. As mentioned previously it is desirable to back up data such as this with invasive resistance measurements, however this is implausible in the case of the LAR as anaesthesia abolishes this endpoint (Raemdonck *et al.* 2012).

This model/endpoint will provide a great opportunity to observe the effect of CS exposure on a key functional feature of allergic asthma to support the inflammation and AHR data.

In this chapter I have demonstrated several endpoints which will now be used to understand the effect of CS exposure in the OVA-driven asthma model (chapter 4). In addition these endpoints will be used as a guide for optimising the HDM model (chapter 5).

Chapter 4. The effect of cigarette smoke on OVA-induced inflammatory status and functional endpoints

4.1. Rationale

In Chapter 3, I characterised the OVA model used in-house and optimised the endpoints that will be used in the present chapter to investigate the effect of CS co-exposure on this model. These include measuring allergic airway inflammation; measurement of airway reactivity to 5-HT using conscious lung function and invasive resistance compliance; measurement of airway reactivity at the level of the isolated trachea; and the late asthmatic response. These endpoints will be utilised to comprehensively assess the effect of CS on the OVA model phenotype and its response to steroid treatment. In-house we currently use an acute CS model in C57Bl/6 mice that has been described previously (Eltom *et al.* 2011). The model is a submaximal CS model consisting of 1 hour exposures to air or cigarette smoke twice per day for three days. This exposure regimen results in robust cellular inflammation (neutrophilia) and up-regulation of multiple inflammatory cytokines including IL-1α, IL-1β and IL-6 (Eltom *et al.* 2011).

The possible ways in which to combine CS exposure with the allergen models are numerous: CS could be given before or after the challenge period, be given in conjunction with the allergen challenge or be given throughout the model protocol (including sensitisation). I was however interested in what effect a background of CS-induced inflammation would have on the allergic response to OVA challenge. It has previously been reported that CS exposure may modulate allergic sensitisation or tolerance (Rumold *et al.* 2001; Robbins *et al.* 2005; Moerloose *et al.* 2006; Van Hove *et al.* 2008; Trimble *et al.* 2009; Lanckacker *et al.* 2012) therefore it was decided not to give CS during the sensitisation phase of the model; it was important the mice were sensitised as normal in order to compare the allergic responses in control and CS exposed mice. Mice were therefore sensitised to OVA plus Alum according to the standard OVA model protocol. Mice were then exposed to air or CS for three days prior to OVA challenge, in accordance with our standard CS model, to generate a background of CS-induced inflammation. CS exposures were maintained during the challenge phase and until endpoint assessment as described in Chapter 3. This aims to be representative of a sensitised patient who smokes, or is exposed to environmental pollution, and undergoes a response to an allergen.

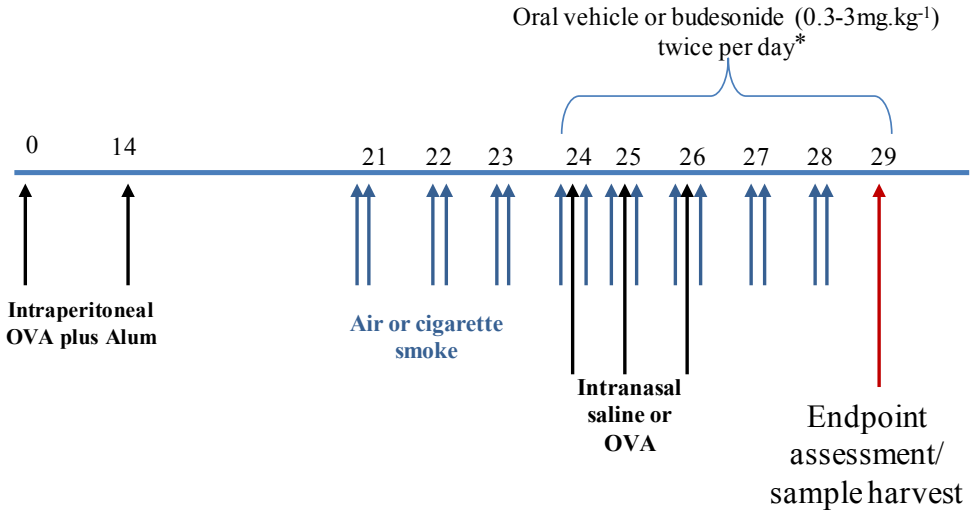
In Chapter 3 it was found that blocking the cyclooxygenase pathway did not modulate the OVA-induced AHR observed in the isolated trachea. However it did provide a general enhancement of contractile responses. Therefore in the present chapter, studies utilising isolated trachea will be performed in the presence of indomethacin to ensure robust

contractions are elicited, but unlike previously will not compare responses with and without indomethacin.

4.2. Methods

4.2.1. Protocol for combining the OVA model and CS exposures

Mice (n=8) were sensitised with Alum plus OVA according to the standard model protocol. Mice were then exposed to air or cigarette smoke twice per day, 4 hours apart, starting on day 21 (3 days prior to OVA challenge). Mice were challenged intranasally with saline or OVA once daily on days 24-26 – approximately halfway between the two CS challenges. Exposures to CS were continued until day 28 (inclusive) and endpoints were assessed on day 29 (as determined in chapter 3). To determine the effect of cigarette smoke exposure on the treatment sensitivity of this model, mice were also dosed with oral vehicle (0.5% methylcellulose plus 0.2% tween80 in water) or budesonide (0.3-3mg.kg^{-1}) twice per day from day 24, receiving a final dose 1 hour prior to endpoint assessment on day 29. This steroid dosing protocol has previously been used in-house and has been effective against OVA-induced inflammation in a Balb/c model (Birrell *et al.* 2003). The protocol for this study is detailed in schematic form in Figure 4.1.



Mice were dosed with budesonide twice per day as indicated,
except on day 29 where mice were dosed once and assessed one hour later.

Figure 4.1: Schematic of the protocol for combining CS and the standard OVA model

4.2.1.1. Evaluating the effect of CS on the OVA model and its treatment: BAL inflammation and non-invasive lung function

In mice exposed to CS combined with OVA challenge as described above, airway reactivity to 5-HT (1, 3 $mg.ml^{-1}$) was evaluated using whole body plethysmography (Penh) 3 days after final challenge. Mice were allowed to recover from spasmogen challenge for at least one hour, after which mice were culled: BAL was performed and levels of inflammatory cells were assessed in the BAL fluid.

4.2.1.2. Evaluating the effect of CS on the OVA model: isolated tracheal responsiveness

In the previous chapter I observed an enhanced response of the isolated trachea to spasmogen challenge in the OVA model. I was therefore interested in the effect of CS exposure on this response; this would help to understand the mechanism behind any effect of CS on OVA-induced AHR. Tracheal rings were harvested from OVA-sensitised mice exposed to air or cigarette smoke and challenged with saline or OVA and were prepared in organ baths. Indomethacin (10μM) was present in KHS throughout the study to enhance the spasmogen-induced contractile responses. Tracheal rings were exposed to increasing doses of 5-HT (1nM- 30μM).

4.2.1.3. Evaluating the effect of CS on OVA-induced LAR

The LAR is an important symptom of allergic asthma in man. Therefore the effect of CS in the OVA-induced LAR model was also evaluated to support the inflammation and AHR data. In the LAR protocol mice are challenged intratracheally with 2% OVA at the end of day 28. Therefore to parallel the inflammation and AHR studies, where mice were exposed to CS for 3 days prior to OVA challenge, mice were exposed to air or cigarette smoke twice per day on days 26-28.

Mice were sensitised with OVA plus Alum according to the protocol for generating the LAR. Mice were then exposed to air or CS twice per day on days 26-28. Mice were also dosed with oral vehicle (0.5% methylcellulose plus 0.2% tween80 in water) or budesonide ($3mg.kg^{-1}$) 1 hour after final CS exposure. Finally mice were challenged intractracheally with 2% OVA 1 hour after budesonide treatment and were immediately placed in whole body plethysmography chambers. Penh was recorded overnight. The protocol for this study is represented in schematic form in Figure 4.2.

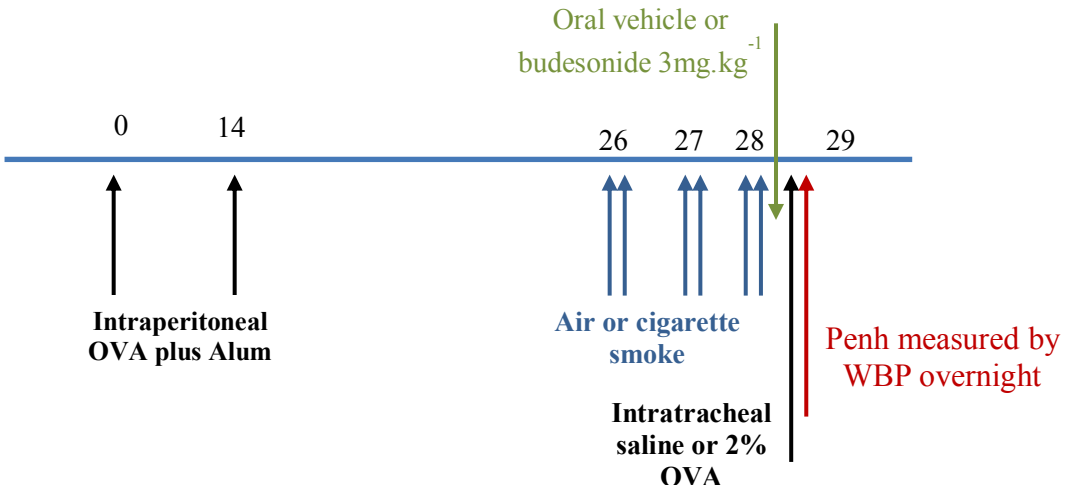


Figure 4.2: Schematic protocol for testing the effect of CS on the OVA-induced LAR

4.3. Results

4.3.1. The effect of CS co-exposure on OVA-induced cellular inflammation

The level of inflammatory cells in the BAL fluid of mice exposed to air/saline, air/ovalbumin, smoke/saline and smoke/ovalbumin were compared (Figure 4.3). Ovalbumin challenge resulted in a significant increase in BAL lymphocytes and eosinophilia (Figure 4.3 A, B, $p<0.005$ and <0.01 respectively, Mann-Whitney U-test) compared to air/saline treated controls. The OVA-induced increase in both BAL lymphocytes and eosinophils was dose-dependently and significantly reduced by steroid treatment (Kruskal-Wallis followed by Dunn's Multiple Comparison post-test, $p< 0.0001$ and $p<0.005$ respectively, 3mg.kg-1 budesonide vs. respective vehicle treated groups). OVA challenge also resulted in an increase in BAL neutrophils (Figure 4.3 D, Mann-Whitney U-test, $p<0.05$) and a small increase in macrophages (Figure 4.3 C). At all doses steroid treatment caused an apparent but non-significant reduction in both neutrophils and macrophages in OVA-challenged mice. Cigarette smoke exposure alone resulted in a significant increase in the levels of BAL neutrophils compared to air/saline treated controls (Figure 4.3 D, $p<0.0001$, Mann-Whitney U-test) but not the other cell types; the CS-induced neutrophilia was not modulated by steroid treatment at any dose.

The combination of CS and OVA resulted in increased levels of neutrophils, macrophages, lymphocytes and eosinophils compared to air/saline controls. CS co-exposure caused an increase in the levels of lymphocytes and macrophages compared to mice challenged with OVA alone ($p< 0.0005$ and $p<0.01$ respectively, Mann-Whitney U-test). The levels of airway eosinophils in CS and OVA co-exposed mice were comparable with those challenged with OVA alone and levels of neutrophils in CS and OVA co-exposed mice were comparable to those exposed to CS alone.

The BAL macrophages, lymphocytes, and eosinophils resulting from the combined exposure to CS and OVA were all dose-dependently inhibited by budesonide treatment. Conversely the neutrophilia observed after CS and OVA co-exposure were not altered by steroid treatment.

From this data it appears that cigarette smoke (at the level used in this study) has little impact on the anti-inflammatory efficacy of steroid treatment. The exception is that CS co-exposure confers a population of steroid resistant neutrophils in the OVA and CS co-exposed mice which are not observed in the mice exposed to OVA alone.

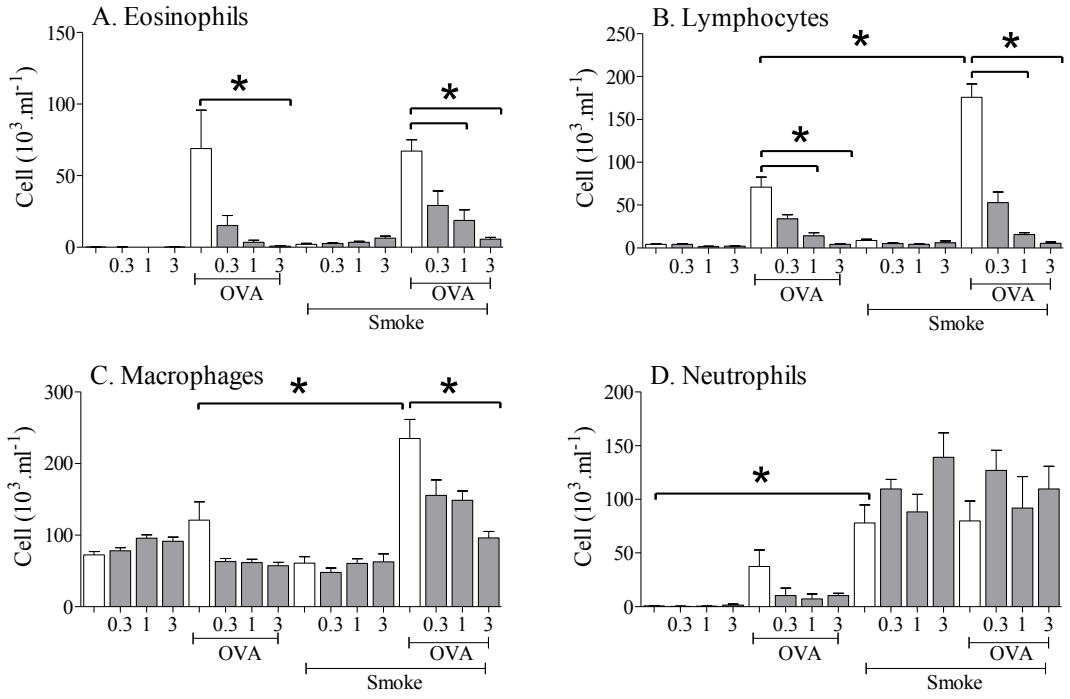


Figure 4.3: The effect of cigarette smoke on OVA-induced cellular inflammation and the anti-inflammatory efficacy of budesonide in this model.
Male C57Bl/6 mice were sensitised with Alum plus OVA, and subsequently challenged with saline or OVA, and exposed to air or cigarette smoke as indicated on the figures. Mice were also treated with vehicle (0.5% methylcellulose plus 0.2% tween80 in water) (open bars) or budesonide (0.3-3mg.kg^{-1}) (closed bars). Cellular inflammation was assessed in BAL fluid 3 days after final challenge. Data expressed as mean cell number (10^3.ml^{-1}) + SEM for n=7-8 per group. *p=<0.05, challenged/budesonide treated groups compared to relevant challenged/vehicle treated controls, Mann-Whitney U-test. Where no symbol is shown on the figure this indicates a non-significant difference.

4.3.2. The effect of CS co-exposure on OVA-induced airway hyperreactivity

4.3.2.1. Conscious WBP

Having investigated the effect of CS on the OVA-induced inflammation I wanted to parallel this by looking at the effect of CS on the OVA-induced functional changes – starting with the AHR. Since 5-HT provided the most reproducible AHR in the OVA model (Chapter 3), this spasmogen was used in the present study.

In OVA-challenged mice a significantly increased response to 5-HT was observed compared to saline-challenged mice (Figure 4.4), $p< 0.05$, Mann-Whitney U-test). OVA-induced AHR to 5-HT was completely inhibited by treatment with budesonide ($3.mg.kg^{-1}$).

Combining CS with the OVA challenge abolished the AHR; the level of Penh in CS plus OVA co-exposed mice after $3mg.ml^{-1}$ inhaled 5-HT was equivalent to the levels seen in the air/saline-challenged controls. Consequently in CS and OVA co-exposed mice there was no longer a window of AHR in which to determine the effect of steroid treatment.

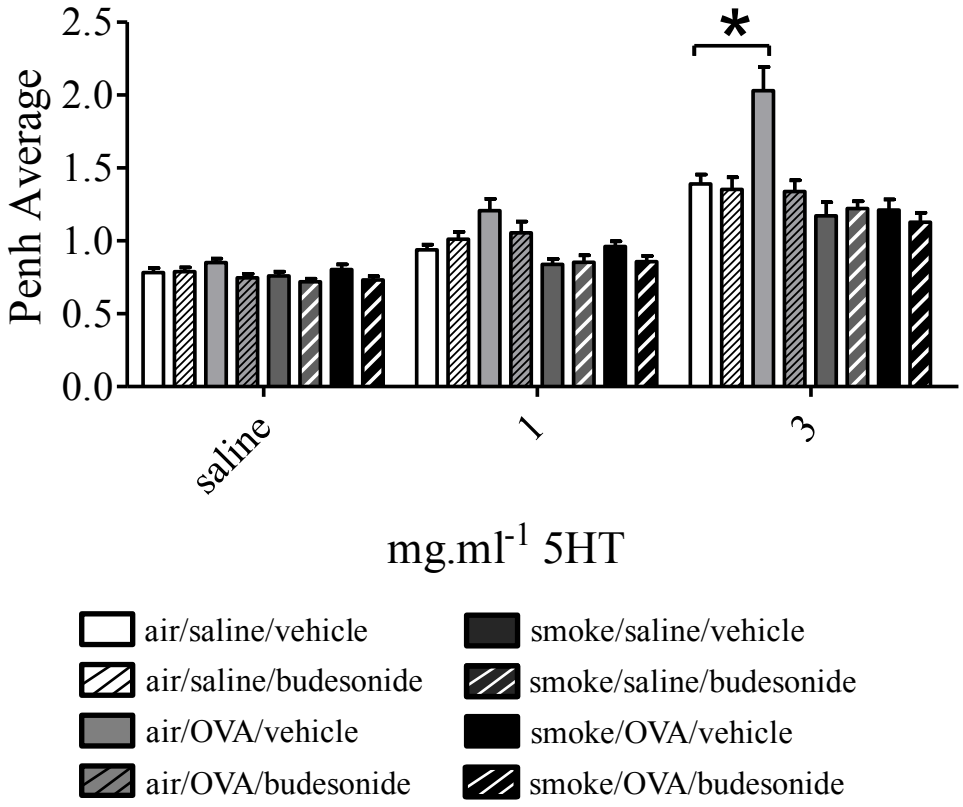


Figure 4.4: Effect of cigarette smoke on OVA-induced airway hyperresponsiveness. Male C57Bl/6 mice were sensitised with Alum and OVA, and subsequently challenged with saline or OVA and exposed to air or cigarette smoke. Mice were also treated with oral vehicle (0.5% methylcellulose plus 0.2% tween80 in water) or $3mg.kg^{-1}$ budesonide. Mice were placed in whole body plethysmography boxes 3 days after final challenge, and response to inhaled 5-HT was recorded as Penh. Data expressed as mean Penh average + SEM for n=12 per group. *=$p<0.05$ relevant OVA challenged groups vs. relevant saline challenged controls, Mann-Whitney U-test. Where no symbol is shown on the figure this indicates a non-significant difference.

4.3.2.2. Isolated trachea

In chapter 3 I showed that the enhanced response to 5-HT in the OVA model was maintained in the isolated trachea. I was then interested to understand whether the inhibitory effect of CS on AHR was dependent on performing measurements in the whole animal, or

whether it would be retained in the isolated tissue. This would help to determine whether the CS exposure had caused a lasting effect on the airway tissue itself or whether this phenomenon was due to processes only observed the whole animal.

In air exposed mice, OVA challenge appeared to cause an increase in the maximum response to 5-HT in addition to an increase in sensitivity; a leftward shift in the response to 5-HT was observed (Figure 4.5 A). The trachea from OVA challenged mice appeared to contract to 5-HT at a log lower dose than the trachea from saline challenged mice. The highest 5-HT concentration induced a contraction of 200mg tension in OVA exposed mice, compared to a contraction of 150mg tension in saline exposed mice; an increase of 33% compared to the contraction in saline challenged mice.

After cigarette smoke exposure, there appeared to be a very small leftward shift in the response to 5-HT in the trachea from OVA challenged mice compared to the trachea from saline challenged controls at the lower 5-HT doses (Figure 4.5). However there was no difference in the responses to higher doses of 5-HT (1μM and above) between saline and OVA challenged mice. The increased maximum tracheal response to 5-HT observed after OVA challenge was lost in the mice which had been co-exposed to CS.

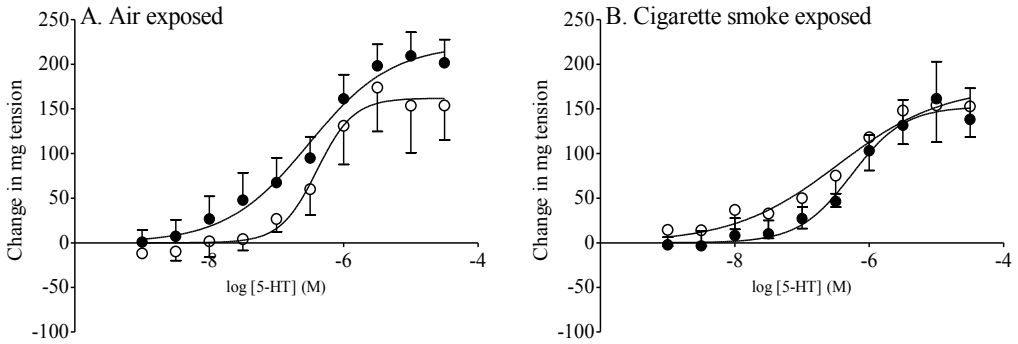


Figure 4.5: Effect of cigarette smoke on OVA-induced *in vitro* airway hyperresponsiveness.
Male C57Bl/6 mice were sensitised with Alum and OVA, and subsequently were challenged with saline (open circles) or OVA (closed circles). In addition mice were exposed to air (A) or cigarette smoke (B). Tracheal rings were subjected to a cumulative dose response to 5-HT (1nM- 30μM). Data expressed as mean change in mg tension plus or minus SEM for n=6 per group. No statistical analysis was performed on this data.

4.3.3. The effect of CS on OVA-induced LAR

Finally I looked at the effect of CS on the OVA-induced LAR. In the OVA-challenged mice there was a robust LAR, illustrated by the increase in Penh occurring approximately 2 hours after challenge (Figure 4.6). This response was substantially reduced

by treatment with budesonide. CS exposure alone did not affect airflow in the mice compared to those exposed to air/saline. In OVA and CS co-exposed mice the late response appeared to be enhanced. But most importantly, the LAR in CS co-exposed mice was no longer impacted upon by the steroid treatment; CS exposure completely blocked the steroid sensitivity of this endpoint.

In order to perform statistical analysis on the LAR I have also plotted the data at the peak in the response, approximately three hours after allergen challenge (Figure 4.7). OVA challenge resulted in a substantial increase in the mean Penh average three hours after allergen challenge, however the LAR in OVA and CS co-exposed mice was significantly greater than that observed in mice challenged with OVA alone ($p<0.01$, Mann-Whitney U-test). Treatment with budesonide ($3.mg.kg^{-1}$) resulted in an apparent reduction in the OVA-induced LAR, but this was not statistically significant ($p=0.0823$, Mann-Whitney U-test). Although the steroid treatment did not elicit a statistically significant reduction in the LAR peak, the increase in Penh in vehicle-treated/OVA-challenged mice compared to vehicle-treated/saline-challenged controls (increase in Penh of 3.4) was almost twice that observed in OVA challenged mice treated with steroid (increase in Penh of 1.8). However it is clear from this figure (4.7) that budesonide had absolutely no effect on the LAR in CS co-exposed mice (Figure 4.7).

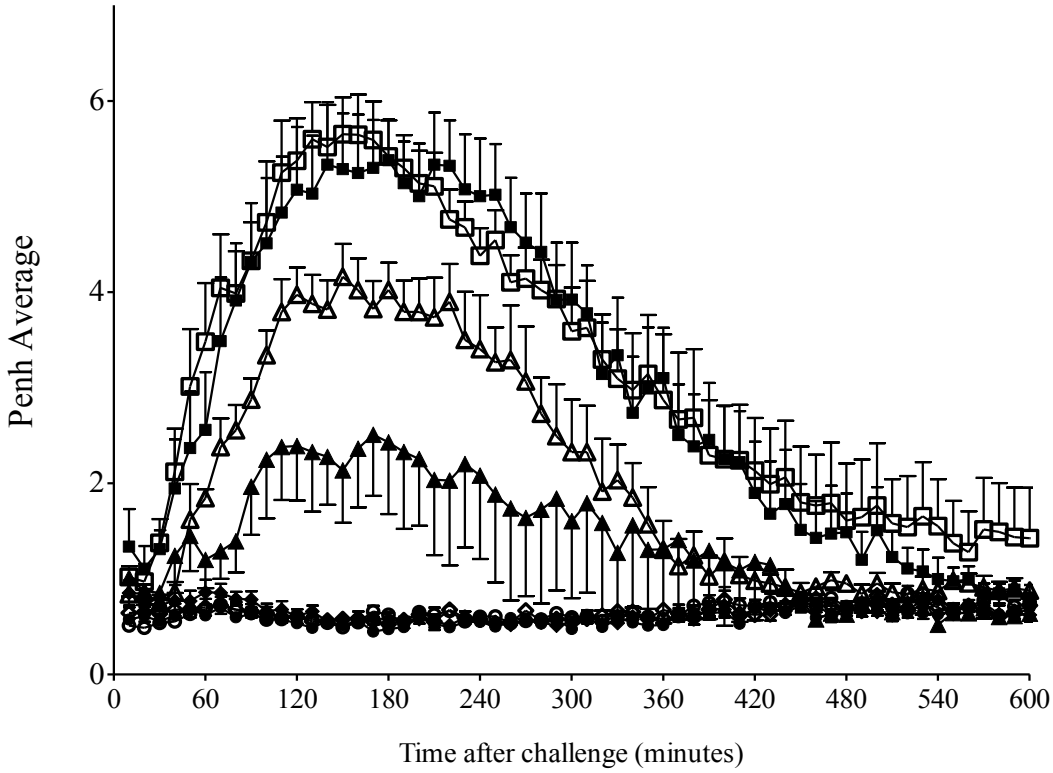


Figure 4.6: Effect of cigarette smoke on OVA-induced late asthmatic response. Male C57Bl/6 mice were sensitised with Alum and OVA and subsequently challenged intratracheally with saline or OVA. In addition mice were exposed to air or cigarette smoke and treated with vehicle (0.5% methylcellulose plus 0.2% tween80 in water) (open symbols) or budesonide (3mg.kg^{-1}) (closed symbols). Circles = air/saline, triangles = air/OVA, diamonds = smoke/saline, squares = smoke/OVA. Immediately after challenge, mice were placed in whole body plethysmography chambers and the LAR recorded as change in Penh. Data expressed as mean Penh average plus or minus SEM for n = 5-8 per group. No statistical analysis was performed on this data.

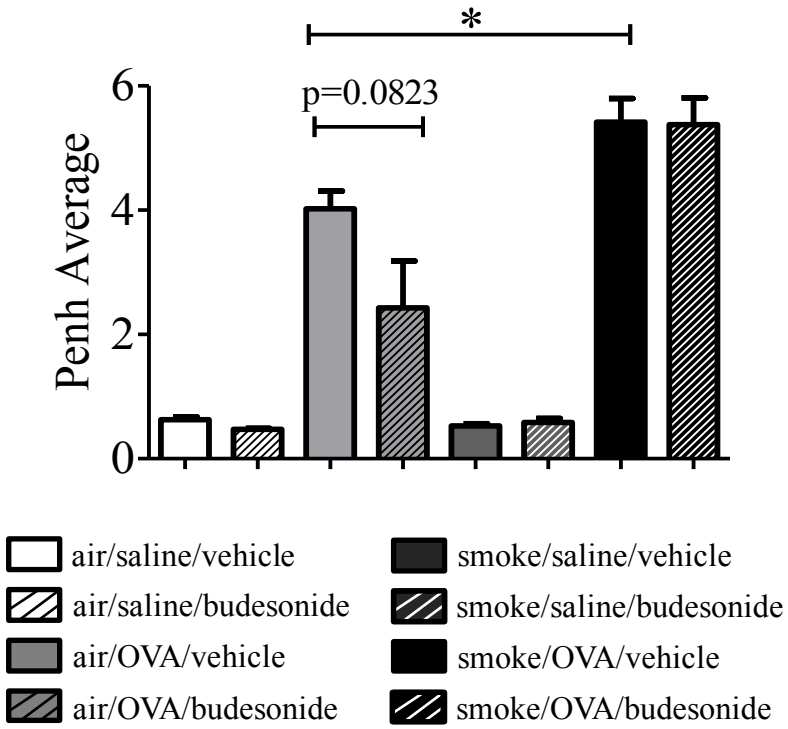


Figure 4.7: Effect of cigarette smoke on OVA-induced late asthmatic response. Male C57Bl/6 mice were sensitised with Alum and OVA and subsequently challenged intratracheally with saline or OVA. In addition mice were exposed to air or cigarette smoke and treated with vehicle (0.5% methylcellulose plus 0.2% tween80 in water) or budesonide ($3mg.kg^{-1}$). Immediately after challenge, mice were placed in whole body plethysmography chambers and the LAR recorded as change in Penh. Data expressed as mean Penh average at the peak of the response (3 hours after challenge) plus or minus SEM for n = 5-8 per group, *=$p<0.01$ Mann-Whitney U-test, CS/OVA/vehicle group vs air/OVA/vehicle group, or relevant OVA challenged/budesonide treated group compared to relevant OVA challenged/vehicle treated group. Where no symbol is shown on the figure this indicates a non-significant difference.

4.4. Discussion

4.4.1. Inflammation

OVA challenge induced an increase in airway lymphocytes and eosinophils but did not induce a significant increase in BAL macrophages or neutrophils at this time point. This is in contrast with the time-course data in the previous chapter (3) where a significant increase in macrophages and a small but significant increase in neutrophils were observed in the BAL fluid 3 days after final challenge. Small variations in responses from study to study are not uncommon and in this case are likely to be due to the increased complexity of this study compared to the time course. This study included far more handling of the animals for CS exposures and also oral dosing of steroids. The OVA-induced increase in BAL eosinophils and lymphocytes were dose-dependently inhibited by steroid treatment. This data is in-line with previously published work where systemic steroid treatment (oral dexamethasone) dose-dependently inhibited OVA-induced eosinophilia in Balb/c mice, reaching an almost complete inhibition at high (1 and 3 $mg.kg^{-1}$) doses (Birrell *et al.* 2003). Presently the BAL macrophages in mice challenged with OVA alone were reduced to levels equivalent to those in saline challenged controls by all doses of budesonide, but this reduction was not statistically significant. This is probably due to the increase in macrophages in OVA-challenged/vehicle-treated mice being small. The OVA-induced BAL neutrophilia appeared to be reduced by steroid treatment, but again this reduction was not statistically significant. The airway cellular inflammation in the OVA model is therefore largely sensitive to steroid treatment. This is not a new finding; others have previously shown steroid treatment including budesonide to be effective against OVA-induced airway inflammation (De Bie *et al.* 1996; Trifilieff *et al.* 2000; Shen *et al.* 2002; Birrell *et al.* 2003; Shen & Wang 2005). However steroid sensitive inflammation is an important feature of asthma models, and shows the model to be clinically relevant.

We have previously shown that the predominant cellular inflammatory response in our CS-driven model is an increase in BAL neutrophil levels (Eltom *et al.* 2011). Presently there was a robust increase in BAL neutrophils but none of the other cell types in response to cigarette smoke alone. This data therefore ties-in with previously published data, and shows that the CS exposure performed as expected. The increase in BAL neutrophils induced by CS alone was completely insensitive to steroid treatment. Steroid-resistant inflammation has been previously reported in CS-driven small animal models (Marwick *et al.* 2004, 2009), thus the lack of effect of steroid on the CS-induced neutrophilia is in-line with previously published data and the clinical phenotype in smokers (Cox *et al.* 1999; Culpitt *et al.* 1999).

CS exposure in conjunction with OVA challenge did not impact on the level of OVA-induced eosinophilia. This is interesting as some publications have suggested that smoking asthmatics are thought to have lower levels of airway eosinophils than non-smoking asthmatics (Broekema *et al.* 2009). However results in murine models of CS and OVA co-exposure have been varied. Some have shown CS co-exposure in mice to augment OVA-induced eosinophilia (Moerloose *et al.* 2005; Min *et al.* 2007) and to have an adjuvant effect on OVA-induced inflammation including eosinophilia (Seymour *et al.* 1997; Rumold *et al.* 2001; Moerloose *et al.* 2006; Trimble *et al.* 2009), but others have shown CS to inhibit OVA-induced eosinophilia (Melgert *et al.* 2004; Robbins *et al.* 2005; Thatcher *et al.* 2008). These disparate findings appear to be largely due to variations in when CS is given – before or after challenge – and whether the CS was treated as an adjuvant in the absence of sensitisation to OVA. Whether hardwood smoke exposure was given before or after allergen challenge also affected the impact of hardwood smoke in the OVA model (Barrett *et al.* 2006).

CS and OVA co-exposure resulted in increased levels of BAL macrophages and lymphocytes compared to levels after either challenge alone. Since CS exposure alone did not change levels of either of these cell types this enhancement is unlikely to be due to an additive effect of the two challenges acting separately; the data therefore appear to show some level of synergy between the OVA and CS challenges in this model. Although CS exposure alone did not result in increased levels of airway macrophages in these studies, smoking is thought to cause increased levels of macrophages in the lungs of smokers; in fact smoking asthmatics have been shown to have higher levels of BAL macrophages than non-smokers (Kane *et al.* 2009). Thus the data presented here appears to be similar to the phenotype observed in the clinic. In addition others have shown the combination of CS and OVA to result in increased levels of airway macrophages in murine models (Moerloose *et al.* 2005). Macrophages are phagocytic and are often attracted to sites of tissue damage to clean up debris and dead cells, especially in states of inflammation. In our standard acute CS model, CS does not induce a significant change in the levels of BAL macrophages, however in a more chronic model significant increases in macrophages are observed (Eltom *et al.* 2011). Thus it may be that the combination of these two inflammatory insults results in accelerated recruitment of macrophages to the lung.

Levels of BAL neutrophils in mice exposed to CS plus OVA were comparable with those observed in mice exposed to CS alone; therefore there did not appear to be an additive increase in neutrophils following the combined exposure.

How the effect of CS on airway neutrophils, lymphocytes or macrophages in the OVA model compares to previously published studies is unclear; these cells types are not frequently included in the articles published to date. Airway eosinophils are the typical inflammatory cellular marker used in asthma models. In Balb/c mice, neither CS exposure nor OVA challenge modulated BAL macrophage levels compared to saline/air-exposed controls, however there was a large increase in the level of BAL macrophages in the mice exposed to CS plus OVA (Moerloose *et al.* 2005); in contrast the BAL lymphocyte levels in mice exposed to CS plus OVA were equivalent to the enhanced levels seen following OVA alone. In the study by Melgert *et al.* (2004) OVA-induced increases in lung tissue macrophages were inhibited by CS exposure. The combination of OVA plus CS resulted in a level of BAL neutrophilia equivalent to levels observed in saline/air exposed controls, whereas CS induced a significant and robust increase in BAL neutrophils (Melgert *et al.* 2004). This is very interesting considering that smoking is thought to cause a neutrophilic inflammatory phenotype in asthmatic patients (Boulet *et al.* 2006; St-Laurent *et al.* 2008).

4.4.2. Anti-inflammatory effects of steroids

The BAL lymphocytes, eosinophils and macrophages in OVA and CS co-exposed mice were dose-dependently and almost completely inhibited by steroid treatment. It may be interpreted that the airway macrophages observed in the OVA and CS co-exposed mice were more responsive to steroid treatment than those in the mice challenged with OVA alone as the treatment caused a statistically significant reduction in macrophage levels in CS and OVA co-exposed mice. However it is likely that the significant response was just due to the greater window of macrophages in these mice. The macrophages in the mice exposed to OVA alone were reduced to baseline levels by steroid treatment at all steroid doses tested. Despite the reduction not being significant all doses appeared to be equally effective. In contrast, in the mice exposed to CS plus OVA the lowest dose of budesonide was only half as effective as the top dose, resulting in an approximate 50% inhibition of macrophage levels in the BAL, while the top dose elicited an almost complete inhibition of the macrophage levels. Therefore it appears that (at low doses) steroid treatment may have been less effective at inhibiting BAL macrophages in the mice exposed to OVA plus CS.

Steroid treatment had no effect on BAL neutrophil levels in mice exposed to CS plus OVA but did appear to have some effect on the neutrophils resulting from OVA exposure alone. The level of BAL neutrophils and the effect of steroid treatment in the mice exposed to OVA plus CS was almost identical to that observed in mice exposed to CS alone. For this reasons it seems likely that the neutrophilia in the co-exposed mice resulted predominantly

from the CS challenge. There was no observed synergy between the two challenges in terms of BAL neutrophilia; rather it appears that CS exposure conferred the addition of a steroid-resistant population of airway neutrophils in the co-exposed mice.

It is generally accepted that in COPD, a disease predominantly induced by cigarette smoking, glucocorticoids fail to inhibit inflammation (Keatings *et al.* 1997; Culpitt *et al.* 1999) or disease progression. Indeed no treatments other than smoking cessation are available that slow disease progression in COPD. Thus one may predict that after CS co-exposure there would be a reduction in the anti-inflammatory efficacy of steroid treatment observed in the OVA model. However in general – with the exception of BAL neutrophils – steroid treatment had a robust anti-inflammatory effect in CS and OVA co-exposed mice. Asthma and COPD do have very distinct pathological features, even in smoking asthmatics so it may well be that the situation in smoking asthmatics is not analogous to that of smoking COPD patients. There are surprisingly few clinical studies which describe the effects of steroid treatment on airway inflammation in smoking asthmatics; the studies tend to report lung function or asthma control as the primary endpoint. In addition if cellular inflammation is described, it is typically only eosinophilia which is reported, therefore there is little direct evidence on the effects of steroids on other inflammatory cells in smoking asthmatics. ICS have been shown to improve sputum eosinophils in asthmatics, but not in smoking asthmatics in short term (Chalmers *et al.* 2002) and long term studies (Pedersen *et al.* 1996), but others have shown that ICS do improve sputum eosinophils and ECP in smokers and non-smokers alike (Lazarus *et al.* 2007). Therefore the effect of smoking on the anti-inflammatory effects of steroids in asthmatics is currently controversial.

To my knowledge there is only one murine study where the effectiveness of systemic (i.p.) steroid treatment has been tested in mice co-exposed to CS and OVA. In this study dexamethasone attenuated the majority of endpoints, including BAL eosinophilia in mice exposed to CS plus OVA (Song *et al.* 2009). The eosinophil was the only BAL cell type described in this study and our cellular inflammation data therefore corresponds with this publication. In this paper however there is no data to show the effect of steroid treatment on mice exposed to OVA alone for either eosinophil data or any of the other endpoints. Although steroids almost completely inhibited the BAL eosinophilia in mice exposed to OVA plus CS the reduction is less complete for other endpoints such as AHR, smooth muscle thickness and α-smooth muscle actin staining. It may be that in these endpoints the steroid treatment would have been less effective in CS/OVA co-exposed mice than those exposed to OVA alone, but this control was not included so it is not possible to assess this.

A hypothesis regarding smoking asthmatics is that the inflammatory phenotype is more neutrophilic than that which is observed in non-smoking asthmatics (Boulet *et al.* 2006; St-Laurent *et al.* 2008; Meghji *et al.* 2011). CS is widely accepted to induce an increase in airway neutrophils (Hunninghake & Crystal 1983; Kuschner *et al.* 1996; Roth *et al.* 1998; Amin *et al.* 2000) which are insensitive to steroid treatment in smokers (Culpitt *et al.* 1999) and this cell type has been associated with a decline in lung function (Stănescu *et al.* 1996). In the above publication by Song et al the effect of CS on neutrophilia in the OVA model and its steroid sensitivity was not described, but steroid treatment inhibited the majority of endpoints measured in mice exposed to OVA plus CS. I would be interested to know what effect the CS exposure described by Song et al had on airway neutrophils. In the present study even after steroid treatment persistent neutrophilia was observed in all mice exposed to CS, including those challenged with OVA. This could indeed be representative of the clinical phenotype; neutrophil markers were shown to be diminished by long term high and low dose ICS, and oral theophylline in non-smoking asthmatics, but not in smokers (Pedersen *et al.* 1996). If smoking asthmatics do exhibit an increase in airway neutrophils, which are more resistant to steroid treatment, this persistent neutrophilia may also be responsible for the alteration in symptoms in asthmatics and reduced steroid responsiveness. The altered level of neutrophilia observed in the present study was evident despite high dose steroid treatment and may be responsible for altered functional symptoms in this model. This will be discussed in more detail later on.

There is a suggestion that in man the inflammatory profile and whether this is most contributed to by smoking or the underlying asthma is dependent on the intensity and duration of smoking history (Polosa & Thomson 2013), indeed in man and murine studies CS exposure has been shown to have dose-dependent inflammatory effects in the lung (Kuschner *et al.* 1996; Clatworthy *et al.* 2009; Eltom *et al.* 2011) thus in both the data described in the present thesis and the data presented by Song et al, a heightened CS exposure may be expected to cause a further effect on OVA model inflammation and its sensitivity to steroid treatment. It may be hypothesised that the level of CS was not enough to observe an effect on the anti-inflammatory effects of steroids; however, dramatic effects on lung function were observed, implying the CS exposure was indeed sufficient to confer phenotypic changes in the model.

As in Chapter 3 I limited my analysis of the pulmonary cellular inflammation in this model to the BAL fluid. Again this has limitations as it restricts the analysis to cells obtained from the airway lumen; however histological samples were also collected, which could be analysed in the future to provide a more comprehensive picture of the cellular inflammatory response in the model. For this thesis rather than performing further analysis of the cellular

inflammation I chose instead to move on to investigating the effect of CS on functional responses in the model.

4.4.3. AHR

In the OVA model, a robust AHR to 5-HT was observed, which replicates the AHR observed in chapter 3, and shows this feature of the model to be reproducible. In OVA-challenged mice, oral budesonide treatment blocked the AHR. This has been shown previously by other groups where steroid treatment diminished OVA-induced AHR in murine models (De Bie *et al.* 1996; Trifilieff *et al.* 2000; Shen *et al.* 2002; Birrell *et al.* 2003, 2008*b*) in organ bath studies, conscious lung function and anaesthetised lung function. AHR in asthmatics is highly sensitive to both inhaled and oral steroid treatment (Djukanović *et al.* 1992, 1997; Laitinen *et al.* 1992; Chalmers *et al.* 2002; Clearie *et al.* 2012). The efficacy of budesonide against OVA-induced AHR in this chapter further demonstrates that the model is clinically representative, and also parallels data in other published murine models.

OVA-induced AHR measured by conscious lung function was abolished by CS exposure and this finding was partially replicated in the isolated trachea. Several clinical papers suggest that smoking will worsen symptoms of asthma (Siroux *et al.* 2000; Apostol *et al.* 2002; Thomson *et al.* 2004, 2013; Eisner & Iribarren 2007; Jang *et al.* 2009; O'Byrne *et al.* 2009) yet the data presented here is at odds with this idea. However this phenomenon has been observed in the clinic in mild asthmatics (Meghji *et al.* 2011) where smoking asthmatics did not develop allergen-induced AHR to MCh. According to the data published by Meghji et al. (2011) the finding in the present thesis may therefore represent a true clinical phenotype, and it would be of great interest to pursue and understand the mechanism behind this effect. In order to elucidate the mechanism by which CS abolished the OVA-induced AHR it is first necessary to understand the mechanisms driving the AHR.

Dogma suggests that airway inflammation in asthma drives AHR; the benefit of ICS on functional features of asthma has been suggested to be due to their ability to reduce airway inflammation (Djukanović *et al.* 1992). The effect of steroid treatment in the OVA challenged mice in this model supports this hypothesis as budesonide reduced the cellular inflammatory response as well as the AHR in OVA treated mice. Therefore it may be expected that a reduction in AHR would be accompanied with a reduction in airway inflammation. In fact the inhibitory effect of CS on AHR has been reported previously by other groups in conscious and anaesthetised lung function studies in mice (Melgert *et al.* 2004; Robbins *et al.* 2005) where the reduction in AHR was associated with a reduction in

airway inflammation including eosinophilia. Melgert et al. (2004) attributed the reduction in AHR to the reduction in eosinophil levels following CS co-exposure, however (Robbins *et al.* 2005) suggested that the loss of AHR could either be attributable to a reduction in inflammation or mucus production, or alternatively due to 'inflammation-independent effects on airway smooth muscle function'. However these possibilities were not explored in more detail. What is interesting about our data is that CS exposure did not impact on BAL eosinophil levels, and enhanced the increase in BAL lymphocytes and macrophages, yet completely abolished AHR. This could mean that in the present model, OVA-induced inflammation in fact does not drive the AHR. Indeed several studies involving model characterisation, pharmacological treatments, antibodies and gene knockouts have suggested a lack of association between cellular inflammation and functional endpoints such as AHR (Hessel *et al.* 1995, 1997, 1998; Nagai *et al.* 1996; Coyle *et al.* 1998; Tournoy *et al.* 2000; Kobayashi *et al.* 2000; Mäkelä *et al.* 2000; Leckie *et al.* 2000; Birrell *et al.* 2003).

If cellular inflammation is not driving the AHR it is likely that airway remodelling, or changes in autonomic regulation may be involved. The CS-induced blockade of OVA-induced AHR observed in Penh studies was backed up by studies in the isolated trachea. The observation that AHR is observed in the isolated tissue in the OVA model alone implies that an intrinsic change in the function of the airway tissue occurs in the OVA model (as discussed in the previous chapter). The blockade of AHR by CS in the isolated tissue implies that the CS-induced inhibition of AHR is due to an inhibitory effect of CS on these tissue level changes in airway function. Adaptation of smooth muscle can lead to changes in its response to stimulation (McParland *et al.* 2005). Smooth muscle hypertrophy and hyperplasia which increases smooth muscle mass has been reported in asthma patients (Ebina *et al.* 1993) and changes in contractile properties of airway smooth muscle such as velocity and extent of muscle shortening have also been documented (Ma *et al.* 2002). The increase in airway smooth muscle mass in asthmatic patients may be explained through an increase in smooth muscle cell proliferation; this has been documented in asthmatic patients (Johnson *et al.* 2001). Therefore CS may act to inhibit the AHR by directly inhibiting ASM proliferation. In the previous chapter I suggested that changes in airway smooth muscle mass in the OVA model could be investigated by staining histological samples for α-SMA. If an increase in smooth muscle was detected in the present OVA model it would be very interesting to determine whether CS inhibited this change. To further support this work the effect of CS on ASM proliferation could also be investigated. The majority of studies into the effect of CS on smooth muscle proliferation have used vascular smooth muscle to investigate the role of CS in atherosclerosis and other cardiovascular conditions. CS has been shown to both reduce and enhance smooth muscle proliferation in these studies across

various species (Stavenow *et al.* 1983; Hu *et al.* 2007; Ferrer *et al.* 2009; Chen *et al.* 2010). However a small number have looked at the effect of CS on proliferation of ASM. While there is some evidence that CS may cause ASM proliferation (Fang *et al.* 1997; Lin *et al.* 2005*a*; Pera *et al.* 2010; Zhang *et al.* 2010; Xu *et al.* 2012), there is also evidence that CS may reduce ASM proliferation, induce cell death and reduce ASM viability (Stavenow *et al.* 1983; Fang *et al.* 1997; Yoon *et al.* 2011). Studies have also looked for a role of CS on airway smooth muscle cell proliferation in asthmatics; passively sensitised human ASMCs and cultured ASMC from asthmatic Brown Norway rats showed accelerated proliferation following exposure to CS (Zhang *et al.* 2010, 2011). CS has also been shown to modulate ASM contractility; CS caused cell death accompanied by ROS generation, reduced cell generation and a reduction in contractile phenotype (Yoon *et al.* 2011). Finally in human ASMCs, CS caused enhanced spasmogen responses, contractility effects and cell proliferation through induction of neurotrophins (Sathish *et al.* 2012). Considering the above data, a consensus on the true effect of CS on airway smooth muscle proliferation or contractility has not yet been established, and to my knowledge no studies have investigated the effect of CS on proliferation of murine ASM. Investigations into the effect of CS on cultured ASM proliferation along with histological investigations into ASM mass from the CS and OVA co-exposure model could therefore prove very interesting and may help to elucidate a mechanism behind the inhibitory effect of CS on AHR in human asthmatics (Meghji *et al.* 2011) and the murine studies described in this thesis.

It is worth noting that one study in mice measured α-SMA staining and showed that CS exposure for 4 months increased airway thickening (Melgert *et al.* 2007). Therefore an inhibition of smooth muscle remodelling may not be the mechanism by which CS inhibited the AHR in the present model. Furthermore histological studies would measure changes in smooth muscle mass in the lower airways. It is thought that there is a large contribution of the upper airways to data obtained using Penh, and the organ bath technique utilises only the trachea. Therefore it would also be prudent to confirm both the finding of OVA-induced AHR, and the CS-induced inhibition of this feature in small airways using PCLS to support the histological proliferation data. If the findings presented in this chapter were not replicated in the lower/smaller airways then histological assessment of lung slices may not be the appropriate tool to investigate smooth muscle proliferation in this model.

In the clinical paper by Meghji et al., and the two murine studies where an inhibition of AHR was observed, MCh was used as the spasmogen, whereas in the present chapter 5-HT was used. In the previous chapter 5-HT was shown to generate the most robust AHR in the models described here, however using *in vitro* studies I showed that the mice were in fact

hyperresponsive to multiple spasmogens (Chapter 3). Confirming whether CS also inhibited OVA-induced AHR to other spasmogens would further support the hypothesis that CS exposure inhibited the AHR through a direct effect on airway smooth muscle function.

Aside from smooth muscle remodelling another type of remodelling which is characteristic of asthma is an increase in collagen deposition in the airway wall (Davies *et al.* 2003). Many papers have suggested that CS exposure or smoking can cause remodelling of the airways. Smokers had increased area of longitudinal bundles consisting of elastic fibres, collagen and myofibroblast matrix (Carroll *et al.* 2000). On its own, long term CS exposure in mice induced features of remodelling such as collagen deposition (Melgert *et al.* 2007). CS has also been shown to impact upon airway remodelling in murine allergen-driven asthma models. CS exposure enhanced remodelling in a murine OVA model (Min *et al.* 2007) and a guinea pig fibrosis model (Cisneros-Lira *et al.* 2003), and increased collagen levels compared to HDM-challenged mice (Botelho *et al.* 2011). Therefore it seems likely that CS may impact on collagen deposition in the OVA model; but what effect would this have on AHR? The elastic properties of the airways may play a role in AHR (Khan *et al.* 2010) and an increase in collage deposition could diminish AHR by opposing smooth muscle contraction. To support this hypothesis, collagenase and elastase treatment to reduce collagen levels resulted in AHR and increased contraction magnitude and velocity in human bronchial strips and murine lung slices (Bramley *et al.* 1995; Khan *et al.* 2010). In addition AHR occurred in an acute BN asthma model, but not a chronic model where collagen deposition occurred (Palmans *et al.* 2000). A CS-induced increase in collagen is therefore another mechanism which could be involved in the CS-inhibited AHR in the present model.

TGF-β is a mediator typically associated with remodelling effects and plays an important role in cell proliferation. CS may promote airway remodelling via an increase in TGF-β and collagen levels (Churg *et al.* 2006; Guo *et al.* 2008; Hizume *et al.* 2012). TGF-β levels were found to be increased in the plasma of COPD patients compared to healthy controls which correlated with a reduction in lung function (Mak *et al.* 1995) and most interestingly TGF-β has been shown to inhibit AHR (Hansen *et al.* 2000; Schramm *et al.* 2003). Furthermore in a murine OVA model CS exposure enhanced collagen deposition and TGF-β expression (Kim *et al.* 2011). Therefore investigating TGF-β and collagen deposition in the present study may also be useful to further understand the effect of CS on AHR. Collagen levels can be assessed in the airways through staining histological lung sections with picro-sirius red as previously described (Last *et al.* 2004), where ovalbumin exposure resulted in an increase in collagen levels in murine airways. In addition TGF-β levels could be assessed by ELISA. It is worth noting that subepithelial collagen deposition induced by TGF-β was associated with AHR in Balb/c mice (Kenyon *et al.* 2003) so there is some evidence to question this hypothesis. Furthermore it may be that that dramatic remodelling

effects will not be observed following the relatively acute CS exposure in this model as remodelling tends to require much longer exposure regimens than inflammatory changes; collagen deposition was noted in a 12 week allergen study in rats, but not in an analogous two week study (Palmans *et al.* 2000).

In a study where CS inhibited both AHR and inflammation another suggested mechanism for the dampening effect of CS was an imbalance between Th1 and Th2 activity (Melgert *et al.* 2004). In this thesis no cytokine measurements have been described in either the OVA model, or the OVA and CS co-exposed mice. The reason for this is that endpoints were assessed at 72 hours after final allergen challenge, in order to optimise AHR measurements. Previous experience measuring cytokine release in allergen models suggested that most of the protein or gene level signal would be lost at the 72 hour time point. Further samples taken at an earlier time point would be needed to perform robust measurements of cytokine levels. However mediator analysis would also be useful to further understand the phenotype in the OVA and CS co-exposed mice. Cytokines of the Th2 phenotype such as IL-13 and IL-5 and also IL-17 are thought to be important in AHR (for example: Foster *et al.* 1996; Hamelmann *et al.* 1997, 1999, 2000; Grünig *et al.* 1998; Mattes *et al.* 2001; Barczyk *et al.* 2003; Barlow *et al.* 2012). An observed reduction in IL-13, IL-5 or IL17 may therefore explain the loss of AHR in CS exposed mice. Interestingly in the study by Robbins et al where CS reduced OVA-induced eosinophilia and AHR the trend was for an increased level of IL-5 and IL-13 in the BAL fluid (Robbins *et al.* 2005) suggesting the loss of AHR and eosinophilia wasn't due to reduced Th2 activity, but in the study by Melgert et al. (2004) no cytokines appeared to be measured. There is not much in the literature on the connection between smoking and Th17 responses in general or in asthma, however a 4 month CS exposure induced a Th17 type phenotype illustrated by high levels of neutrophils, macrophages, B cells, IL-17, IL-6, G-CSF and GM-CSF (Melgert *et al.* 2007). Interestingly IL-17 may influence airway remodelling through promoting the release of pro-fibrotic cytokine release from fibroblasts (Molet *et al.* 2001) and exogenous IL-17 reduced allergen-induced AHR (Schnyder-Candrian *et al.* 2006), so Th17 cells may also be involved in the CS mediated inhibition of the AHR in this model. Measuring cytokines such as IL-13, IL-5 and IL-17 may aid further understanding of the effect of CS in the OVA model described in this chapter.

Inducible nitric oxide synthase (iNOS) was another suggested mechanism behind the dampening of inflammation by cigarette smoke (Melgert *et al.* 2004) which is also an interesting possibility. iNOS is thought to be the most important NOS isoform involved in

the generation of nitric oxide (NO) under pathological conditions and cigarette smoke can induce iNOS expression (Chang *et al.* 2001). NO has been shown to relax airway smooth muscle *in vitro* (Munakata *et al.* 1990) and has been shown to relax guinea pig airways *in vivo* (Dupuy *et al.* 1992). NO is also important in nerve-dependant bronchodilation as it has been shown to be the neurotransmitter of human iNANC responses *in vitro* (Belvisi *et al.* 1992*a*, *b*; Bai & Bramley 1993; Ward *et al.* 1995), the only endogenous neuronal bronchodilator mechanism in man. Therefore it is indeed possible that CS-induced NO production could be a mechanism by which CS inhibits AHR. Measurements of NO, or iNOS expression may therefore be worthwhile to see if this is involved in the CS-induced inhibition of AHR in the present studies. This could also be investigated by treatment with a broad spectrum nitric oxide synthase inhibitor such as L-NAME. Fraction of exhaled NO (FeNO) is regularly used as a diagnostic and experimental biomarker of asthma, but interestingly publications have suggested that smoking asthmatics have reduced FeNO (McSharry *et al.* 2005; Spears *et al.* 2011) and CS has been shown to reduce pulmonary eNOS expression in guinea pigs (Ferrer *et al.* 2009). This implies that in the present model CS is unlikely to be inhibiting the AHR through NO release.

All of the above factors would be worth investigating to help elucidate the mechanism by which CS inhibited the AHR in this model, and may provide clues as to the mechanism behind the effect of CS in smoking asthmatic patients.

4.4.4. LAR

The LAR induced by OVA-alone was diminished by steroid treatment. Although at the peak of the response this inhibition was not statistically significant, the increase in Penh after OVA challenge was reduced by approximately one half in mice treated with steroids. The OVA-induced late response was significantly increased following CS co-exposure, which correlates with clinical studies where smoking has been shown to worsen asthma symptoms (Siroux *et al.* 2000; Apostol *et al.* 2002; Thomson *et al.* 2004, 2013; Eisner & Iribarren 2007; Jang *et al.* 2009; O'Byrne *et al.* 2009). However none of these papers directly measure the effect of CS on the response to allergen challenge in asthmatics. Most studies measure AHR or FEV_1 as the functional markers of asthma. Although allergen inhalation challenge is generally well tolerated it may induce severe side effects such as anaphylaxis, acute bronchoconstriction and prolonged disease exacerbation (Gauvreau & Evans 2007). The preference for measurement of inflammation, FEV_1 and AHR may be reflected in these difficulties associated with allergen challenge. As I have previously

mentioned, a recent publication suggests that smoking asthmatics may not exhibit OVA-induced AHR (Meghji *et al.* 2011). This implies that AHR may not be the most appropriate functional endpoint for studying asthmatics, as this paper clearly shows that AHR is not observed in all asthmatics. This also suggests that it is important to measure allergen-induced bronchoconstrictive responses in addition to the AHR. None of the previous murine studies which have investigated the effect of CS in OVA-driven models have studied the LAR; most research groups have opted for AHR as the functional endpoint for murine studies. Therefore the CS-induced increase in the OVA-induced LAR observed in this chapter is a novel finding. Only one study has looked at the effect of CS on the response to allergen challenge in asthmatics (Meghji *et al.* 2011); however in this study the magnitude of the fall in FEV_1 during the early and late responses were similar in both smokers and non-smokers. This study concerned mild asthmatic patients and the smoking and non-smoking groups were matched for baseline FEV_1, methacholine PC_{20} and basal eosinophil levels. It may be that a difference in the EAR and LAR in normal and smoking asthmatics was not observed because the subjects were mild asthmatics, matched for asthma severity.

An accelerated decline in lung function is observed in COPD patients compared to healthy subjects (Fletcher & Peto 1977), where the predominant cause is smoking. Smoking is also associated with a reduced lung function in asthmatics (Grol *et al.* 1999; Harmsen *et al.* 2010). However no change in baseline Penh was detected in the CS-exposed animals; the smoke exposure in the present chapter therefore did not cause a reduction in lung function, however due to the relatively short nature of the CS regimen this is not surprising.

In the present studies, and in previously published work (Raemdonck *et al.* 2012) the OVA-induced LAR was sensitive to steroid treatment. Steroid treatment has previously been shown to be effective against the allergen-induced LAR in the clinic (Cockcroft & Murdock 1987; Paggiaro *et al.* 1994; Kidney *et al.* 1997; Inman *et al.* 2001; Leigh *et al.* 2002). The most striking finding from the data presented in this chapter was that CS exposure rendered the OVA-induced LAR completely insensitive to steroid treatment; to my knowledge this has not been previously reported. The two primary differences observed in the Meghji study between smokers and non smokers were an increase in airway neutrophils, and the lack of AHR in the smokers. Our model therefore appears to parallel the characteristics observed in the smoking cohort described by Meghji et al. (2011). I would therefore be very interested to know what effect CS would have had on the efficacy of steroid treatment in these patients. Given the general acceptance that smoking is associated with a reduction in steroid sensitivity, the present model may provide a clinically relevant, functional parameter of an allergic asthmatic response, which could be further used to understand the steroid resistance observed in smoking asthmatics. If our results concerning the lack of efficacy of steroid

treatment in CS co-exposed mice were replicated in the clinic, this could have implications for the treatment of smoking asthmatics.

There are several possible mechanisms for steroid resistance that have been implicated in asthma. One of the mechanisms by which glucocorticoids reduce inflammatory gene expression is through recruitment of HDACs (Ito *et al.* 2006; Tsaprouni *et al.* 2007), and subsequent modification of the histone complex and chromatin structure of DNA. CS exposure has been shown to reduce HDAC2 activity (Marwick *et al.* 2004) and impaired HDAC2 activity has also been suggested as a mechanism for a reduced effect of steroids on NF-κB mediated inflammation (Ito *et al.* 2006). Impaired GR binding to GRE or to AP-1 or an increased level of GR-β have been observed in steroid insensitive asthmatics (Adcock *et al.* 1995*a*, *b*; Leung *et al.* 1997; Hamid *et al.* 1999). All of these factors may play a role in steroid resistance in asthma and in the present model, however since steroid treatment significantly reduced the levels of airway eosinophils, macrophages and lymphocytes in CS co-exposed mice it could be argued that the above mechanisms are not involved. In contrast the airway neutrophils in CS or CS and OVA exposed mice did not respond to steroid treatment therefore different cell types appear to respond differently to CS exposure and/or steroid treatment.

CS is widely accepted to induce an increase in airway neutrophils (Hunninghake & Crystal 1983; Kuschner *et al.* 1996; Roth *et al.* 1998; Amin *et al.* 2000) and increased levels of neutrophils are observed in the lungs of smoking asthmatics. Furthermore the neutrophil has been associated with worsened lung function (Stănescu *et al.* 1996; Boulet *et al.* 2006; St-Laurent *et al.* 2008; Meghji *et al.* 2011). Neutrophils and neutrophil markers are thought not to respond to steroid treatment, or in some cases to even be increased by it (Tanizaki *et al.* 1993; Pedersen *et al.* 1996; Culpitt *et al.* 1999; Gauvreau *et al.* 2002); steroids inhibit neutrophil apoptosis (Cox 1995). Furthermore neutrophils were also found to be increased in the BAL fluid from steroid-dependent intractable asthma patients (Tanizaki *et al.* 1993) and high levels of airway neutrophils were associated with a poor response to ICS treatment (Green *et al.* 2002). Interestingly levels of sputum neutrophils were inversely correlated with the level of inhibition of allergen-induced sputum eosinophils, and the inhibition of the LAR correlated with the level of reduction in sputum eosinophils by steroid treatment (Gauvreau *et al.* 2002). This finding implies that in the clinic there may be a connection between the level of pulmonary neutrophils and the ability of steroid treatment to inhibit the LAR. Therefore the persistent CS-induced neutrophilia in the CS and OVA co-exposed mice may be responsible for the steroid-insensitive and enhanced LAR in this group; the neutrophil levels in the OVA model and the response of these cells to steroid treatment parallel the LAR and its response to steroids. A model has been proposed whereby C3a drives the LAR

after allergen challenge by production of IL-1β and neutrophil recruitment into the lungs (Mizutani *et al.* 2009) which supports a role for neutrophils in the LAR in the present model.

In the past T helper responses have been thought to be polarised to either Th1 or Th2 type, however this is now thought to be too straight-forward due to the identification of further Th subsets which have been implicated in diseases such as asthma. One such subset is Th17 (Lindén 2001; Hellings *et al.* 2003; Prause *et al.* 2004; Zhou *et al.* 2005*b*; McKinley *et al.* 2008; Wakashin *et al.* 2008; Wilson *et al.* 2009; Kawaguchi *et al.* 2009; Souwer *et al.* 2010; Bajoriūnienė *et al.* 2012; Kudo *et al.* 2012) of which the two primary mediators are IL-17 and IL-22. IL-17 has been detected in asthma patients and may be related to asthma severity (Molet *et al.* 2001; Barczyk *et al.* 2003; Hashimoto *et al.* 2005; Agache *et al.* 2010). Currently, however the role of Th17 in murine models is controversial. Some data suggests IL-17 to be involved in both allergen-induced late responses (Nakae *et al.* 2002) and allergen induced eosinophilia and neutrophilia (Schnyder-Candrian *et al.* 2006), however in contrast exogenously applied IL-17 inhibited OVA-induced AHR and reduced recruitment of eosinophils and lymphocytes (Schnyder-Candrian *et al.* 2006). In this study it was suggested that IL-17 may have opposing roles during initiation (sensitisation) compared to challenge (effector phase). IL-17 has been implicated in the recruitment of neutrophils to the airways (Lindén 2001; Zhou *et al.* 2005*b*) and transfer of Th17 cells to mice resulted in influx of neutrophils and AHR which were both insensitive to steroid treatment (McKinley *et al.* 2008). Conversely Th2 cell transfer resulted in steroid sensitive lymphocyte and eosinophil induction and AHR (McKinley *et al.* 2008). Furthermore IL-17 has been shown to reduce glucocorticoid sensitivity of mediator production by airway epithelial cells via an PI3K activation and a reduction in HDAC activity (Zijlstra *et al.* 2012). Although there is not much evidence to connect smoking and Th17 responses one chronic murine study documented CS exposure to induce a Th17 type response including high levels of neutrophils (Melgert *et al.* 2007). Thus a Th17 type response and associated neutrophilia may be involved in the steroid-resistant LAR in this model.

Aside from the profile of cellular inflammation there are several other reasons which may explain why steroids were less effective in the LAR in CS co-exposed mice. Our group has previously shown that the LAR may be mediated by allergen-induced release of a TRPA1 ligand which results in airway sensory nerve activation and a reflex cholinergic contraction (Raemdonck *et al.* 2012). The TRPA1 channel has also been previously implicated in other allergen-driven asthma models (Caceres *et al.* 2009) where TRPA1 knockout or pharmacological inhibition attenuated allergen-induced cellular influx, cytokine

production, mucus production and AHR. Although we have not fully elucidated the mechanism behind the LAR in this model, it is likely that endogenous transcription of a TRPA1 activator from an as yet unidentified cell type is responsible for the TRPA1 activation. Gene expression and inflammation are controlled by transcription factors such as NF-κB and AP-1 and the efficacy of both steroids and an IκK inhibitor in the LAR support the above hypothesis (Birrell *et al.* 2005; Raemdonck *et al.* 2012). In the present studies the LAR began approximately 1 hour after challenge. For TRPA1 mediator synthesis to be involved in the LAR this would need to occur rapidly. Transcription factor activation in cultured cells can be detected as soon as 1 hour after stimulation (Birrell *et al.* 2005, 2008*a*) and increased levels of inflammatory cytokines and transcription factor activation can be detected as soon as 2 hours after stimulation *in vivo* (Eltom *et al.* 2011; Rastrick *et al.* 2013) so it is indeed possible for mediators to be released and stimulate airway sensory nerves within the timescale of the LAR.

TRPA1 on sensory nerves has been shown to be a sensor of oxidative stress and ROS, which results in sensory neuronal activation in mice (Andersson *et al.* 2008; Bessac *et al.* 2008). CS contains multiple oxidants (reviewed in (Pryor & Stone 1993) and exposure to CS is widely known to induce oxidative stress and tissue damage. It also causes TRPA1 activation (Andrè *et al.* 2008) via toxins such as acrolein, crotonoaldehyde and hydrogen peroxide (Simon & Liedtke 2008). CS may therefore provide an exogenous TRPA1 activator, or stimulate release of ROS which activate TRPA1. This would circumvent the need for mediator transcription, the step in which steroids are likely act to inhibit the LAR. As mentioned neutrophils are highly responsive to CS exposure (Hunninghake & Crystal 1983; Kuschner *et al.* 1996; Roth *et al.* 1998; Amin *et al.* 2000), and are capable of releasing multiple reactive oxygen species. Therefore in line with previous discussions the neutrophil may play a role in TRPA1 activation in the LAR, and as such may be responsible for the steroid insensitivity of this endpoint. There are several ways to investigate the above hypotheses and I will discuss these in the future studies in Chapter 7.

What does this mean for the treatment of smoking asthmatics? Importantly TRPA1 blockers have been suggested as a treatment for allergic asthmatics (Belvisi *et al.* 2011), however if the above proposed mechanism for the effect of CS in the LAR is correct, this treatment approach may not be appropriate in patients that smoke. One of the important arms of the mechanism for the LAR devised by Raemdonck *et al.* (2012) is a cholinergic contraction of ASM. Therefore a long acting muscarinic antagonist (LAMA) such as tiotropium may be a more appropriate option, and this drug is already being evaluated as an add-on therapy for treatment resistant asthmatics (Bateman *et al.* 2008; Barnes 2010*b*;

Nogami *et al.* 2012; Antoniu & Antohe 2013). It would be interesting to see whether a LAMA proved to be effective in the LAR in CS co-exposed mice where steroids were not.

Another hypothesis regarding CS-induced steroid resistance is that CS inhibits HDAC recruitment and activity, and thus negates one of the mechanisms by which steroids act (Marwick *et al.* 2004; Adenuga *et al.* 2009). Theophylline is thought to restore steroid sensitivity (Cosio *et al.* 2009; To *et al.* 2010; Sun *et al.* 2012) and has been suggested as a treatment approach in COPD patients (Barnes 2003, 2010*a*) with combined treatment showing efficacy in some studies (Cosio *et al.* 2009; Ford *et al.* 2010). This hypothesis and treatment approach may also be of relevance in the asthma field. Indeed theophylline has shown to inhibit allergen-induced symptoms in asthmatics (Crescioli *et al.* 1991) and has shown benefit as an add-on therapy (Spears *et al.* 2009). Although theophylline is thought to have many targets its effect on steroid sensitivity is thought to be through PI3K-δ inhibition (To *et al.* 2010), which lead to the proposal of using PI3K-δ inhibition to restore glucocorticoid responsiveness (Marwick *et al.* 2009, 2010). It would therefore be interesting to determine whether theophylline or a PI3K-δ inhibitor could restore steroid sensitivity of the LAR in the present model. This would provide further support for the use of theophylline as an add-on therapy in severe asthmatics. Leukotriene antagonists are another class of drugs with potential as an add-on therapy for asthmatics. It would also be interesting to test the efficacy of a leukotriene antagonist such as monteleukast in the present model. It is possible that this would not be effective here as leukotriene mediators were shown not to be involved in the group's rat LAR model (Raemdonck *et al.* 2012). However they may show efficacy in the smoke-enhanced LAR because previously, monteleukast improved symptoms of asthma in smokers but not in non-smokers (Lazarus *et al.* 2007). In smoking asthmatics there is some debate as to whether increasing the ICS dose or an add-on therapy such as a LABA is more effective (Tomlinson *et al.* 2005; Clearie *et al.* 2012). Thus I would also like to test the effect of a LABA in addition to steroid treatment in this model. It is however noted that differences in sensitivity to LABAs are likely to be observed in this model compared to the clinical response because bronchodilation in the mouse is mediated via the β_1-adrenoceptor (Henry & Goldie 1990), whereas in man it is mediated by β_2. Inclusion of the appropriate controls (LABA treated/air exposed/OVA challenged subjects) would therefore be vital to obtain meaningful data from these studies.

Comprehensive profiling of therapeutic responses in the CS enhanced LAR model described here could help to further understand the clinical predictivity of the model and further support its use for evaluation of potential therapies. The studies in mouse which have combined CS and OVA have not generally tested the efficacy of any treatments in these models. Thus investigating this in our model would provide some very novel data. Given the

lack of effect of steroid in the CS-enhanced LAR, it may well be an exciting model in which to search for compounds which may be effective in treatment-resistant asthma. The LAR is a key feature of allergic asthma in the clinic, so this model may indeed provide a clinically relevant model in which to investigate the processes behind steroid resistance in smoking asthmatics.

Chapter 5. Development of an allergic HDM-driven model of asthma

5.1. Rationale

In Chapter 4 I showed CS to have divergent effects in the OVA-driven asthma model both inhibiting the OVA-induced AHR and rendering the LAR insensitive to steroid treatment. I then wanted to parallel this work in a model driven by a more clinically relevant allergen: HDM. The multiple-challenge, topical HDM model has become increasingly used over the last 10 years (i.e. (Johnson *et al.* 2004). Our group has doubts about the relevance of these models because despite the clinical relevance of the allergen model endpoints concentrate on inflammation, remodelling and AHR rather than classical allergen responses such as EAR or LAR in mice following HDM challenge. Considering that the asthmatic phenotype in these models is generated as a composite response to multiple exposures over a period of time it is difficult to know how an LAR in this model would be measured. In man and our murine OVA model a single allergen challenge in sensitised mice (or asthmatics) results in a clear late phase bronchoconstriction – the LAR (Booij-Noord *et al.* 1971; Robertson *et al.* 1974; Raemdonck *et al.* 2012). In addition it is possible that much of the airway inflammation observed in response to HDM is due to innate mechanisms rather than allergic mechanisms (De Alba *et al.* 2010; Birrell *et al.* 2010). Finally all challenged mice typically respond to HDM, whereas in man, only certain individuals become sensitised to an allergen, presumably because of genetic factors which promote atopy. The above factors draw into question the relevance of these HDM-driven models with regards to allergic asthma, despite using a clinically relevant allergen.

In protocols such as the OVA model described in Chapter 3 a separate sensitisation and challenge phase is used and only sensitised mice respond to the OVA challenge. I therefore set out to develop a HDM-driven allergic model based around the separate sensitisation and challenge phases of the in-house OVA model, where only the mice which had been sensitised would respond to HDM challenge. This would hopefully reduce the component of acute inflammatory response in the model. In addition it is hoped that the approach of sensitisation followed by challenge would make it more likely that the LAR would be observed in this model.

In the field data has been reported using both recombinant HDM allergenic protein such as Der p 1, and whole mite extract. HDM extract has been shown to achieve Th2 sensitisation, eosinophilia and AHR in Balb/c mice but intranasally delivered recombinant Der p 1 alone failed to induce inflammation in the airways (Cates *et al.* 2004). In humans, although recombinant Der p 1, Der p 2 and mite extract caused a similar EAR, the LAR and

AHR were much stronger in those challenged with HDM extract. This implies that constituents of HDM other than the major allergens Der p 1 and 2 may be important in the allergic responses to HDM in asthmatics (Van Der Veen *et al.* 2001). In addition although Der p 1 and Der p 2 are thought to be the major allergens in HDM, multiple other protein components have been shown to have IgE binding capabilities and biological activity (Thomas *et al.* 2002). Therefore I have opted to use HDM extract for the studies in this thesis rather than isolated or recombinant proteins.

The content of commercially available HDM extract varies from batch to batch in terms of its content of major allergenic proteins Der p 1 and Der p 2 (Meyer *et al.* 1994), and other constituents such as LPS. Different HDM batches have been shown to induce different responses in murine comparison studies, possibly based on their biochemical properties (Post *et al.* 2012). The majority of murine studies using HDM extract utilise a specific concentration of total protein in their models, for example a dose of 25μg per mouse (Fattouh *et al.* 2008; Phipps *et al.* 2009; Botelho *et al.* 2011; Chen *et al.* 2013). Consequently as batches vary one may expect to see variations in results due to the varied level of Der p 1 delivered. Because of this I have decided to calculate HDM doses based on the concentration of Der p 1 in the extract. In this thesis all studies will use the same batch of HDM obtained from Greer (lot number 124632, the details of which are provided below).

In order to develop the model the first step would be to optimise a sensitisation dose and route. The majority of publications on murine OVA models utilise systemic sensitisation to OVA in conjunction with an adjuvant. In contrast, the recent publications using intranasal HDM models are generated in the absence of adjuvant or even any systemic sensitisation, which suggests that an adjuvant is not required (Johnson *et al.* 2004; Cates *et al.* 2004; Phipps *et al.* 2009). To develop the sensitisation phase of the model the ability of intranasal HDM administration to induce sensitisation will be compared with systemic sensitisation which is the route used for sensitisation in the OVA model. I will also compare the efficacy of systemic sensitisation in the absence and presence of Alum. In allergy diagnosis, clinical studies and murine models, IgE levels are commonly used as a marker of allergic sensitisation, therefore I will measure plasma total and HDM-specific IgE following sensitisation to determine the optimum sensitisation dose and route. The next phase would be to perform a dose response to allergen challenge in sensitised mice to establish a challenge dose which caused airway inflammation only in mice previously sensitised to HDM. Then, utilising the endpoints optimised in the OVA model (Chapter 3) I will determine whether allergic AHR and the LAR can also be demonstrated in this model. These endpoints are all important to establish the clinical relevance of the model.

5.2. Methods

5.2.1. HDM

HDM extract, purified from *Dermatophagoides pteronyssinus* (Der p; GREER laboratories, USA) was used for all experiments (lot number 124632, see table 5.1 for details). HDM concentrations are calculated based on the HDM (Der p 1) content of the batch used rather than total protein. Concentrations of HDM therefore refer to the quantity of Der p1.

Component	Content
Der p 1	121.06µg/vial
Dry weight	14.83mg/vial
Protein	4.79mg/vial
Endotoxin	125 EU/vial

Table 5.1:Details of the HDM extract used for all studies in this thesis

5.2.2. Optimising a HDM sensitisation protocol

The first stage towards developing the acute HDM model was to establish an optimum sensitisation dose and route using a regimen adapted from the previously described OVA model (figure 5.1).

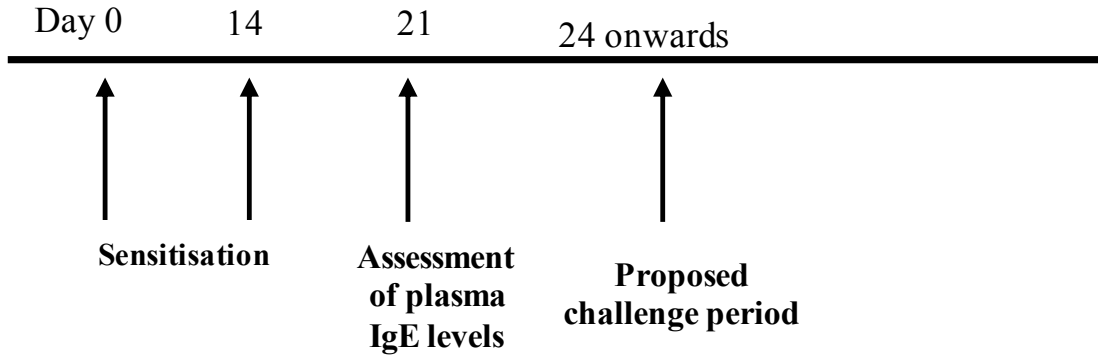


Figure 5.1: Schematic diagram of HDM sensitisation dose selection study

For topical sensitisation (i.n.) mice received saline or HDM (0.005-500µg/kg) on day 0 and 14. For systemic sensitisation (i.p.) mice received saline, Alum (diluted 1:1 with saline), HDM (0.005-500µg/kg,) or Alum + HDM (0.005-500µg/kg) on day 0 and 14.

Mice were given an overdose of sodium pentobarbitone (200 mg/kg, i.p.) on day 21 and heparinised blood samples were taken by cardiac puncture. Total and HDM-specific plasma IgE levels were measured by ELISA. The measurement of IgE levels 7 days after final sensitisation has been reported previously (Hessel *et al.* 1995).

5.2.3. HDM challenge dose response

Having selected a sensitising dose and route I then performed a dose response to HDM challenge in sensitised mice. The goal was to select a dose which only caused inflammation in mice which had previously been sensitised with HDM, and not in those sensitised with vehicle. Mice were sensitised on day 0 and 14 with saline in Alum or HDM in Alum (0.5µg.kg^{-1} i.p). Mice were challenged intranasally with saline or HDM on days 24-26: paralleling the OVA model. In a published model a dose of 25µg HDM per mouse has been shown to cause non-allergic inflammation (Eltom *et al.* 2010). HDM aliquots may contain as little as 10% Der p 1, therefore this dose is equivalent to around 125µg.kg^{-1} Der p 1. A dose response of 0.125-125µg.kg^{-1} was performed to encompass this published dose and several lower doses. The highest dose would serve as a positive control, to enable selection of a dose which did not induce this non-allergic inflammation. It was important that any non-allergic inflammation would be detected, therefore I chose to assess inflammation in this dose response 24 hours after final challenge as this is a common time point used for assessing response to an innate challenge such as LPS (Hardaker *et al.* 2010) in vivo. Mice were therefore culled by overdose with sodium pentobarbitone (200 mg/kg, i.p.) and inflammation was assessed in the BAL fluid 24 hours after final HDM challenge. A schematic for this study is shown in figure 5.2.

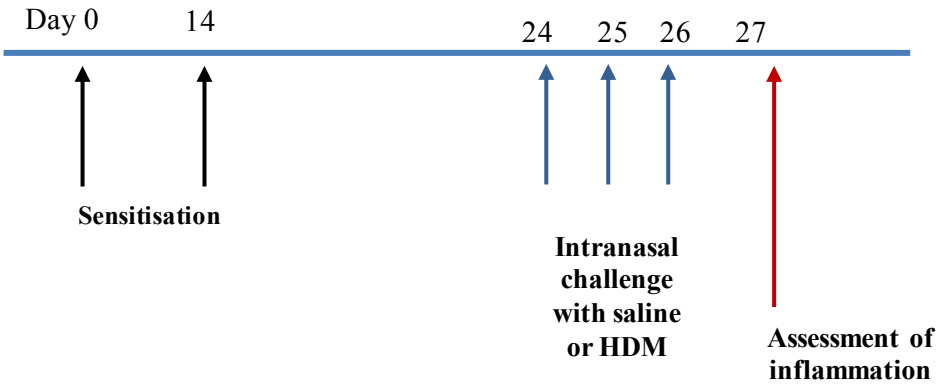


Figure 5.2: Schematic diagram of HDM challenge dose selection study

5.2.4. Assessing the requirement for Alum during sensitisation in the allergic response to HDM-challenge

I was interested to determine whether the presence of Alum during sensitisation was necessary to observe an allergic response to HDM challenge. To investigate this, mice were sensitised (i.p.) on day 0 and 14 with saline, Alum, HDM ($0.5\mu g.kg^{-1}$) or Alum plus HDM ($0.5\mu g.kg^{-1}$). Mice were then challenged intranasally once daily on days 24-26 with saline or HDM ($1.25\mu g.kg^{-1}$). In the previous study, inflammation was assessed 24 hours after final challenge, however in-lieu of performing a comprehensive time course in the HDM model, this study also provided the opportunity to assess the inflammatory profile in this model 3 days after final challenge – the time point used in the OVA model. If this time point proved suitable to assess the allergic inflammation in this model it would be used for subsequent studies. I would then utilise this time point to determine whether the inflammation in this model was also accompanied by AHR.

5.2.5. Does sensitisation and challenge with HDM induce airway hyperresponsiveness to 5-HT?

I sought to determine whether airway hyperresponsiveness was observed in the HDM model. AHR to 5-HT was most reproducibly observed in the OVA model compared to ACh or MCh. Therefore a dose response to 5-HT was performed in HDM-sensitised and –challenged mice.

Having shown that Alum was dispensable in this model, this study was performed without Alum during sensitisation. Mice were sensitised on day 0 and 14 with saline or HDM ($0.5\mu g.kg^{-1}$) i.p. and then challenged with saline or HDM ($1.25\mu g.kg^{-1}$). Mice were

then placed in whole body plethysmography chambers 3 days after final HDM challenge, and airway responsiveness to 5-HT (1, 3, 10mg.ml^{-1}) was assessed (Penh).

5.2.6. Does topical sensitisation promote an allergic response to HDM challenge despite not inducing a detectable increase in IgE?

In earlier experiments I investigating which sensitisation route was most effective for inducing an increase in HDM-specific and total plasma IgE. Intranasal sensitisation failed to induce an increase in either of these endpoints compared to vehicle-sensitised mice, whereas an increase in IgE was observed in systemically-sensitised mice. Throughout the field topical sensitisation has become the preferred route of sensitisation for allergic asthma models, therefore I was interested to determine whether HDM challenge would induce an allergic inflammatory response in mice sensitised to HDM intranasally, despite the lack of IgE.

Mice were sensitised on days 0 and 14 with saline or 0.5µg.kg^{-1} HDM both intranasally and systemically (i.p.) without Alum. On days 24-26 mice were challenged intranasally once daily with saline or 1.25µg.kg^{-1} HDM and inflammation was assessed in the BAL fluid 3 days after final challenge.

5.2.7. HDM-induced late asthmatic response

A key symptom of allergic asthma in the clinic is the late asthmatic response which occurs after allergen exposure. In the OVA model this was not observed after any of the three standard OVA challenges in the model used to generate allergic inflammation and AHR. A modified protocol is therefore used to achieve the LAR as detailed in table 5.2. An LAR was also not detected using whole body plethysmography after any of the three intranasal challenges in the HDM model. Thus in an attempt to generate the LAR in the HDM model a modified protocol was employed, analogous to that used for the OVA-LAR model. Sensitisation and challenge doses were titrated from the allergic HDM model as described in table 5.2.Mice were sensitised with HDM in Alum (2.5µg.kg^{-1} in 500µl saline (i.p.)) on days 0 and 14 and subsequently challenged with HDM (12.5µg.kg^{-1} in 25µl saline (i.t.)) on day 28 between 4 and 5pm. Mice were immediately placed in whole body plethysmography chambers and Penh was recorded overnight

	Allergic inflammation and AHR	LAR
OVA sensitisation	10µg.mouse^{-1} in 100µl Alum in saline (i.p.)	50µg.mouse^{-1} in 500µl Alum in saline (i.p.)
OVA challenge	50µg.mouse^{-1} in 50µl saline (i.n.)	25µl of 2% OVA in saline (i.n.)
HDM sensitisation	50µg.kg^{-1} in 100µl saline (i.p.)	2.5µg.kg^{-1} in 500µl saline (i.p.)
HDM challenge	1.25µg.kg-1 in 50µl saline (i.n.)	12.5µg.kg^{-1} in 25µl saline (i.t.)

Table 5.2: Sensitisation and challenge doses used for the standard OVA and HDM models in comparison with the LAR models

5.2.8. Investigating the role of Alum in the LAR

A HDM-induced LAR was not detected in the study described in section 5.2.8. The difference between the OVA model used for LAR and the HDM model employed above was the presence of Alum. I therefore wanted to determine whether Alum was required for the generation of the OVA-induced LAR, and whether the use of Alum in the HDM model would promote the generation of the LAR.

Mice were sensitised with 50µg OVA (with and without Alum) in 500µl i.p. or with 2.5µg.kg^{-1} HDM (with and without Alum) in 500µl i.p. on days 0 and 14. Mice were then challenged with their respective allergen (25µl of 2% OVA or 12.5µg.kg^{-1} HDM (in 25µl) i.t.) on day 28 between 4 and 5pm. Immediately after challenge mice were placed in whole body plethysmography chambers and Penh was recorded overnight.

5.2.9. Investigating the role of Alum in the OVA-induced asthma model

Whilst developing the allergic HDM-induced asthma model, it became apparent that the use of Alum was not required to generate allergic inflammation and airway hyperreactivity in this model. Interestingly however the LAR appeared to be dependent on the presence of Alum during sensitisation in both the HDM and OVA models. I was therefore interested to determine whether Alum was indeed required for the allergic inflammation and airway hyperreactivity seen in the standard OVA model. Traditionally the in-house OVA model has utilised an adjuvant but we have never tested whether this is a requirement for the development of asthmatic features in this model.

Mice were sensitised with saline or OVA (10µg per mouse in 100µl i.p.) in saline or Alum on days 0 and 14 and then challenged intranasally with saline or OVA (50µg per

mouse) on days 24-26. On day 29, 3 days after final OVA challenge, mice were placed in whole body plethysmography chambers and exposed to saline followed by increasing concentrations of 5-HT (1-10 $mg.ml^{-1}$). Once recovered from the 5-HT challenge the mice were culled by overdose with sodium pentobarbitone. Plasma and BAL fluid samples were obtained and were assessed for total and OVA-specific IgE, and levels of cellular inflammation respectively.

5.3. Results

5.3.1. Optimising a HDM sensitisation protocol

The first stage of developing the HDM-driven model was to optimise the sensitisation phase: firstly the dose route, and secondly the HDM concentration for sensitisation. Mice were sensitised with saline or various concentrations of HDM, either intranasally or intraperitoneally (with and without Alum). Total and OVA-specific IgE were then measured in the plasma by ELISA as a marker to evaluate the effectiveness of sensitisation.

Firstly, intranasal sensitisation with HDM at any dose did not induce a change in levels of either total or HDM-specific IgE compared to mice sensitised intranasally with saline (Figure 5.3). This dose route was therefore discounted as a method of sensitisation for the present model.

A bell-shaped increase in total IgE was observed with increasing HDM dose in mice sensitised systemically both with and without Alum compared to respective saline or Alum sensitised mice (Figure 5.3). The increase in total IgE was statistically significant in mice sensitised with $0.5\mu g.kg^{-1}$ HDM (both with and without Alum). At higher HDM doses ($5.500\mu g.kg^{-1}$) levels of total IgE were comparable with levels observed in saline or alum sensitised controls.

The trend for HDM-specific IgE appeared to parallel the trend seen with total IgE. Both sensitisation with and without Alum induced a substantial increase in HDM-specific IgE with the central HDM doses. Although none of the doses of HDM caused a statistically significant increase in HDM-specific IgE compared to levels in saline challenged mice when dosed without Alum (Mann-Whitney U-test), the $5\mu g.kg^{-1}$ HDM dose more than doubled the level of HDM-specific IgE compared to saline sensitised controls. In mice sensitised systemically with HDM in Alum, 5 of the 6 HDM doses appeared to induce a robust increase in the levels of HDM-specific IgE compared to the Alum sensitised control. The increase in HDM-specific IgE reached statistical significance at the $5\mu g.kg^{-1}$ dose.

Having discounted intranasal sensitisation the choice was then between systemic sensitisation with or without Alum; these two sensitisation protocols appeared to induce similar results, especially for total IgE. However it is specific allergy to HDM that is most important in this model, rather than just a general increase in non-specific IgE levels. Sensitisation with HDM in Alum appeared to generate a more reproducible increase in HDM-specific IgE across the various HDM doses than sensitisation without Alum. Therefore despite the criticisms of the use of exogenous adjuvants I decided to opt to take forward a systemic sensitisation protocol including Alum for further model development.

Due to the apparent bell-shaped nature of the response to systemically-dosed HDM-sensitisation I chose the 0.5μg.kg^{-1} HDM dose as the optimum dose to achieve allergic sensitisation in this model. The selected dose is indicated by the black arrows on Figure 5.3.

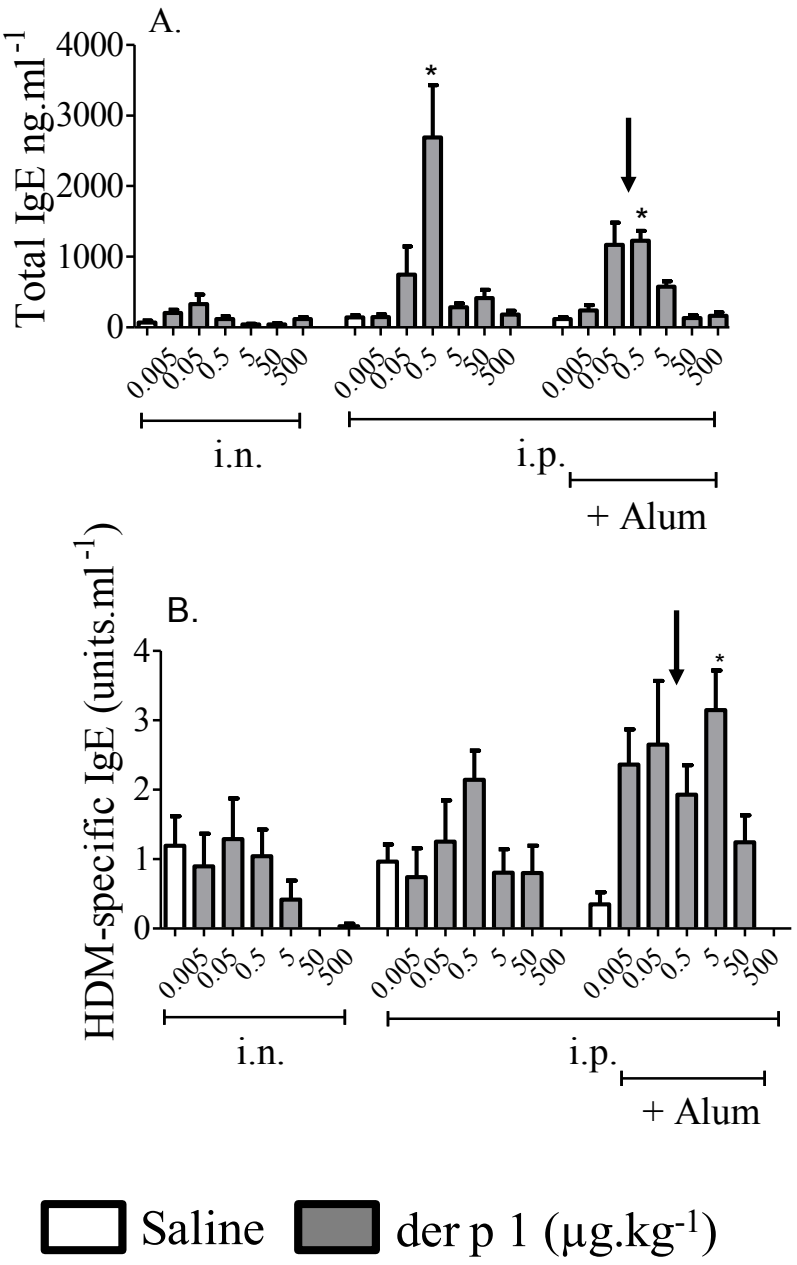


Figure 5.3: IgE levels in response to sensitisation with HDM via different routes. Male C57Bl/6 mice were sensitised with vehicle or HDM (0.005-500μg.kg^{-1} Der p 1) either intranasally or intraperitoneally (with and without Alum) as indicated below the figures. Levels of total (A) and HDM-specific (B) IgE in plasma were measured by ELISA. Data expressed as mean total IgE levels (ng.ml^{-1}) (A), or mean HDM-specific IgE (units.ml^{-1}) (B) + SEM for n=6-12 per group. *=$p<0.05$ HDM sensitised groups vs. respective saline challenged controls, Kruskal-Wallis one-way ANOVA followed by Dunn's Multiple Comparison post-test. Where no symbol is shown on the figure this indicates a non-significant difference.

5.3.2. HDM challenge dose response

Having chosen a dose of 0.5μg.kg^{-1} HDM with Alum dosed intraperitoneally as the sensitisation regimen for the allergic HDM model, the next stage was to select the HDM dose for the challenge phase of the model. To do this I performed a dose response to HDM in mice sensitised with vehicle or HDM and then measured cellular inflammation to determine the efficacy of the different challenge doses (Figure 5.5).

In mice which were sensitised with vehicle (Alum) only, no changes in the levels of BAL fluid eosinophils or macrophages were seen after HDM challenge compared to the saline challenged controls (Figure 5.4 A, C). Levels of lymphocytes in the BAL fluid (Figure 5.4.B) were significantly increased compared to saline-challenged controls after challenge with 12.5 and 125μg.kg^{-1} house dust mite ($p<0.05$ vs. Alum-sensitised/saline-challenged controls, Kruskal Wallis one-way ANOVA followed by Dunn's Multiple Comparison post-test). The top two HDM doses also caused an increase in the levels of BAL neutrophils (Figure 5.4 D) compared to levels in saline challenged controls. This increase was statistically significant after challenge with 125μg.kg^{-1} HDM ($p<0.05$ vs. Alum-sensitised/saline-challenged controls, Kruskal Wallis one-way ANOVA followed by Dunn's Multiple Comparison post-test).

HDM challenge appeared to induce a much greater increase in BAL cellular inflammation in mice sensitised to HDM (plus Alum) compared to those sensitised with Alum alone. Levels of BAL eosinophils, lymphocytes, and macrophages were significantly increased compared to levels observed in saline-challenged controls after 1.25, 12.5 and 125μg.kg^{-1} HDM (Figure 5.4 A, B, C); levels of BAL neutrophils (Figure 5.4 D) were significantly increased compared to saline challenged controls after 12.5 and 125μg.kg^{-1} HDM ($p<0.05$ vs. saline-challenged (HDM-sensitised) controls, Kruskal Wallis one-way ANOVA followed by Dunn's Multiple Comparison post-test). Levels of eosinophils appeared to plateau at a dose of 1.25μg.kg^{-1} HDM, which caused a 115 fold increase in BAL eosinophilia compared to levels seen in saline-challenged controls. This dose caused a significant increase in the levels of eosinophils, lymphocytes and macrophages, but importantly did not cause a change in the levels of any of the measured cell types in the mice which had not previously been exposed to HDM. The aim of developing this allergic model was to titrate a HDM dose that only induced an inflammatory response in mice which had previously been exposed to the allergen, and thus were 'sensitised'. Therefore the dose of 1.25μg.kg^{-1} HDM was selected as the challenge dose for the allergic HDM model. This dose induced a substantial but sub-maximal level of cellular inflammation in the BAL fluid, and

importantly was low enough not to induce any non-allergic cellular inflammation. The selected dose is indicated by the black arrows on Figure 5.4

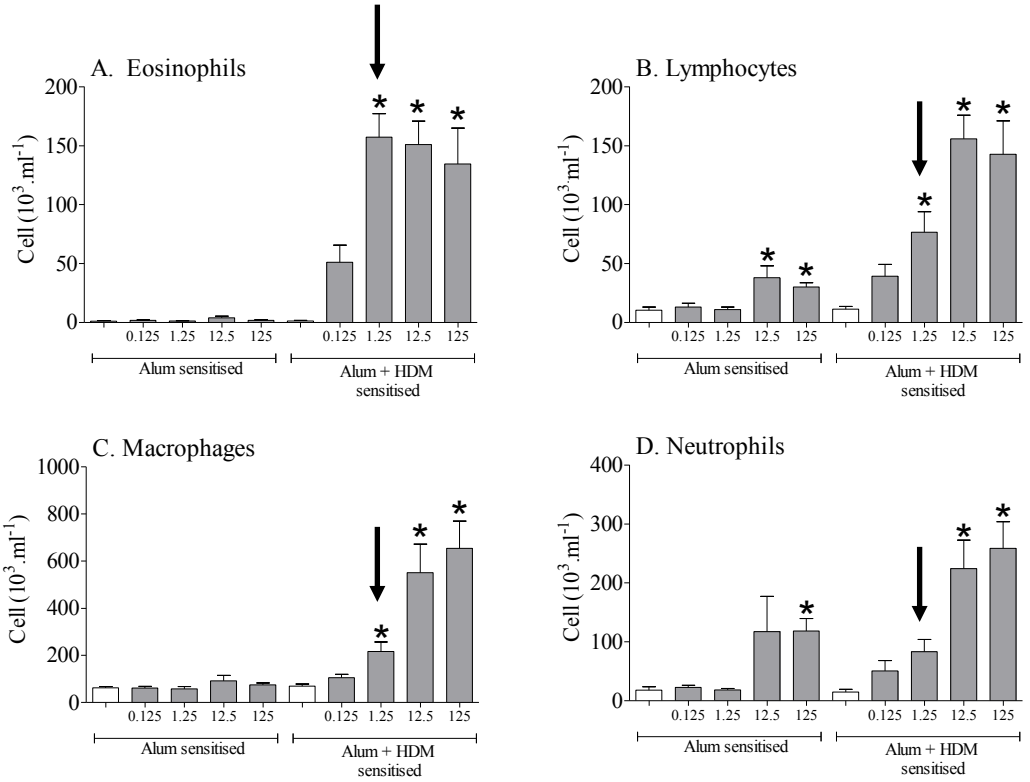


Figure 5.4: The effect of intranasal HDM challenge in vehicle and HDM-sensitised mice
Male C57Bl/6 mice were sensitised with vehicle (Alum) or HDM and challenged intranasally with saline (open bars) or HDM (0.125-125µg.kg^{-1} Der p 1) (grey bars). Cellular inflammation was assessed in BAL fluid 24 hours after final HDM challenge. Data expressed as mean cell numbers (10^3.ml^{-1}) + SEM for n= 7-8 per group. *=$p<0.05$ Kruskal-Wallis one-way ANOVA followed by Dunn's Multiple Comparison post-test, HDM challenged group vs. relevant saline-challenged controls. Where no symbol is shown on the figure this indicates a non-significant difference.

5.3.3. Assessing the role of Alum during sensitisation on the response to HDM-challenge

Previously (Figure 5.3) I showed that an exogenous adjuvant was not necessary to achieve an increase in plasma total IgE or HDM-specific IgE after systemic sensitisation with HDM. However, sensitising the mice to HDM in conjunction with Alum appeared to lead to a more reproducible increase in HDM-specific IgE compared to sensitisation without Alum. Consequently a dose response to topical HDM challenge was performed in mice sensitised to HDM in the presence of Alum. HDM challenge in mice sensitised with HDM in Alum induced a robust increase in airway cellular inflammation.

An interesting question then arose however as to whether it was actually necessary to include Alum during sensitisation in this model in order for an allergic response to occur after HDM challenge. To address this question, mice were sensitised with saline or HDM formulated in vehicle (saline) or Alum and were then challenged with either saline or HDM according to the doses selected above. Inflammation was assessed in this study 3 days after final HDM challenge. This is the time-point which was used for the OVA model, and would be a likely time-point to investigate AHR in this model. Thus this study also provided the opportunity to determine the airway inflammatory profile at this later time-point.

Firstly, HDM challenge did not induce a significant change in the levels of BAL eosinophils, lymphocytes, macrophages or neutrophils in mice which were sensitised with saline (either with or without Alum) compared to relevant saline-challenged controls (Figure 5.5). This supports the data in Figure 5.4 as again the HDM dose chosen did not induce any non-allergic inflammation. Secondly, in mice sensitised with HDM plus Alum, HDM challenge induced robust and statistically significant increases in BAL eosinophils, lymphocytes, and neutrophils (Figure 5.5 A, B D, $p<0.05$, Mann-Whitney U-test vs. HDM-sensitised/saline-challenged controls). Interestingly however, HDM challenge also resulted in statistically significant increases in the levels of BAL eosinophils, lymphocytes and neutrophils (Figure 5.5 A, B, D, $p<0.05$, Mann-Whitney U-test vs. HDM-sensitised/saline-challenged controls) in mice sensitised to HDM in the absence of Alum compared to HDM-sensitised/saline challenged controls. This very clearly shows that Alum is not required to sensitise the mice to respond to a subsequent HDM challenge, so all further model work in this model will now be conducted without this or any other exogenous adjuvant.

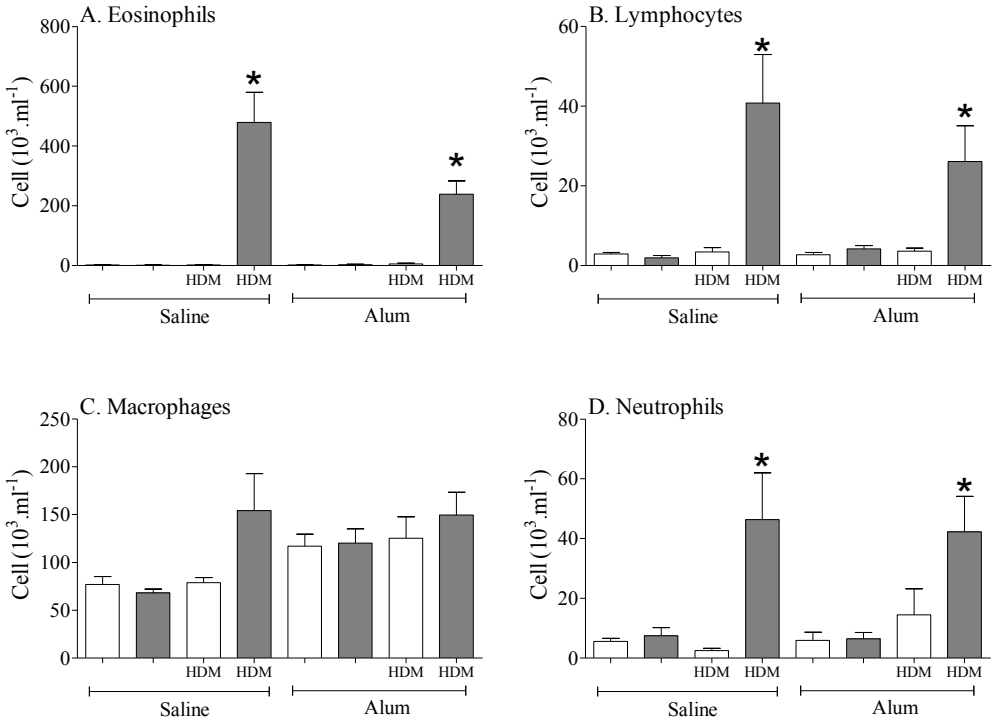


Figure 5.5: The requirement for Alum during sensitisation in the allergic inflammatory response to HDM challenge

Male C57Bl/6 mice were sensitised with saline or HDM made up in saline or Alum as indicated below the figures. Mice were then challenged with saline (open bars) or HDM (grey bars). Inflammatory response to challenge was assessed in the BAL fluid 3 days after challenge. Data expressed as mean cell number (10^3.ml^{-1}) + SEM for n=6 per group. *=$p<0.05$ Mann-Whitney U-test, HDM challenged groups vs. relevant HDM-sensitised/saline-challenged controls. Where no symbol is shown on the figure this indicates a non-significant difference.

5.3.4. Does sensitisation and challenge with HDM induce airway hyperresponsiveness to 5-HT?

Inflammation is just one of the endpoints which should be measured and used to validate a new allergic asthma model. Functional endpoints such as AHR are also very important to determine the usefulness of the model. Therefore I next had to determine whether AHR was observed in the newly developed HDM model.

Mice were sensitised with saline or HDM (without Alum) and subsequently challenged with saline or HDM according to the newly developed dosing regimen. AHR was then assessed 3 days after final HDM challenge. In saline-sensitised/saline-challenged mice, inhaled 5-HT caused a dose-dependent increase in airflow obstruction measured by Penh (Figure 5.6). The standard control used in-house for the allergic asthma models is the

allergen-sensitised/saline-challenged group. The response to inhaled 5-HT in this group was indistinguishable from the response in saline-sensitised/saline-challenged mice. In HDM-sensitised/HDM-challenged mice the increase in Penh was significantly enhanced compared to HDM-sensitised/saline-challenged controls (Figure 5.6, $p<0.05$ and 0.005 for 1 and 3 $mg.ml^{-1}$ dose respectively, Mann-Whitney U-test vs. HDM-sensitised/saline challenged controls). This is a striking AHR, and the data clearly show that an exogenous adjuvant is not required to observe AHR in the HDM-driven allergic asthma model; the caveat to this is that the AHR may be stronger in mice sensitised to HDM plus Alum, but having obtained a substantial AHR in the absence of Alum it did not seem necessary to investigate this further. The increased response to $10mg.ml^{-1}$ 5-HT in the HDM/HDM group compared to the HDM/saline controls was not statistically significant, and appeared to be less robust than the increased response to 3mg.ml. It is noted that some animals in the HDM/HDM group responded extremely strongly to the $10mg.ml^{-1}$ 5-HT dose and had to be removed from the experiment due to respiratory distress. This is likely to have lowered the response for this group which would explain the lack of significant AHR. The $10mg.ml^{-1}$ dose will not be used for subsequent HDM model studies to avoid these adverse effects.

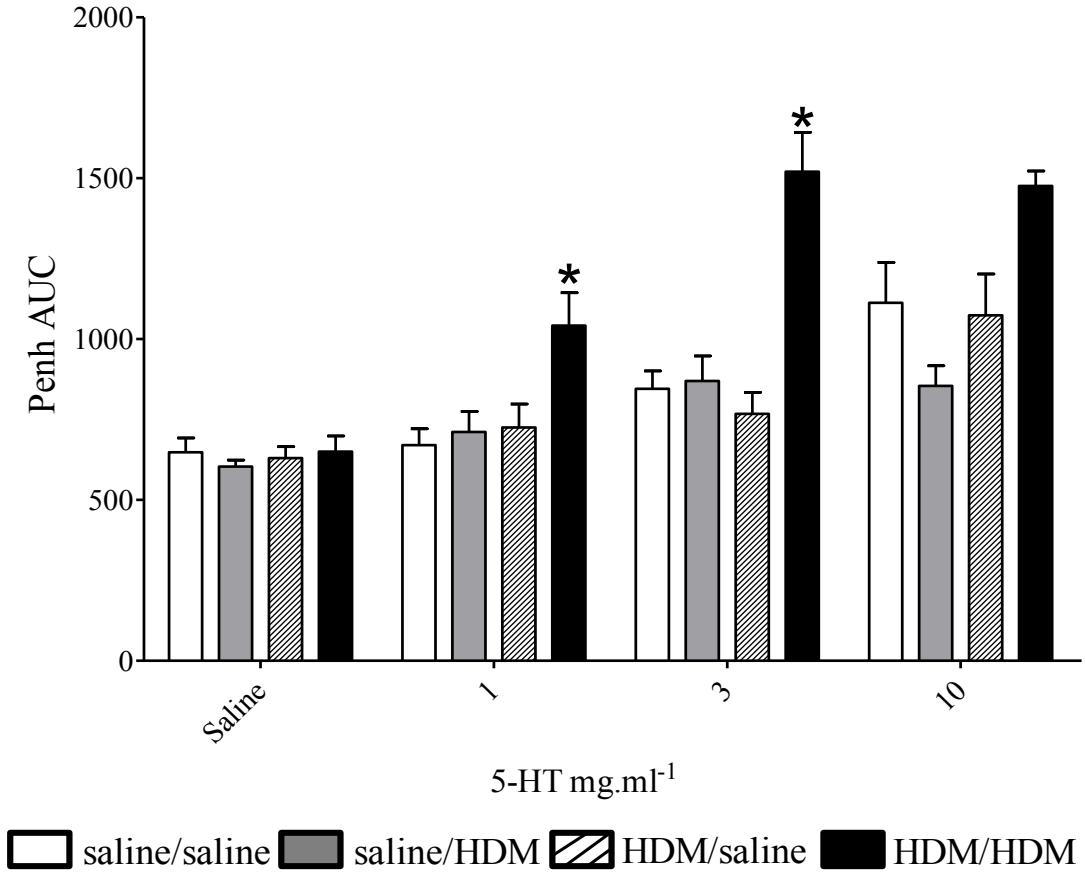


Figure 5.6: Effect of HDM sensitisation and challenge on airway responsiveness to inhaled 5-HT

Male C57Bl/6 mice were sensitised with vehicle (saline) or HDM and subsequently challenged with saline or HDM. Open bars = saline sensitised/saline challenged, grey bars = saline sensitised/HDM challenged, striped bars = HDM sensitised/saline challenged, black bars = HDM sensitised/HDM challenged mice. Mice were placed in whole body plethysmography chambers 3 days after final HDM challenge and airway responsiveness to inhaled 5-HT was assessed as Penh. Data expressed as mean Penh area under curve + SEM for n = 4-6 per group. *=$p<0.05$, Mann-Whitney U-test HDM challenged groups vs. HDM-sensitised/saline-challenged controls. Where no symbol is shown on the figure this indicates a non-significant difference.

5.3.5. Does topical sensitisation promote an allergic response to HDM challenge despite not inducing a detectable increase in IgE?

The trend has been for researchers to switch to using models consisting of repeated topical sensitisation with HDM, rather than the more traditional models with distinct sensitisation and challenge phases. In the sensitisation studies earlier in this chapter, I showed that intranasal sensitisation with HDM failed to induce changes in total or HDM-specific IgE despite using doses which induced marked increases in these endpoints when

given systemically. However, with such a widespread use of topical HDM models I was interested as to whether topical sensitisation in our model could actually induce sensitisation to HDM despite the lack of IgE production.

To investigate this, mice were sensitised with saline or HDM ($0.5\mu g.kg^{-1}$) either intranasally or intraperitoneally and were then challenged intranasally with saline or HDM. Cellular inflammation was then assessed in the BAL fluid 3 days after final HDM challenge. As previously, in mice sensitised systemically to HDM, HDM challenge induced a robust and statistically significant increase in the levels of BAL fluid eosinophils, lymphocytes, and neutrophils (Figure 5.7, A, B, D) compared to saline-challenged mice ($p<0.05$, Mann-Whitney U-test vs. relevant HDM-sensitised/saline-challenged controls). Conversely HDM challenge failed to induce any changes in the levels of these cell types (or macrophages) in the BAL fluid of mice sensitised intranasally with HDM (Figure 5.78 A, B, C, D).

This data therefore supports the use of systemic sensitisation in the allergic HDM model developed in this thesis.

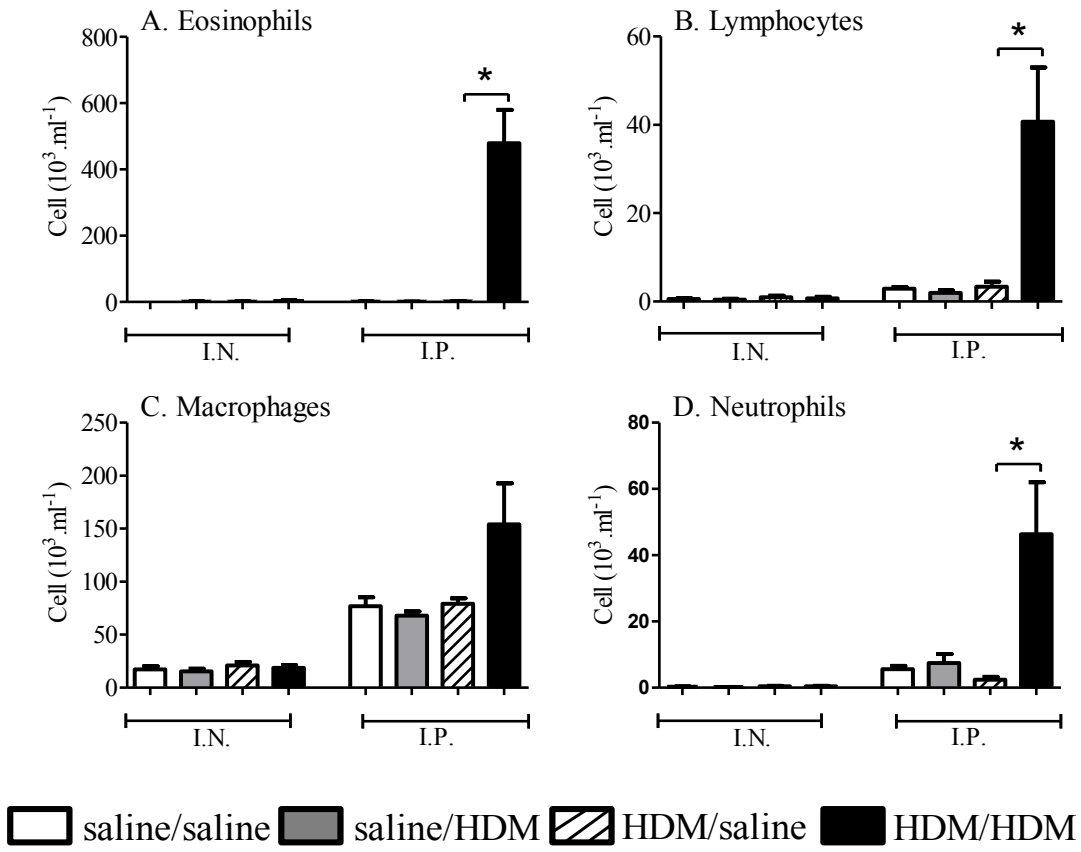


Figure 5.7: The effect of intranasal HDM challenge in topically sensitised mice
Male C57Bl/6 mice were sensitised intranasally or intraperitoneally as indicated below the figures with saline or HDM. Mice were subsequently challenged with saline or HDM: open bars = saline-sensitised/saline-challenged, grey bars = saline-sensitised/HDM-challenged, striped bars = HDM-sensitised/saline-challenged, black bars = HDM-sensitised/HDM-challenged mice. Cellular inflammation was assessed in BAL fluid 3 days after final HDM challenge. Data expressed as mean cell number (10^3/ml^{-1}) + SEM for n = 6-8 per group. *=$p<0.05$, HDM challenged groups compared to relevant HDM-sensitised/saline-challenged controls, Mann-Whitney U-test. Where no symbol is shown on the figure this indicates a non-significant difference.

5.3.6. HDM-induced late asthmatic response

The final endpoint that I was interested to model was the late asthmatic response. In the OVA model none of the three intranasal challenges used to generate allergic inflammation cause any change in airflow obstruction (Penh). The same was found in the HDM model: mice were placed in whole body plethysmography boxes immediately after each of the three intranasal challenges and Penh levels remained stable in all mice, included those which were HDM-sensitised and –challenged. In order to generate the OVA-induced

LAR the doses used for the standard OVA model are scaled up, as described in table 5.2. I implemented a similar approach to try to obtain a HDM-induced LAR. The doses of HDM used for the allergic model were scaled up accordingly to determine doses for HDM-LAR study; the resultant doses are explained in table 5.2.

Mice were therefore sensitised systemically with 2.5µg.kg^{-1} HDM and challenged intratracheally with 12.5µg.kg^{-1} HDM in 25µl saline. Immediately after challenge, mice were placed in whole body plethysmography boxes and Penh was monitored overnight. From previous studies with the OVA model and naive mice I was confident that saline-sensitised and -challenged mice would demonstrate stable Penh values so in this case just the HDM/HDM group was tested for generation of LAR. After HDM-sensitisation and -challenge there was no change in the Penh levels throughout the 15 hours after HDM challenge (Figure 5.8). Thus this approach failed to induce a HDM-induced LAR.

5.3.7. Investigating the role of Alum in the LAR

The key difference between the OVA-LAR model and the HDM-LAR model is the use of Alum. Although I have shown clearly that HDM-induced inflammation and AHR does not require an exogenous adjuvant during sensitisation, the late response may be different: Alum may be key for the LAR. To address this I monitored Penh levels in OVA- or HDM- challenged mice which had been sensitised to their respective allergen according to the extrapolated LAR protocols – with and without Alum.

In mice sensitised with OVA plus Alum, OVA challenge resulted in a robust LAR (figure 5.9 A) as described in Chapter 3. However in mice sensitised with OVA without Alum, OVA challenge failed to induce any change in airflow obstruction over the 10 hour monitoring period. This means that Alum is essential for the OVA-induced LAR in our model. HDM challenge failed to result in any change in airflow obstruction (Penh) in mice sensitised to HDM without Alum, however did cause a small LAR in mice which were sensitised to HDM with Alum (Figure 5.9 B). This was initially quite encouraging; although smaller than the OVA-induced LAR there is a clear response to HDM challenge in the HDM-Alum sensitised mice. However when this response is viewed as traces for individual subjects (Figure 5.10) it is clear that this response is very poorly reproducible. In fact only 1 of the 5 mice showed a strong response to the HDM challenge; the rest either failed to respond or responded only very minimally. Although there is a hint that this challenge protocol may induce a HDM-induced LAR it would definitely not be a viable model to test the effect of pharmacological interventions or indeed the effect of cigarette smoke.

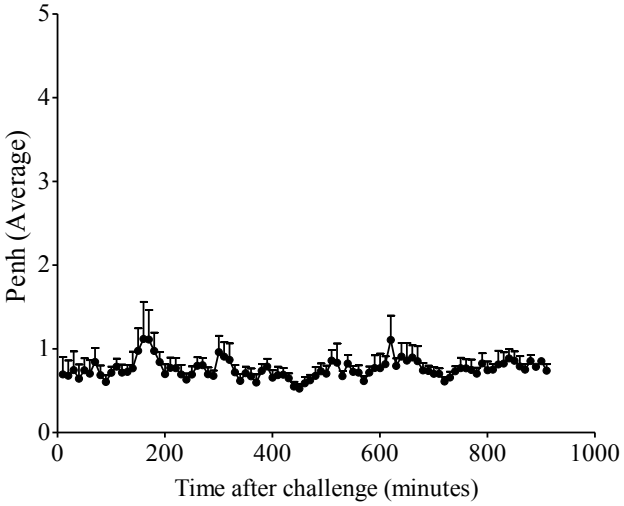


Figure 5.8: Penh levels in HDM-sensitised and -challenged mice over time after challenge

Male C57Bl/6 mice were sensitised with HDM and subsequently received a single i.t. challenge with HDM. Mice were immediately placed in whole body plethysmography chambers and Penh was recorded overnight. Data expressed as mean Penh average + SEM for n=7 per group. No statistical analysis was performed on this data

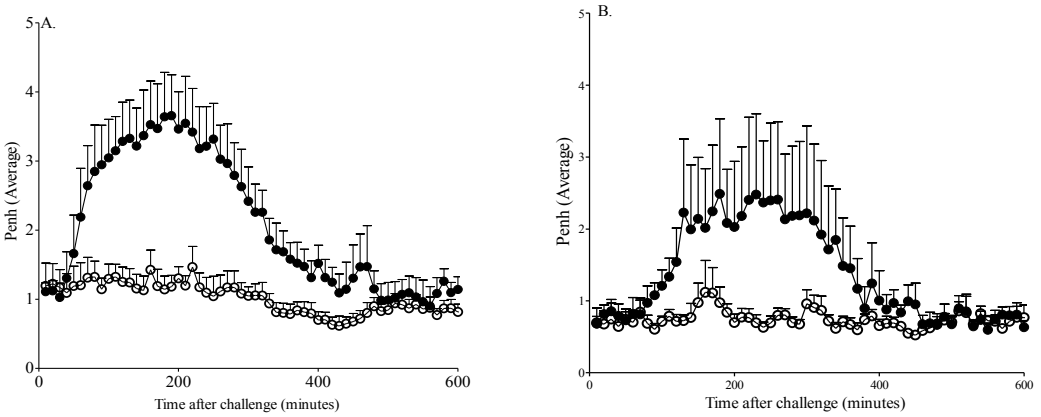


Figure 5.9: The role of Alum in the OVA- and HDM- driven LAR

Male C57Bl/6 mice were sensitised with OVA (A) or HDM (B) made up in saline (open circles) or Alum (closed circles) and subsequently intratracheally challenged with the relevant allergen. Immediately after challenge mice were placed in whole body plethysmography chambers and the LAR recorded as change in Penh. Data expressed as mean Penh average + SEM for n= 6-8 per group. No statistical analysis was performed on this data

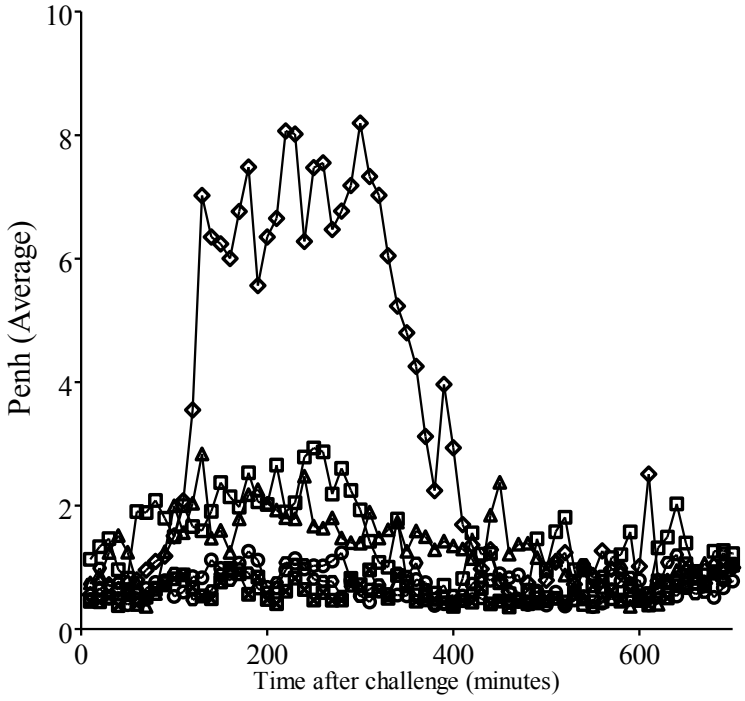


Figure 5.10: Whole body plethysmography traces of individual HDM-sensitised mice after HDM challenge

HDM (plus Alum)-sensitised male C57Bl/6 mice were intratracheally challenged with HDM. Mice were immediately placed in whole body plethysmography chambers and late response was recorded as change in Penh. Data show separate responses to HDM of 7 individual mice from a single experiment, expressed as Penh average. No statistical analysis was performed on this data.

5.3.8. Investigating the role of Alum in the OVA-induced asthma model

This chapter is predominantly concerned with the development of a HDM-driven model of allergic asthma, however the data presented so far has highlighted a divergence in the requirement for an adjuvant in the generation of different HDM-induced pathologies. This spurred an interest in the role of Alum in the OVA-induced responses seen in our models, especially as this surrogate allergen is typically used in conjunction with an adjuvant. I was therefore next interested to determine whether the inflammation and AHR observed in the OVA model was dependent on the presence of exogenous adjuvant, and accordingly whether sensitisation to OVA could induce IgE production in the absence of Alum.

Mice were sensitised with saline or OVA in the absence or presence of Alum and were then challenged with saline or OVA. Inflammation in the BAL fluid and AHR were

then assessed 3 days after final OVA challenge. Total and OVA-specific IgE levels were assessed in plasma by ELISA.

OVA challenge resulted in a significant increase in airway eosinophils, lymphocytes, macrophages and neutrophils (Figure 5.11 A, B, C, D) in mice which were sensitised with OVA formulated in Alum. Interestingly, OVA challenge also resulted in significant increases in all 4 cell types in the BAL fluid in mice sensitised with OVA in the absence of Alum. With the exception of BAL neutrophils, the OVA-induced cellular inflammatory response in the BAL fluid was equivalent in mice sensitised with OVA with and without Alum. OVA appeared to induce a greater increase in the level of BAL neutrophils in mice sensitised with OVA plus Alum compared to OVA alone, however this difference was not statistically significant (Mann-Whitney U-test).

One interesting observation to note is that OVA challenge itself caused a (comparatively small) increase in the levels of airway lymphocytes and neutrophils compared to saline challenged mice in mice which are sensitised to saline only (both when Alum is absent and present during sensitisation). This means that a small component of the increased neutrophil and lymphocyte levels in the BAL fluid of the 'allergic mice' may have resulted from an innate, non-allergic response.

Next I looked at the role of Alum in the OVA-induced AHR to 5-HT (Figure 5.12). Interestingly, the OVA-induced AHR was still observed even if the mice are sensitised to OVA without Alum: OVA-sensitisation and –challenge induced a significant increase in the response to 1, 3 and 10mg.ml^{-1} 5-HT in mice sensitised with Alum ($p<0.05$, Mann-Whitney U-test vs. Alum-OVA-sensitised/saline-challenged group). The response to 1, 3 and 10mg.ml^{-1} 5-HT in OVA/OVA mice sensitised without Alum also appeared to be enhanced compared to the OVA/saline group, but this increase was only significant at 3mg.ml^{-1} 5-HT ($p<0.05$, Mann-Whitney U-test vs. Alum-OVA-sensitised/saline-challenged group). In conclusion however, an enhanced response to inhaled 5-HT was still observed in OVA-sensitised and –challenged mice in the absence of Alum during sensitisation.

Compared with levels observed after OVA challenge, OVA-sensitisation alone resulted in very little change in the levels of plasma total or OVA-specific IgE (Figure 5.13). However in the presence of Alum, OVA sensitisation (OVA/saline group) did cause a significant increase in the levels of both total and OVA specific IgE compared to levels in saline-sensitised/saline-challenge mice ($p=0.0059$ and 0.0013 respectively compared to relevant saline-sensitised/saline-challenged mice, Mann-Whitney U-test). However these increases appear somewhat masked by the dramatic increases in both total and OVA-specific IgE induced by subsequent OVA challenge. This however did not occur in the mice sensitised to OVA without Alum. In OVA-sensitised mice, OVA challenge resulted in a significant increase in the levels of total plasma IgE compared to levels in saline challenged

mice regardless of whether Alum was present during sensitisation (Figure 5.13 A, $p<0.05$ vs. relevant saline-challenged controls). The same trends were also observed for plasma OVA-specific IgE (Figure 5.13 B).

Alum is therefore not required for OVA-sensitisation and -challenge to induce increased levels of plasma total and OVA-specific IgE. However there may be some requirement for Alum for the initial increase in total and OVA-specific IgE after sensitisation alone. This may explain the requirement for Alum in the OVA-induced LAR model.

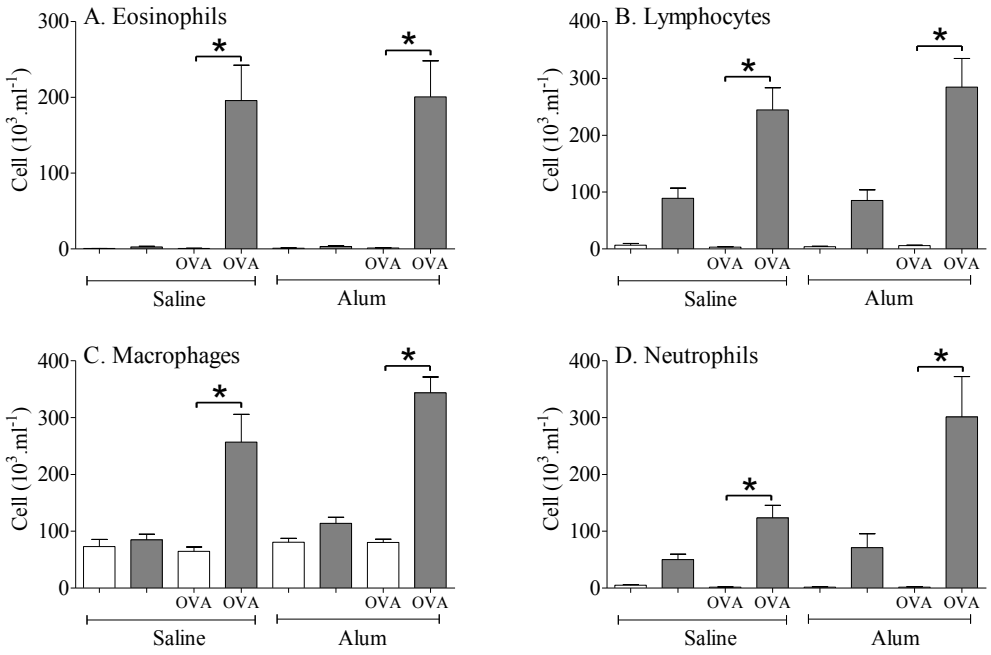


Figure 5.11: The role of Alum during sensitisation in the cellular inflammatory response to OVA challenge

Male C57Bl/6 mice were sensitised with saline or OVA made up in saline or Alum as indicated below the figure. Mice were subsequently challenged with saline (open bars) or OVA (grey bars) and cellular inflammation assessed in BAL fluid 3 days after final challenge. Data expressed as mean cell number (10^3.ml^{-1}) + SEM for n=7-9 per group. *=$p<0.05$ OVA challenged mice compared to relevant OVA-sensitised/saline-challenged controls, Mann-Whitney U-test.

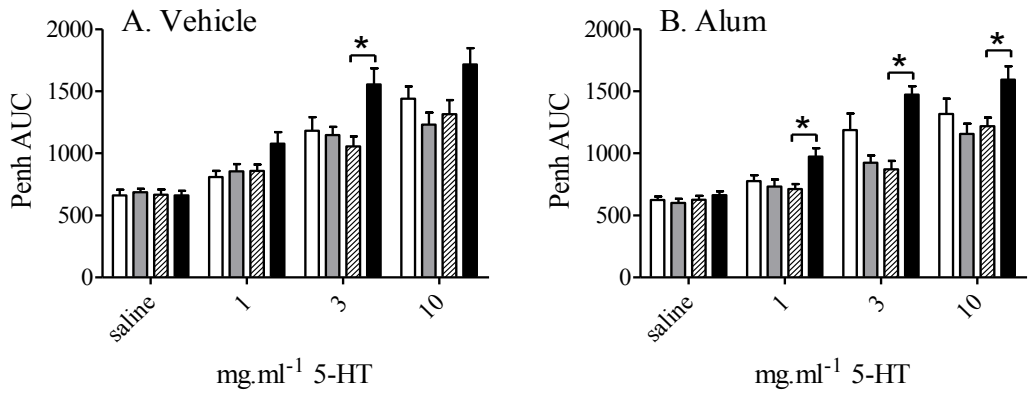


Figure 5.12: The role of Alum in OVA-induced airway hyperresponsiveness to 5-HT
Male C57Bl/6 mice were sensitised with saline or OVA made up in saline (A) or Alum (B) and subsequently challenged with saline or OVA. Open bars = saline-sensitised/saline-challenged, grey bars = saline-sensitised/OVA-challenged, striped bars = OVA-sensitised/saline-challenged, black bars = OVA-sensitised/OVA-challenged. Animals were placed in whole body plethysmography chambers and airway responsiveness to 5-HT was assessed 3 days after final challenge. Data expressed as mean Penh area under the curve + SEM for n=7-9 per group. *=p<0.05 Mann-Whitney U-test, OVA challenged mice compared to relevant OVA sensitised/saline challenged controls. Where no symbol is shown on the figure this indicates a non-significant difference.

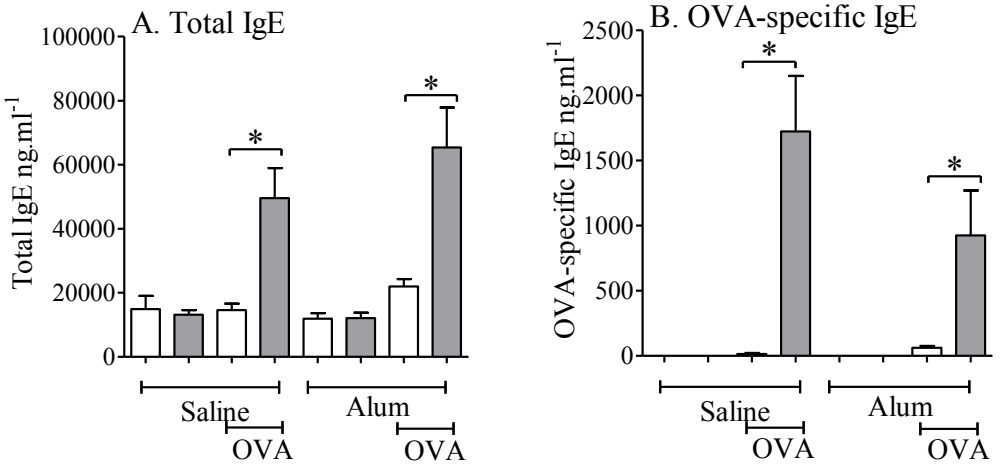


Figure 5.13: The role of Alum during sensitisation in the production of IgE in the allergic OVA model
Male C57Bl/6 mice were sensitised with saline or OVA made up in saline or Alum as detailed below the figures. Subsequently mice were challenged with saline (open bars) or OVA (grey bars). Plasma levels of total IgE (A) and OVA-specific IgE (B) were assessed by ELISA, 3 days after final OVA challenge. Data expressed as mean + SEM for n=7-9 per group. *=p<0.05, OVA challenged mice compared to relevant OVA-sensitised/saline-challenged controls, Mann-Whitney U-test.

5.4. Discussion

In this chapter I have described the development of an allergic, HDM-driven murine model of asthma where AHR and a robust airway cellular inflammation consisting of eosinophils, lymphocytes and neutrophils are observed; these features are only detected in the presence of prior sensitisation so I can be confident that the responses result entirely from an allergic response to the topical HDM challenge.

Firstly I established an optimum sensitisation protocol for the model. It was found that intranasal HDM sensitisation over a wide range of HDM doses did not induce any change in the level of systemic total or HDM-specific IgE. I used this immunoglobulin as a marker of allergic sensitisation because of its historical use in the clinic as a marker of allergy (such as the RAST test) (Ebner & Kraft 1975; Brückner *et al.* 1977), and because it is the most frequently used immunoglobulin to measure allergic sensitisation in murine models of asthma. It could be argued that the immunization protocol was not sufficient to induce sensitisation to HDM; however a wide range of sensitisation doses were used and, a robust increase in total and HDM-specific IgE was observed following systemic sensitisation. It is interesting that a bell-shaped IgE response to HDM sensitisation was observed – one may expect that increasing doses of HDM allergen would induce increased sensitisation however this is often not the case. This lack of a linear sensitisation dose response in terms of IgE production has been published previously where sensitisation of Balb/c mice with 10µg OVA plus Alum resulted in high levels of OVA-specific IgE while sensitisation with 1000µg resulted in low levels of OVA-specific IgE (Sakai *et al.* 1999). In the clinic several papers have also shown that risk of sensitisation to HDM does not always increase with increasing HDM levels (Cullinan *et al.* 2004; Schram-Bijkerk *et al.* 2006; Torrent *et al.* 2006, 2007; Tovey *et al.* 2008). Thus the effects of HDM levels on IgE production and sensitisation shown here may well reflect the clinical situation.

Having selected the optimum sensitisation dose and route, a challenge dose response was performed. In line with one of the main aims for the newly developed model, a challenge dose was selected which induced airway inflammation only in the mice which had previously been sensitised with HDM. Initially this was performed in the presence of Alum, as sensitisation to HDM plus Alum appeared to induce a more robust increase in HDM-specific IgE. However it was subsequently found that Alum during sensitisation was not required for the induction of allergic airway inflammation in response to HDM challenge. This is a great advantage for the model as it avoids the use of a non-physiological and Th2 polarizing adjuvant (Comoy *et al.* 1998). Having established a dose of HDM which induced

allergic airway inflammation in HDM-sensitised mice I then showed that HDM challenge also induced a robust AHR to 5-HT.

The majority of research groups that utilise HDM-driven murine models favour topical sensitisation rather than systemic sensitisation (Johnson *et al.* 2004, 2011; Cates *et al.* 2004; Ulrich *et al.* 2008; Phipps *et al.* 2009), although murine models which utilise systemic sensitisation have been described (Clarke *et al.* 1999; Tournoy *et al.* 2000; Kikuchi *et al.* 2006; Kelada *et al.* 2011). Subcutaneous HDM in Alum in C57Bl/6 mice followed by intranasal HDM challenge induced production of HDM-specific IgE and airway eosinophilia (Clarke *et al.* 1999). In Balb/c and C57Bl/6 mice, i.p. sensitisation to HDM without adjuvant followed by a single topical HDM challenge induced airway eosinophilia and production of Th2 cytokines, however AHR was only observed in Balb/c mice (Kelada *et al.* 2011). Finally a single i.p. administration of purified Der p 1 followed by 7 daily aerosolised exposures to HDM extract in C57Bl/6 mice induced airway eosinophilia and AHR (Tournoy *et al.* 2000).

Thus a HDM model such as the one described in this chapter is not completely novel. However to my knowledge a model has not previously been published which uses systemic HDM extract without adjuvant in C57Bl/6 mice which induces both inflammation and AHR; the great advantage of using this strain is the ability to use genetically modified mice to investigate mechanisms in the model. Since little further work has been published utilising the above models, the model developed in this chapter is a great addition to the field. The fact that it parallels the OVA model will allow the two models to be used side by side for comparison, and will allow any HDM-specific effects to be noted. So far the two models have appeared to induce similar responses.

5.4.1. Mechanisms involved in the response to HDM

Because of the temporal overlap between the sensitisation and challenge phases of the topical HDM models, mechanistic investigations tend not to distinguish between sensitisation and allergic response processes, generally investigating the model phenotype as a whole. There are currently a few prevailing theories as to what may be driving the responses to HDM in the topical challenge models. These often centre around the effects of HDM on epithelial cells. Group 1 HDM allergens (Der p 1 and Der f 1) are cysteine or serine proteases (Chua *et al.* 1988; Ino *et al.* 1989; Ando *et al.* 1991; Stewart *et al.* 1991; Dilworth *et al.* 1991; Hewitt *et al.* 1995, 1997). This protease activity may promote the

allergenicity of HDM by inducing epithelial barrier dysfunction and increasing epithelial permeability; causing disruption to epithelial intercellular tight junctions by cleavage of occludin or by inducing E cadherin delocalisation (Herbert *et al.* 1995; Wan *et al.* 1999*a*, 2000; Heijink *et al.* 2010; Post *et al.* 2012). Given that the initial sensitisation in the present model is via the peritoneum this mechanism is unlikely to be involved in allergic sensitisation in the model. It may be that after three intranasal challenges the epithelium is damaged enough to allow further penetration of the allergen to promote the allergic response to challenge; however a single intranasal HDM dose in Balb/c mice was shown not to induce altered barrier function (Turi *et al.* 2011).

The proteases found in allergens such as HDM may also promote allergenicity through cleavage of cell surface markers. Through cleavage of CD23 on B cells, HDM may cause increased production of IgE as CD23 on B cells is involved in the negative regulation of IgE production (Sherr *et al.* 1989; Flores-Romo *et al.* 1993; Yu *et al.* 1994; Schulz *et al.* 1995, 1997; Hewitt *et al.* 1995). Der p 1 can cleave CD25 (IL-2 receptor) from human peripheral blood T cells, and as the IL-2R is important in generation of Th1 cells, this may mean Der p 1 in HDM can promote the Th2 environment of asthmatics (Schulz *et al.* 1998). Der p 1 may also promote a Th2 environment by altering the balance between IL-4 and IFN-γ production through cleavage of CD40 on DCs or by promoting loss of Th1 activity through cleavage of C-type lectins on DCs (Comoy *et al.* 1998; Ghaemmaghami *et al.* 2002; Furmonaviciene *et al.* 2007). The protease activity in house dust mite may also promote cytokine release from epithelial cells via PAR-2 receptor activation. PAR-2 stimulation promoted DC uptake of antigen and DC migration; Th2 sensitisation, airway inflammation and AHR in response to allergen challenge (Ebeling *et al.* 2007). In addition PAR-2 activation may cause release of cytokines such as GM-CSF, IL-6, IL-8 and eotaxin from epithelial type which may promote and polarise the adaptive immune system (King *et al.* 1998; Sun *et al.* 2001; Asokananthan *et al.* 2002; Kauffman *et al.* 2006).

In addition to the effects on epithelial cells HDM allergens have also been shown to directly affect other cell types including dendritic cells, basophils and mast cells and T cells causing release of asthma relevant mediators such as IL-5, Il-5 and IL-13 (Hammad *et al.* 2001; Phillips *et al.* 2003). Der p 1 treatment of DCs resulted in an increased capacity to induce T cell proliferation, and release of Th2 chemokines CCL17 (TARC) and CCL22 (MDC) (Hammad *et al.* 2001, 2003). These effects may all promote antigen presentation and the generation of a Th2 environment leading to IgE production, and eosinophilia typical of asthmatics (Phillips *et al.* 2003).

Another signalling pathway which has received much attention is that of the Toll-like receptors. Der p 2 has been shown to possess structural and functional homology with MD-2 (the LPS binding component of TLR4) which may facilitate activation of TLR4 (Trompette *et al.* 2009). The TLR pathway has been implicated in HDM-induced eosinophilia, Th2 responses, AHR and the recruitment and maturation of mDCs in the lung (Phipps *et al.* 2009) and also in the migration of DCs and IL-4 competent basophils to the draining mediastinil lymph nodes (Hammad *et al.* 2010). Airway response to HDM may be mediated through activation of TLR4 on epithelial cells, resulting in the release of TSLP, GM-CSF, IL-25 and Il-33 which go on to interact with DCs to promote Th2 immunity and asthma-like responses (Hammad *et al.* 2009).

Other factors implicated in the effects of HDM include GM-CSF, cysteinyl-leukotrienes, Dectin-1 and -2 and Syk (Cates *et al.* 2004; Barrett *et al.* 2009; Nathan *et al.* 2009). GM-CSF may drive Th2 sensitisation to intranasal HDM (Cates *et al.* 2004). HDM extracts can release cysteinyl-leukotrienes from dendritic cells via activation of Dectin-2 receptors and subsequent activation of Syk (Barrett *et al.* 2009). Or β-glucan moieties on HDM can release CCL20 (MIP-3α) – a chemokine for immature DCs – from human epithelial cells through activation of Dectin-1 and Syk (Nathan *et al.* 2009).

As few studies in the literature have utilised systemically sensitised HDM models little work has been done to investigate mechanisms driving systemic sensitisation to HDM. Since the sensitisation is not via the airway it seems unlikely that epithelial release of mediators which interact with DCs in the lung would be involved in sensitisation in the model, however some of the mechanisms described above may well be involved in the present model. In the Chapter 7 I will discuss possible future studies to investigate pathways involved in the response to HDM in the present model.

5.4.2. Intranasal sensitisation and HDM

It has been suggested that topical sensitisation is a more appropriate, clinically relevant sensitisation method (Renz *et al.* 1992), and accordingly several models are now described which utilise solely topical challenges such as (Johnson *et al.* 2004). However these models – where the sensitisation and challenge phases are blurred – make it difficult to distinguish between features of sensitisation and the allergic response to challenge. Thus it may be that the ideal model would utilise an intranasal sensitisation phase followed by a separate intranasal challenge phase as published by (Phipps *et al.* 2009) in Balb/c mice.

Although intranasal sensitisation in our hands did not induce a change in systemic IgE levels I was interested in whether this intranasal sensitisation would still prime the mice to respond to a subsequent HDM challenge. However in contrast to the Phipps model intranasal sensitisation with HDM did not prime the mice to respond to a subsequent HDM challenge and this supports the use of systemic sensitisation in the model. It also suggests that the increased levels of IgE observed in the model may be a driving factor in the inflammation and AHR, as intranasal sensitisation – which failed to induce an increase in either total or HDM-specific IgE – also failed to induce a change in cellular inflammation.

Why intranasal dosing in the model did not induce sensitisation in our hands remains to be determined. The Phipps study was conducted in Balb/c mice whilst C57/BL6 mice were used in this thesis. However, C57Bl/6 mice have been utilised for solely topical HDM models in the past (Hammad *et al.* 2009) so the strain should not have been prohibitive. Secondly this may be due to the doses used. The intranasal sensitisation dose used in the present chapter was based around the dose used for systemic sensitisation. Due to the different dosing procedures (i.p. vs i.n) it is almost impossible to compare the level of antigen which reaches the target in the two cases and it may be that the mechanisms of topical vs systemic sensitisation are different enough that different doses would be required. The Phipps study used intranasal sensitisation on days 0, 1 and 2 whereas I sensitised the mice on day 0 and 14; perhaps the repeated dosing at close intervals is important. This would make sense given the multitude of studies using 5 times per week exposures (Johnson *et al.* 2004, 2011; Fattouh *et al.* 2010).

It is also interesting that no IgE was detected in the Phipps study, rather IgG1 was measured. In addition to IgE, IgG has been suggested to play a role in allergic asthma and other diseases which may share similar mechanisms such as anaphylaxis (Jacoby *et al.* 1984; Ito *et al.* 1986; Pelikan & Pelikan-Filipek 1986*a*; Out *et al.* 1991; Oettgen *et al.* 1994; Hamelmann *et al.* 1997*a*, 1999*a*; Miyajima *et al.* 1997; Dombrowicz *et al.* 1997; Korsgren *et al.* 1997; Kitz *et al.* 2000; Crosby *et al.* 2002; Strait *et al.* 2006; Tsujimura *et al.* 2008; Ishikawa *et al.* 2010; Castro *et al.* 2011; Williams *et al.* 2012). Allergen-specific IgG1 and 4 are found in BALF of asthmatics (Out *et al.* 1991; Kitz *et al.* 2000) and risk of asthma has been associated with level of HDM-specific IgG (Platts-Mills *et al.* 2001). In studies in atopic individuals IgE levels and IgG levels were well paralleled; increased risk of asthma was found in children with both IgE and IgG (Lau *et al.* 2005). Furthermore several murine models of asthma have highlighted a role for IgG. In an OVA model increased IgE, IgG1, Th2 cytokine production and eosinophil accumulation was observed (Hamelmann *et al.* 1999*a*) and IgE was not essential for eosinophilia or AHR in this systemic model (Mehlhop *et al.* 1997; Hamelmann *et al.* 1999*a*). Antigen-specific IgG (1) followed by allergen

challenge has been shown to induce hypersensitivity reactions and AHR (Oshiba *et al.* 1996; Miyajima *et al.* 1997) while OVA-specific IgG-immune-complexes promote features of allergic asthma (Hartwig *et al.* 2010). A model has been proposed whereby IgG promotes secondary Th2 responses by binding to FcγRs on immune cells such as DCs, monocytes, macrophages, basophils, eosinophils, neutrophils, B cells, NK cells and mast cells (Williams *et al.* 2012).

Thus although IgE has received the most attention for role in asthma, IgG subclasses may also be key players and may well be involved in the immune responses in the newly developed HDM model. It would be interesting to measure levels of total and HDM specific IgG subclasses both after sensitisation and after allergen challenge in the HDM model to further understand the mechanisms driving then model. In addition it would be prudent to parallel this with measurements of IgGs in the OVA model. This could easily be done by ELISA as described for IgE. It has been shown in murine OVA models that IgG detection may be delayed compared to IgE (Renz *et al.* 1992) for example only being detected 40 days after sensitisation while IgE was detected after 1 week (Hessel *et al.* 1995), thus it may be necessary to take out measurements to later time points to detect this.

Some may question the validity of the present model as dogma in the field is that asthmatics become sensitised to HDM and other aeroallergens through the airways. This has resulted in the suggestion that topical sensitisation is preferable (Renz *et al.* 1992) due to being more clinically relevant. However I do not feel that there is currently enough evidence to make this claim. In fact there are several other ways in which patients may become sensitised and ultimately develop asthma including in-utero sensitisation, or as a result of early atopic dermatitis and the atopic march. As outlined in the introduction several papers have suggested that infants may have some features of allergy at birth through prenatal sensitisation, rather than becoming sensitised through inhalation exposure (Holloway *et al.* 2000; Miller *et al.* 2001; Nambu *et al.* 2003; Hagendorens *et al.* 2004; Schönberger *et al.* 2005; Peters *et al.* 2009). The atopic march is a phenomenon noted in certain atopic individuals whereby atopic dermatitis is developed early on, followed by allergic rhinitis and subsequently atopic asthma (Gustafsson *et al.* 2000; Ricci *et al.* 2006; van der Hulst *et al.* 2007; Kapoor *et al.* 2008). In these cases it is highly likely that sensitisation is systemic, rather than through airway exposure (Spergel 2010). Considering this evidence, it may be that airway sensitisation is not the driving mechanism in allergic asthma. Thus as this has yet to be established, the trend to opt for solely topical models of asthma may not be appropriate. Furthermore not every person who is exposed to HDM develops allergic sensitisation or asthma. This fact implies that there is a difference in these patients, be it due to genetic factors, environmental factors, or the interaction of both. This idea is largely

ignored in the chronic, topical models such as (Johnson *et al.* 2004) because control mice receive saline and do not develop 'asthma' while those which do receive HDM all develop the model phenotype. This may be because the response is indeed a build-up of innate responses to the multiple HDM challenges (De Alba *et al.* 2010; Birrell *et al.* 2010). While the chronic topical models are very useful to investigate HDM-induced chronic inflammation and remodelling the model developed in the present chapter circumvents the issue that much of the response may be made up of repeated innate responses and also provides the opportunity to assess the effect of HDM challenge on a background of systemic allergy. In addition the ability to separate the sensitisation and challenge phases of the model will be highly useful to probe mechanisms driving allergic asthma.

Having developed a HDM-driven model of asthma which demonstrates two key features of asthma – allergic airway inflammation and AHR – I am now in a position to parallel the previous work in the OVA model investigating the effect of CS co-exposure. These studies make up the following chapter, where I will also evaluate the efficacy of steroid treatment in the HDM model.

5.4.3. LAR

One caveat to the HDM model as it stands currently is that I was not able to observe a robust LAR. It is possible that an LAR may have been induced in certain subjects but not robustly across all subjects in the study. It may therefore be that with further adjustments to the HDM sensitisation and challenge protocol that a robust LAR could be observed and this is something I would be keen to pursue. However further work on this was unfortunately beyond the scope of this thesis. To my knowledge nobody has yet published on a HDM-induced LAR in the mouse, so if achieved this would be a highly useful tool to supplement the allergic HDM-driven model and a great addition to the field; the LAR is a very clinically relevant, functional feature of asthma. I have not yet established why an LAR was not observed in the HDM model when it was observed in the OVA model despite the two models using very similar protocols; other features such as AHR and airway inflammation are also highly replicated between the two models. In published work our group has previously shown TRPA1 channel activation to be involved in the OVA-induced LAR. In addition other groups have shown TRPA1 to be involved in ovalbumin driven responses including airway inflammation and AHR (Caceres *et al.* 2009). However to my knowledge nobody has investigating whether a role exists for TRPA1 in the response to HDM challenge. It may therefore be interesting to investigate the ability of HDM challenge to induce activation of TRPA1 channels or other airway sensory nerve components. Further

understanding of the effect of these allergens on airway neuronal activity could help with development of a HDM-driven LAR model in the future.

In contrast to allergic inflammation and AHR, the LAR in the OVA-driven model was completely dependent on the presence of Alum during sensitisation. Indeed other groups which have shown LAR in murine OVA models have utilised Alum during sensitisation (Crosby *et al.* 2002; Nabe *et al.* 2005), while another group observed an EAR, AHR and airway eosionophilia but no LAR in a murine OVA model which lacked an adjuvant (de Bie *et al.* 2000). This is a very interesting finding: further understanding of the mechanisms driving the OVA-induced LAR may aid work towards development of the HDM-induced LAR model.

Why is Alum required for the OVA-induced LAR? Initially I hypothesised that Alum would be required for OVA-induced IgE production and that IgE was essential for the LAR. This hypothesis would be supported by the finding that anti-IgE treatment inhibited the allergen-driven LAR in the clinic (Fahy *et al.* 1997). There may be some requirement for Alum for the initial increase in total and OVA-specific IgE after sensitisation alone (in the standard OVA model) and this may explain the requirement for Alum in the LAR. However these changes in IgE are small in comparison with the changes observed after both OVA sensitisation and challenge and Alum was not required for these changes. Thus it is may be that IgE does not drive the LAR and other immunoglobulins such as IgGs are involved. The role of IgG in allergic airway inflammation has been reviewed recently (Williams *et al.* 2012), but no mention of the late asthmatic response was made; this therefore appears to be a relatively new idea. In the clinic it is likely that IgE mediates the early response (Boulet *et al.* 1997*a*), however several studies have tried to determine the contribution of IgE or IgG1 to the development of the LAR using allergen challenge protocols and comparing IgE and IgG levels in those which develop an LAR compared to those which do not. Development of an LAR after allergen challenge was associated with increased serum levels of both total IgE and IgG (Pelikan & Pelikan-Filipek 1986*a*) but IgG1 rather than IgE was predictive of a patient developing an LAR after HDM challenge (Ito *et al.* 1986). Allergen-specific IgG and IgG1 to candida or mite allergens were higher in LAR positive asthmatics, while IgE did not differ (Ogurusu *et al.* 1991). In contrast Der f specific IgE was higher in dual responders than isolated early responders, while IgG levels did not differ (Hong & Park 1989). In addition although not frequently cited as an asthmatic mediator IgA may also be involved in asthma; both allergen-specific IgA and IgE in the BAL fluid/serum were found to be determinants of the LAR (Peebles *et al.* 2001).

In a BN rat OVA model, neither levels of OVA-specific IgE or IgG were found to correlate with the magnitude of the LAR (Waserman *et al.* 1992). However in mouse little has been done to determine the immunoglobulin dependence of the LAR, probably in part because of the dearth of murine models of this endpoint. The EAR in C57Bl/6 mice may be driven by IgG rather than IgE (Crosby *et al.* 2002); an LAR is also observed in this model so it would be very interesting to know the IgE or IgG dependence of the LAR. This work is based on a model which utilises C57Bl/6 mice systemically sensitised with OVA plus Alum so these findings may well be relevant to the present model. In mice which were passively sensitised with OVA-specific IgE and then topically challenged with OVA, a late response occurred after 4 challenges, and interestingly IgG1, Il-4 and Il-13 were also increased at this time-point (Mizutani *et al.* 2012). Neutrophils and the complement mediator C3a have been implicated in the LAR (Mizutani *et al.* 2009, 2012; Nabe *et al.* 2011) and IgG1 may drive C3a cleavage and complement activation (Mizutani *et al.* 2012). Most interesting in terms of the present data is systemic sensitisation with Alum in mice induced more OVA-specific IgG1 production than adjuvant-free sensitisation, but no difference was found in IgE levels (Conrad *et al.* 2009). This implies that IgG1 may be the Alum dependent factor which promotes the LAR in the present model. An IgG-C3a-neutrophil axis in the LAR may therefore be interesting to investigate and I will discuss this further in the Chapter 7.

5.4.4. OVA model and adjuvant

While developing the HDM model it was also found that the presence of Alum during sensitisation was not required for the allergic response to OVA in mice previously sensitised to this allergen, or for the production of OVA-specific IgE. However the presence of Alum during sensitisation was necessary for the induction of the LAR.

Dogma and the majority of historical models suggest that the OVA model requires the use of a systemic adjuvant to induce allergic sensitisation in this model. However in this chapter I have shown that an adjuvant is not required for the production of OVA-specific IgE or the induction of airway inflammation or AHR in the OVA model. While most OVA models used in the field incorporate an adjuvant, several adjuvant-free OVA models have however been described in the past which describe systemic sensitisation with OVA (Renz *et al.* 1992; Hessel *et al.* 1995; Blyth *et al.* 1996; De Bie *et al.* 1996; Besnard *et al.* 2011), predominantly in Balb/c mice. These protocols have been shown to induce several of the hallmark features of asthma including allergen-specific IgE, the EAR, AHR, airway inflammation and airway remodelling including airway epithelial thickening, reticular basement membrane fibrosis, GC hyperplasia.

What I have shown is therefore not a new finding however it has been largely ignored in the field. This is surprising since the use of an exogenous adjuvant has been widely criticised for not being clinically relevant. The next question to ask is how does OVA induce allergic sensitisation? Many publications have investigated the allergenic properties of HDM suggesting that TLR4 activation and the endogenous proteinase activity may be important (as described above). However parallel work has not really been conducted for the OVA model; this is likely to be because HDM is a clinically relevant allergen for asthma and OVA is not. It has been noted that OVA is contaminated with LPS (Watanabe *et al.* 2003). Organic molecules such as LPS possess adjuvant activity (Eisenbarth *et al.* 2002), thus this may be a mechanism as to how sensitisation to OVA in the absence of adjuvant occurs (Dabbagh *et al.* 2002; Eisenbarth *et al.* 2002; Piggott *et al.* 2005) in the present experiments and those of (Renz *et al.* 1992; Hessel *et al.* 1995; Blyth *et al.* 1996; De Bie *et al.* 1996). It may be that some people do not see the allergenic effects of OVA without an adjuvant due to utilising a batch containing less LPS, however I think it more likely that this adjuvant-free regimen has not been tested: very few publications have actually compared the effectiveness of sensitisation to OVA with or without adjuvant. One such study (Conrad *et al.* 2009) showed that systemic sensitisation with Alum induced more OVA-specific IgG1 production than adjuvant-free sensitisation, but no difference was found in IgE levels.

One mechanism through which Alum is thought to work is through the inflammasome-IL-1β axis promoting an adaptive immune response (Kool *et al.* 2008*a*; Eisenbarth *et al.* 2008). Several studies have suggested that Alum can induce release of the danger signal uric acid, and that this is the mechanism by which Alum activates the inflammasome and promotes allergenicity (Goto & Akama 1982, 1984; Goto *et al.* 1997; Shi *et al.* 2003; Martinon *et al.* 2006; Kool *et al.* 2008*a*, 2011; Hornung *et al.* 2008). Recently it has been shown that the NLRP3 inflammasome and downstream mediators IL-1β and IL-1α may also be involved in an allergic OVA model in the absence of Alum (Besnard *et al.* 2011) via an effect on DC function. Another mediator implicated in the effects was TSLP as levels of this cytokine were reduced in the NLRP3$^{-/-}$ mice in the OVA model (Besnard *et al.* 2011). Due to the involvement of TSLP in Th2 cell differentiation by regulation of OX40L on DCs and IL-4 synthesis (Ito *et al.* 2005; Omori & Ziegler 2007), this is highly likely to be involved in promotion of Th2 immune responses in this model. It would be very interesting to determine whether the same processes were involved in the present OVA model, and also in the HDM model. I will discuss possible future studies to investigate this in Chapter 7.

It may be that in some cases such as (Conrad *et al.* 2009) immunoglobulin production may be bolstered by the addition of adjuvant, however OVA-specific IgE, airway cellular inflammation, Th2 type response (IL-5 and IL-13, and AHR to MCh were all

observed in the absence of Alum in this model. Thus it is actually not advantageous to include this un-physiological agent in the OVA model if key asthmatic features can be induced without it. If this practice was adopted in the field, this might allay some of the criticisms of this widely useful model. For this thesis it was decided to continue using Alum in the OVA model to allow consistency between the experiments that had already been performed and subsequent experiments which were to be conducted after this finding.

Chapter 6. The effect of cigarette smoke on HDM-induced airway inflammation and AHR

6.1. Rationale

In Chapter 5, I developed a HDM-driven model of allergic asthma which features allergic inflammation and AHR. To parallel the work described in Chapter 4, I will now combine CS exposure with the newly developed HDM-driven asthma model. As the sensitisation and challenge regimen of the HDM model replicate that of the OVA model, I will use the CS exposure regimen described in Chapter 4 and combine this with the HDM model.

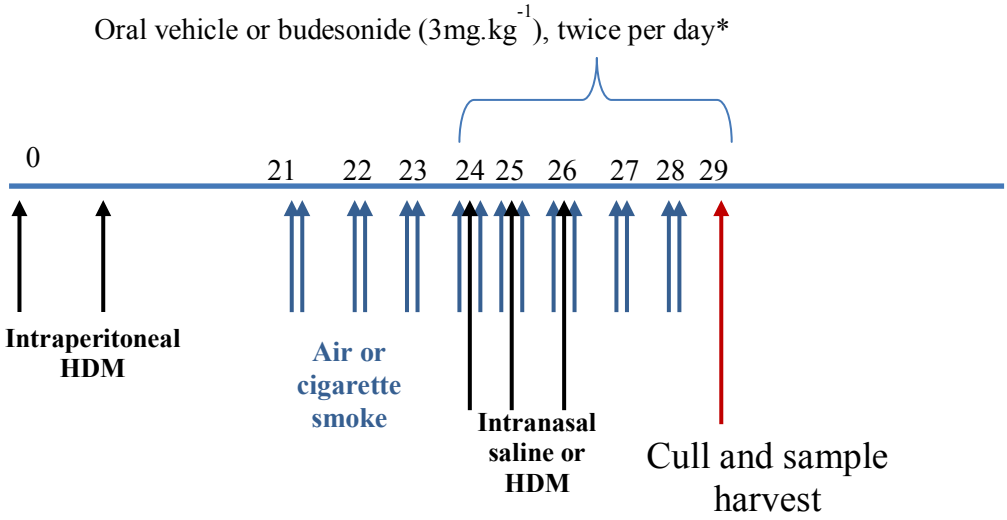
In addition these studies provided the opportunity to assess the sensitivity of the HDM model to steroid treatment with steroid and how this is impacted on by the co-exposure with CS. The steroid treatment regimen was based on that described for the OVA model in Chapter 4. To limit the number of animals used for these studies I selected a dose of steroid which effectively attenuated OVA-induced AHR and inflammation: $3mg.kg^{-1}$ budesonide, rather than performing a full dose response.

6.2. Methods

Mice were sensitised intraperitoneally with HDM (0.5µg.kg^{-1} in 0.1ml saline) on days 0 and 14. Mice were then exposed to air or cigarette smoke twice per day starting on day 21 (4 hours apart). Mice were challenged intranasally with saline or HDM (1.25µg.kg^{-1} in 50µl saline) once daily on days 24-26, approximately halfway between the two CS challenges. Exposures to CS were continued until day 28 (inclusive) and endpoints were assessed on day 29. To determine the effect of cigarette smoke exposure on the treatment sensitivity of this model, mice were also dosed with oral vehicle (0.5% methylcellulose plus 0.2% tween80 in water) or budesonide (3mg.kg^{-1}) via oral gavage twice per day from day 24 and also received a final dose 1 hour prior to endpoint assessment on day 29. The protocol for this study is detailed in schematic form in figure 6.1.

6.2.1. Evaluating the effect of CS on the HDM model and its treatment: inflammation and conscious lung function

In mice exposed to CS plus HDM as described above airway responsiveness to inhaled 5-HT (0.3, 1, 3, mg.ml^{-1}) was assessed 72 hours after final HDM challenge using whole body plethysmography (Penh). Mice were allowed to recover from spasmogen challenge for at least one hour, after which mice were culled: BAL was performed and levels of inflammatory cells were assessed in the BAL fluid.



* Mice were dosed with budesonide twice per day as indicated, except on day 29 where mice were dosed once and culled one hour later.

Figure 6.1: Schematic of the protocol for combining CS and the HDM model

6.3. Results

6.3.1. The effect of CS on HDM-induced airway inflammation and the anti-inflammatory effects of steroid

HDM challenge alone resulted in significant increases in the levels of eosinophils, lymphocytes, macrophages and neutrophils (Figure 6.2 A-D) compared to saline challenged controls (p= 0.0001, 0.0001, 0.0147 and 0.0046 respectfully, Mann-Whitney U-test). Treatment with ($3mg.kg^{-1}$) budesonide (Figure 6.2 A-D) almost completely inhibited the HDM-induced increases in BAL eosinophils, lymphocytes, and neutrophils (p=0.0002, 0.0001 and 0.0405 respectfully, Mann-Whitney U-test). In addition steroid treatment significantly reduced the HDM-induced increases in BAL macrophages (p=0.0147, Mann-Whitney U-test).

CS co-exposure caused an apparent additive increase in the level of BAL lymphocytes and a significant increase in the level of BAL macrophages (p= 0.0321, Mann-Whitney U-test) compared to HDM-challenged/air-exposed controls. CS exposure however had little effect on the level of HDM-induced BAL eosinophils. After CS and HDM co-exposure the levels of BAL neutrophils were comparable with those observed after CS exposure alone. These results therefore largely parallel the cellular inflammation data observed in Chapter 4 where CS and the OVA model were combined. CS co-exposure did not have a striking effect on levels of airway inflammation compared to levels in mice exposed to HDM alone.

After CS and HDM co-exposure budesonide significantly reduced the levels of BAL eosinophils, lymphocytes, and macrophages (p= 0.0001, 0.0002 and 0.0008 respectively, Mann-Whitney U-test). Budesonide however had no effect on the level of BAL neutrophils in mice exposed to CS alone, or CS plus HDM. Again these data are very similar to the data obtained in the OVA model.

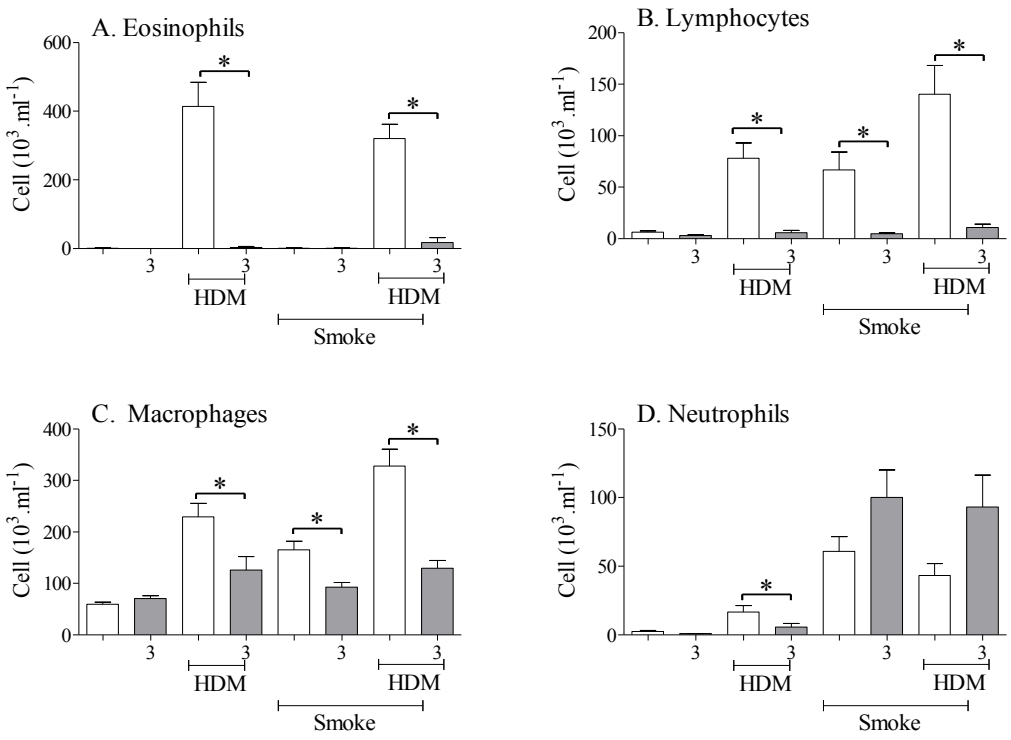


Figure 6.2: The effect of cigarette smoke on HDM-induced cellular inflammation and the anti-inflammatory efficacy of budesonide in this model
Male C57Bl/6 mice were sensitised with HDM, and subsequently challenged with saline or HDM, and exposed to air or cigarette smoke as indicated on the figures. Mice were also treated with vehicle (0.5% methylcellulose plus 0.2% tween80 in water) (open bars) or budesonide (3mg.kg^{-1}) (closed bars). Data expressed as mean cell number (10^3.ml^{-1}) + SEM n=11-12 per group. *=p<0.05 HDM challenged/budesonide treated mice vs. relevant challenged/vehicle-treated controls, Mann-Whitney U-test.

6.3.2. The effect of CS on HDM-induced AHR

In HDM-sensitised mice, 5-HT induced a greater response in HDM challenged mice than in those which were challenged with saline (Figure 5.3). This AHR was pronounced at 1mg.ml^{-1} 5-HT and significant at a dose of 3mg.ml^{-1} 5-HT (p=0.0051, Mann-Whitney U-test). This replicates the data obtained in Chapter 5 which shows that the AHR in this model is robust and reproducible. In HDM-challenged mice treated with budesonide the AHR observed to 1 and 3mg.ml^{-1} 5-HT was completely abolished. In the mice exposed to CS plus HDM, the AHR appeared to be almost abolished at 1mg.ml^{-1}, but a significant AHR was observed with 3mg.ml^{-1} 5-HT (p=0.0009, Mann-Whitney U-test). Although the AHR was not completely blocked after CS exposure as was observed in the OVA model, the response to 5-HT appeared to be shifted to the right by CS; this is most clearly observed after 1mg.ml^{-1} 5-HT. In air exposed mice, the increase in Penh area under the curve (AUC) induced by

1mg.ml^{-1} 5-HT was more than double the response to 5-HT in the saline challenged mice. In smoke exposed mice, the increase in Penh AUC induced by 1mg.ml^{-1} 5-HT was only marginally greater in HDM-challenged mice than in saline challenged mice. Therefore only a very small enhancement in the response to 1mg.ml^{-1} 5-HT was observed in CS-exposed/HDM-challenged mice compared to CS-exposed controls. In mice exposed to CS plus HDM the AHR observed to 3mg.ml^{-1} 5-HT was completely abolished by steroid treatment (Figure 6.3).

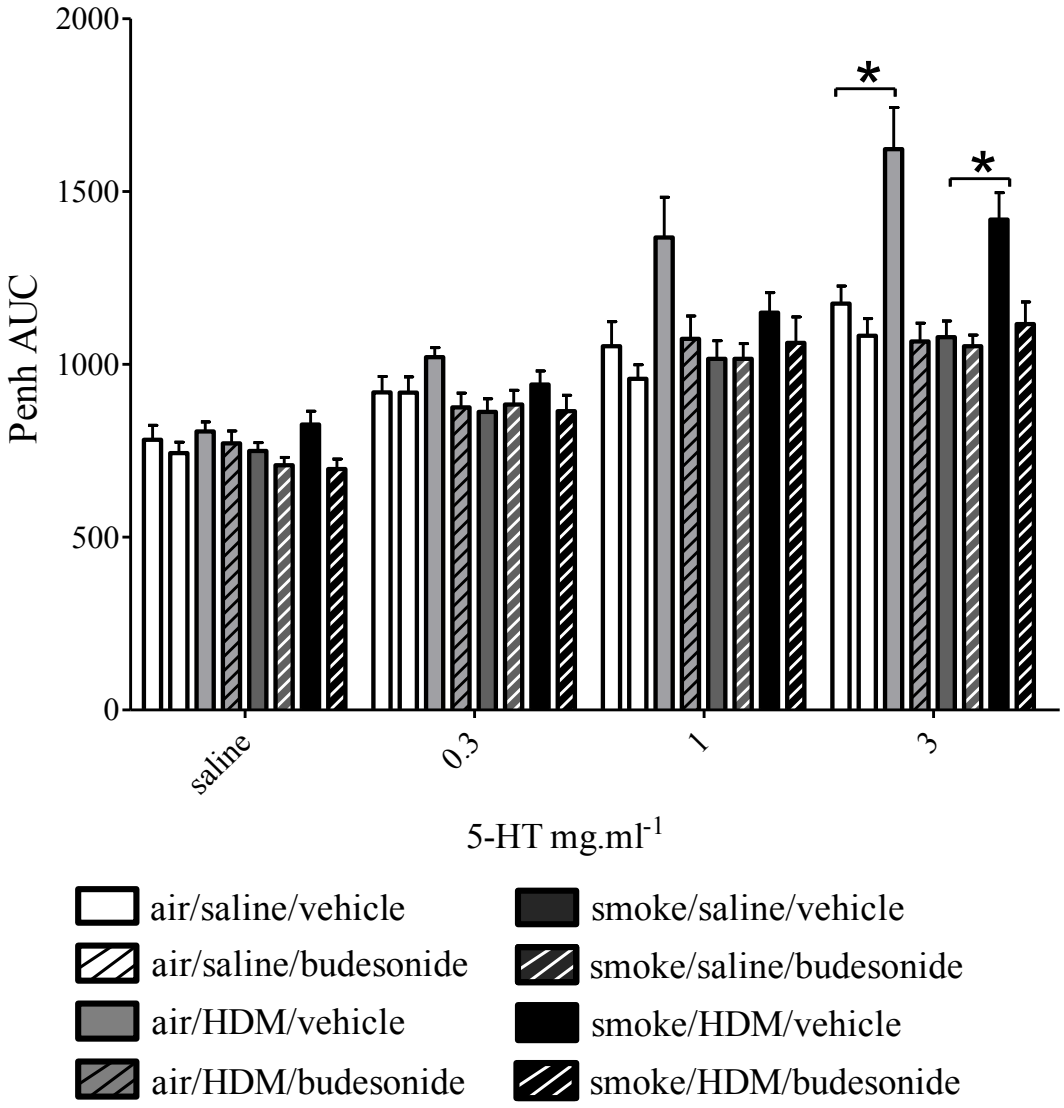


Figure 6.3: Effect of cigarette smoke on HDM-induced airway hyperresponsiveness
Male C57Bl/6 mice were sensitised with HDM, and subsequently challenged with saline or HDM and exposed to air or cigarette smoke. Mice were also treated with oral vehicle (0.5% methylcellulose plus 0.2% tween80 in water) or 3mg.kg^{-1} budesonide.
White bars = air/saline challenged, light grey bars = air/HDM challenged, dark grey bars = smoke/saline challenged, black bars = smoke/HDM challenged. Plain bars = vehicle treated mice, striped bars = budesonide treated mice. Mice were placed in whole body plethysmography boxes 3 days after final challenge and response to inhaled 5-HT recorded as Penh AUC. Data expressed as mean Penh AUC + SEM for n=11-12 per group. *=$p<0.05$ HDM challenged/vehicle treated mice compared to relevant air/saline/vehicle or smoke/saline/vehicle controls, Mann-Whitney U-test. Where no symbol is shown on the figure this indicates a non-significant difference.

6.4. Discussion

In this chapter I firstly showed that the airway inflammation and AHR observed in the HDM model was highly sensitive to treatment with oral steroid (budesonide). With current interest in the field centring on probing the immunological features of HDM models, few publications have actually determined whether murine HDM models are sensitive to steroid treatment. In a model which incorporates multiple topical challenges with HDM (Ulrich *et al.* 2008) showed that the airway and lung tissue inflammation was sensitive to treatment with both topical and systemic steroids. However systemic dexamethasone failed to impact on AHR or BAL total airway cellular inflammation in a HDM model utilising systemic sensitisation with HDM followed by intranasal HDM challenges (Mushaben *et al.* 2013). It is important that any models of a clinical disease which are utilised to test the efficacy of novel therapeutic entities respond to the standard existing therapies for that disease. As pulmonary inflammation and AHR are attenuated by steroid treatment in the clinic (Djukanović *et al.* 1992, 1997) the finding that steroid treatment abolishes both airway inflammation and AHR in the current HDM-driven model provides further evidence that the model demonstrates features consistent with clinical asthma.

The main aim of the chapter was to assess the effect of CS co-exposure on the newly developed HDM-driven asthma model. The results observed here largely replicated those observed in the OVA model. With the exception of a small additive enhancement of lymphocyte and macrophage numbers in the BAL fluid in CS co-exposed mice compared to mice challenged with OVA alone, CS did not appear to dramatically impact on HDM-induced airway inflammation. In addition CS exposure did not dramatically impact on the sensitivity of the HDM-induced airway inflammation to treatment with oral steroid. Budesonide was able to significantly inhibit the HDM-induced airway neutrophilia but not in mice co-exposed to CS plus HDM; however the neutrophilia induced by CS alone was not inhibited by budesonide treatment either. It therefore seems likely that the neutrophilia in the co-exposed mice resulted predominantly from the CS challenge and that CS exposure conferred the addition of a steroid-resistant population of airway neutrophils to the HDM model.

The enhanced neutrophilic phenotype may well be representative of the phenotype of smoking asthmatics: A common line of thought regarding smoking asthmatics is that the inflammatory phenotype is more neutrophilic than that which is observed in non-smoking asthmatics (Boulet *et al.* 2006; St-Laurent *et al.* 2008; Meghji *et al.* 2011). CS is widely accepted to induce an increase in airway neutrophils (Hunninghake & Crystal 1983;

Kuschner *et al.* 1996; Roth *et al.* 1998; Amin *et al.* 2000) which are resistant to steroid treatment in smokers (Culpitt *et al.* 1999) and this cell type has been associated with a decline in lung function (Stănescu *et al.* 1996). Neutrophil markers were shown to be diminished by long term high and low dose ICS in non-smoking asthmatics, but not in smokers (Pedersen *et al.* 1996). There is a suggestion that in man the inflammatory profile and whether this is most contributed to by smoking or the underlying asthma is dependent on the intensity and duration of smoking history (Polosa & Thomson 2013), indeed in man and murine studies CS exposure has been shown to have dose-dependent inflammatory effects in the lung (Kuschner *et al.* 1996; Clatworthy *et al.* 2009; Eltom *et al.* 2011) thus it may be worth investigating whether further CS exposure may induce more striking effects in the present model such as a reduction in lung function

Similarly to the OVA model, it may be predicted that CS co-exposure would reduce the anti-inflammatory efficacy of steroid treatment in the HDM model because in COPD – a disease predominantly induced by cigarette smoking – glucocorticoids fail to inhibit inflammation (Keatings *et al.* 1997; Culpitt *et al.* 1999). Smoking is also thought to reduce asthmatic patients' responses to steroid treatment (Chalmers *et al.* 2002; Chaudhuri *et al.* 2003; Lazarus *et al.* 2007). However in general steroid treatment had a robust anti-inflammatory effect in CS and HDM co-exposed mice. Surprisingly few clinical studies have described the effects of steroid treatment on airway inflammation in smoking asthmatics; the studies tend to report lung function or asthma control as the primary endpoint. In addition if pulmonary cellular inflammation is described, it is typically only eosinophilia which is reported, therefore there is little direct evidence on the effects of steroids on other inflammatory cells in smoking asthmatics. ICS have been shown to improve sputum eosinophils in asthmatics, but not in smoking asthmatics in short term (Chalmers *et al.* 2002) and long term studies (Pedersen *et al.* 1996), but others have shown that ICS do improve sputum eosinophils and ECP in smokers and non-smokers alike (Lazarus *et al.* 2007). Therefore the effect of smoking on the anti-inflammatory effects of steroids in asthmatics is currently controversial.

In terms of AHR in the HDM model, CS exposure resulted in a shift in HDM-induced AHR. This is in contrast with the OVA model where CS resulted in a complete abrogation of OVA-induced AHR. It might be that in the OVA model the AHR only appeared to be completely blocked at the doses of 5-HT used in the study, and that with higher doses of 5-HT a shifted response would be revealed. I have not yet determined whether AHR in the HDM model can be detected using classical resistance measurements, or in the isolated trachea. However if these features were observed it would be important to parallel the whole

body plethysmography work using these techniques. Several clinical papers suggest that smoking will worsen symptoms of asthma (Siroux *et al.* 2000; Apostol *et al.* 2002; Thomson *et al.* 2004, 2013; Eisner & Iribarren 2007; Jang *et al.* 2009; O'Byrne *et al.* 2009) yet if anything in the studies presented here, CS exposure attenuated HDM-induced AHR. In the clinic smoking mild asthmatics did not develop allergen-induced AHR to MCh (Meghji *et al.* 2011). According to the data published by Meghji et al. (2011) the finding in the present thesis may therefore represent a true clinical phenotype, and it would be of interest to pursue and understand the mechanism behind this effect. However the inhibition of allergen-induced AHR was clearest in the OVA model and the OVA-induced AHR has been more widely characterised to date; non-specific AHR was observed and the AHR has been backed up with resistance measurement and studies using the isolated trachea. It may therefore be better to investigate this phenomenon in the OVA model initially. I have already discussed in detail in Chapter 4 the investigations that I would like to perform to further understand the CS-induced inhibition of AHR. Initially these would centre on measuring α-SMA and collagen levels in the airways using histological samples obtained from the OVA plus CS studies. If changes were detected in these endpoints in the OVA model, I would also parallel this work in the HDM model.

In the OVA model the AHR was completely abolished by CS exposure, but in the HDM model AHR was still observed with high 5-HT doses. Interestingly and in contrast with the LAR data, this AHR in CS and HDM co-exposed mice was still sensitive to steroid treatment. Considering this and the loss of AHR in smokers described by Meghji et al. (2011) it may be that the asthma symptoms which are both worsened in smokers and resistant to steroid treatment do not relate to AHR. This has implications for pre-clinical models of asthma, where AHR is frequently used to model the lung function changes observed in the disease.

Multiple publications have investigated the effect of CS co-exposure in murine OVA models as described in Chapter 4; however to my knowledge at the time of writing only two studies have investigated the effect of CS co-exposure in murine HDM-driven models. Firstly (Lanckacker *et al.* 2012) showed that CS can facilitate sensitisation to HDM through enhancement of HDM uptake and DC migration to mediastinal lymph nodes, or by promoting a Th2 environment. This work is based on multiple intranasal doses with HDM in low doses that did not cause a dramatic airway phenotype when given alone; however the combination of CS and HDM promoted airway and lung tissue inflammation and HDM-induced IgG1 production. In this model little AHR was observed after HDM or CS alone but the combination exposure resulted in enhanced AHR (Lanckacker *et al.* 2012). The primary

aim of that study was to investigate the role of HDM on the sensitisation to HDM. This is in contrast to the present study where the aim was to determine the role of CS on the response to HDM challenge in mice already sensitised to HDM, thus the two models are not particularly comparable.

The second study which investigates the effect of CS in a murine HDM model was published by Botelho et al. (2011). In this model mice were exposed to HDM intranasally for 3 weeks followed by a combination of HDM and CS for a further two weeks. CS exposure resulted in a reduction in HDM-induced eosinophilia in the airway in mice challenged with both standard dose and low dose HDM. This was purported to be due to a reduction in eosinophil trafficking due to reduced expression of ICAM-1 and eotaxin-1 rather than inhibiting eosinophil survival. CS also caused an increase in collagen deposition without affecting AHR (Botelho *et al.* 2011). In Chapter 4 I described a possible role for collagen in attenuating AHR (Bramley *et al.* 1995; Khan *et al.* 2010) and CS has been reported to increase collagen deposition (Carroll *et al.* 2000; Melgert *et al.* 2007; Kim *et al.* 2011; Botelho *et al.* 2011). This may well be relevant to the CS-induced attenuation of AHR observed in this chapter. As I have described for the work in the OVA model, it may be interesting to measure collagen depositions and α-SMA levels in lung tissue from this study to determine whether increased collagen is present, and whether this may be impacting on the AHR.

The results in the present model do not particularly replicate the results currently available in the literature. This is most likely to be due to the fundamental differences in experimental design between all three studies and the different HDM models used. In the OVA and CS co-exposure models already described in earlier chapters, strikingly different results have been observed in different models depending on the exposure regimens used (Melgert *et al.* 2004, 2007; Moerloose *et al.* 2005, 2006; Robbins *et al.* 2005; Min *et al.* 2007; Van Hove *et al.* 2008; Trimble *et al.* 2009; Song *et al.* 2009).

Surprisingly few studies have mechanistically investigated the interaction between HDM and cigarette smoke – aside from those investigating the relationship between CS and risk of asthma. Many papers suggest that CS increases risk of asthma and promotes sensitisation (Flodin *et al.* 1995; Torén & Hermansson 1999; Cook & Strachan 1999; Jarvis *et al.* 1999; Plaschke *et al.* 2000; Chen *et al.* 2002; Piipari *et al.* 2004; Polosa *et al.* 2008). Der p 1 may induce sensitisation by cleaving CD23 from B cells and resulting in increased IgE production (Hewitt *et al.* 1995). Natural anti-proteases exist in the lung which have been shown to inhibit Der p 1 cleavage of CD23 (Hewitt *et al.* 1995) and pollutants such as CS can inactivate α-1 antiprotease (Evans & Pryor 1994). Thus Hewitt et al. (1995) suggest that

indoor pollutants such as CS may promote HDM sensitisation by potentiating the effect of HDM proteolytic activity. Cigarette smoke has also been shown to damage the respiratory epithelium (Jones *et al.* 1980; Burns *et al.* 1989) and induce increased epithelial permeability (Li *et al.* 1994), thus increasing permeability to allergens. This may potentiate the effects of HDM which is known to induce epithelial barrier dysfunction (Herbert *et al.* 1995; Wan *et al.* 1999*b*, 2000; Heijink *et al.* 2010; Post *et al.* 2012). CS alone did not affect the permeability of HBECS, however augmented the Der p-induced increase in permeability. CS also promoted the passage of Der p across human bronchial epithelial cell (HBEC) cultures (Rusznak *et al.* 1999). This interaction is most likely to be of relevance for sensitisation to HDM. However one may hypothesise that if CS induces epithelial damage this may promote passage of allergen across the airway epithelium as shown by (Rusznak *et al.* 1999) and that this might lead to an increased response to HDM challenge. However an enhanced HDM-induced phenotype was not observed in the present study.

There are other cases where CS and HDM may act through the same mechanisms, for example through activation of TLR4 (Doz *et al.* 2008; Trompette *et al.* 2009; Hammad *et al.* 2009, 2010; Phipps *et al.* 2009) so I would naturally expect that CS would increase severity of responses to HDM. However this did not occur. The AHR was inhibited and the inflammation did not appear to be dramatically altered, however clinical studies predominantly quote lung function or healthcare parameters when reporting worsened disease in smoking asthmatics (Siroux *et al.* 2000; Apostol *et al.* 2002; Austin *et al.* 2005; Eisner & Iribarren 2007; Shavit *et al.* 2007; Polosa *et al.* 2008; Jang *et al.* 2009). It would be very interesting to model further lung function changes in the HDM model with the goal to determine whether CS worsened these. For example it may be worthwhile to utilise a more chronic dosing regimen which may induce chronic remodelling and a reduction in lung function.

The two published HDM and CS co-exposure models I have described were both conducted in BALB/c mice, thus the model described in this thesis is the first to combine CS and HDM in C57Bl/6 mice. This is a great advantage as it means that in the future genetically modified mice strains can be tested in the model to investigate mechanisms driving the combined phenotype, such as the attenuation of the AHR. In addition an advantage of HDM model having distinct sensitisation and challenge phases is that it can be ensured that the CS exposure does not impact upon sensitisation, ensuring that the only effect observed is that of CS on the response to HDM challenge.

As I have mentioned the result of CS co-exposure in the OVA and the HDM model were largely duplicated. Thus it is a reproducible model, and the results are therefore unlikely to be allergen-specific. The advantage of having the two models is that the HDM model is more clinically relevant, whilst the OVA model features a robust allergen-induced LAR. The two models are therefore complementary and can both be utilised in the future to further investigate the effect of CS in allergic asthma.

Chapter 7. Summary and future studies

7.1. Thesis summary

Asthma is a highly prevalent disease globally, and with the increase of the modern, urbanised lifestyle worldwide incidence continues to increase. Although symptoms in most patients with mild disease are well controlled, a proportion of asthmatics, often with severe disease are poorly controlled, despite treatment with high doses of ICS, OCS or add-on therapy. These patients contribute the majority of the economic burden of the disease, and asthma in these patients is more likely to impact upon their quality of life, including their ability to work. Many cases of severe or treatment resistant asthma are associated with smoking or passive exposure to pollutants or cigarette smoke. Preclinical models which closely replicate key features of disease are highly useful to investigate disease mechanisms and also to test the efficacy of new therapeutic entities. The aim of this thesis was therefore to develop a murine model of CS and allergen co-exposure which could be used in the future to investigate the mechanisms behind the effect of smoking in asthmatics. I chose to develop a murine model due to the availability of genetically modified murine strains which will be utilised in the future to investigate disease mechanisms. These mice were generated on the C57Bl/6 strain, so all work in this thesis was conducted in this strain.

Our group has previously established an in-house murine CS model (Eltom *et al.* 2011; Rastrick *et al.* 2013) which I was able to utilise in this thesis. Ovalbumin has been used for several decades as a surrogate allergen in pre-clinical asthma models. The group had previously established a murine ovalbumin-driven model based on previously published work in the BALB/c mouse (Birrell *et al.* 2003). In chapter 3 I performed a detailed characterisation of the in-house OVA model. Temporal increases in airway eosinophils, lymphocytes, macrophages and neutrophils were observed in mice sensitised and challenged with OVA. In conjunction with this a robust AHR was observed using non-invasive lung function, which was backed up using classical resistance measurements and also in the isolated trachea. In a slightly modified protocol, a late asthmatic response was also observed in OVA sensitised mice following OVA challenge. This model therefore recapitulates several of the key features of allergic asthma: allergic airway inflammation, allergic airway hyperresponsiveness, and the late asthmatic response.

Having established the response to OVA alone I then combined the OVA model with the in-house CS model. Cigarette smoke co-exposure caused a significant increase in the levels of BAL macrophages and lymphocytes compared to OVA alone, but did not modulate the levels of OVA-induced airway eosinophilia. CS exposure did not alter the anti-inflammatory efficacy of steroids on OVA-induced inflammation; airway eosinophils,

lymphocytes and macrophages were all inhibited by steroid treatment in mice co-exposed to CS. In contrast the levels of airway neutrophils in mice exposed to CS combined with OVA were comparable with those observed in mice exposed to CS alone. Steroid treatment failed to impact upon the airway neutrophilia in the mice co-exposed with OVA plus CS; however the airway neutrophils resulting from CS alone were themselves insensitive to steroid treatment. In terms of the OVA-induced lung function changes, CS completely abolished the OVA-induced AHR measured by non-invasive lung function, and a similar observation was observed in the isolated trachea. Although this is somewhat counter intuitive considering several papers have shown smoking to worsen asthma symptoms (Siroux *et al.* 2000; Apostol *et al.* 2002; Thomson *et al.* 2004, 2013; Eisner & Iribarren 2007; Jang *et al.* 2009; O'Byrne *et al.* 2009), this finding has actually been observed in the clinic (Meghji *et al.* 2011). In contrast, the OVA-induced LAR was enhanced by CS co-exposure and was rendered completely insensitive to steroid treatment. Thus CS appeared to have disparate effects on the various endpoints of the OVA model.

The OVA model has received criticism in the past for several reasons: firstly it is not a clinically relevant allergen for asthma. Secondly, in many cases tolerance develops to chronic exposure. Thirdly the model typically requires systemic sensitisation along with an adjuvant. Not only does the use of an adjuvant add an additional non-physiological manipulation to the model, but as dogma suggests that sensitisation in asthma occurs via the airways, systemic sensitisation may not be appropriate. There is however data concerning the atopic march and prenatal allergen sensitisation which suggest that airway sensitisation is not the only mechanism for allergen sensitisation that may be involved in asthma development. Because of the described issues, many groups have switched to using the clinically relevant allergen: HDM. These models also provide the advantage of being adjuvant free and also utilise multiple topical challenges with HDM. I decided that it would be important to parallel the work performed in the OVA model with work in a HDM-driven model. I therefore next set out to develop an in-house HDM-driven model. In house we have shown that HDM can induce an innate inflammatory response in the airways (De Alba *et al.* 2010). Therefore we are concerned about the true allergic nature of the topical HDM models, as the inflammation may just be made up of multiple responses to an innate insult (De Alba *et al.* 2010; Birrell *et al.* 2010). On top of this, nobody has yet demonstrated an EAR or LAR in mouse in response to HDM. Because of these issues I set out to develop a HDM-driven murine model in which airway inflammation only occurred in animals which had previously been sensitised to HDM.

To develop the sensitisation phase of the model I measured plasma total and OVA-specific IgE in mice treated with various doses of HDM via the intranasal and intraperitoneal

routes; the latter with and without Alum. None of the HDM doses induced a change in total or OVA-specific IgE when given intranasally. In contrast a bell shaped IgE dose response was observed in response to systemically dosed HDM both with and without Alum. The 0.5μg.kg^{-1} HDM dose which induced the highest level of IgE production was selected for further model development. I next went on to optimise the challenge phase of the HDM model. In non-sensitised mice, challenge with high HDM doses resulted in a small increase in airway neutrophils and lymphocytes, however in mice sensitised with HDM plus Alum, HDM challenge induced a robust, dose-dependent increase in airway eosinophils, lymphocytes, macrophages and neutrophils compared to mice challenged with saline. This was not observed in non-sensitised mice. I therefore selected a dose of HDM (1.25μg.kg^{-1}) which resulted in robust allergic airway inflammation but no inflammation in non-sensitised mice. Using direct comparison studies I then determined that Alum during sensitisation was not required for the allergic airway inflammation observed in the HDM model. Subsequently I demonstrated a robust allergic AHR in the HDM model which also did not require the use of Alum. Thus a HDM-driven model was developed in which allergic airway inflammation and AHR was observed. The use of a non-physiological and artificially Th2 polarising exogenous adjuvant was not needed to induce these responses, which improves the clinical relevance of this model.

Due to the trend for using intranasally dosed HDM for models in the field I was interested to determine whether intranasally dosed HDM could sensitise the mice to respond to a subsequent HDM challenge despite the lack of increased total or HDM-specific IgE. While HDM challenge induced allergic airway inflammation as previously in mice sensitised to HDM systemically, no change in airway inflammation was observed following HDM challenge in mice sensitised intranasally. This finding supports the use of intraperitoneal sensitisation in this model.

The late asthmatic response is a key feature of allergic asthma therefore I was keen that the HDM model should also demonstrate this endpoint. Unfortunately no evidence of airflow obstruction was observed after any of the three HDM challenges used to induce allergic airway inflammation. I therefore modified the HDM model protocol in line with the adapted protocol used to generate OVA-induced AHR, but again this failed to generate an LAR. Apart from the use of a different allergen the primary difference between the HDM model and the OVA-LAR model is the use of Alum. Therefore I hypothesised that Alum may be required for the generation of the LAR. To investigate this I compared HDM and OVA challenge in mice sensitised to the relevant allergen both with and without adjuvant. Interestingly the absence of Alum completely abolished the OVA-induced LAR. In addition a small LAR was observed in the HDM model when mice were sensitised with HDM plus

Alum; however this was neither reproducible nor robust. Therefore Alum appeared to be essential for the LAR in both models. This then led me to question the role of adjuvant in the allergic OVA model as it was not required for inflammation or AHR in the HDM model. Indeed Alum was not actually required for allergic inflammation, AHR or the production of OVA-specific IgE in the OVA model. This is a very interesting finding because it means that one of the major criticisms of the OVA model can be circumvented. This is actually not an entirely new finding as others have reported adjuvant free OVA models in the past (Renz *et al.* 1992; Hessel *et al.* 1995; Blyth *et al.* 1996; De Bie *et al.* 1996; Besnard *et al.* 2011) and the fact that this has been observed by other groups supports the data presented here. The use of an adjuvant free OVA model in the future could alleviate some of the criticism of the use of this model.

To summarise the HDM model development work – a model was developed in which allergic airway inflammation and AHR was observed, which was dependent on prior allergic sensitisation to the allergen but not the presence of an exogenous adjuvant. However to date it has not been possible to generate a robust LAR in this model. Although other similar HDM models have been described in the literature (Clarke *et al.* 1999; Tournoy *et al.* 2000; Kikuchi *et al.* 2006; Kelada *et al.* 2011) very little work has been performed in these models beyond the initial description of the model. Thus this model will be a great addition to the tools available to the group. Further work planned in the group using this model will hopefully generate important insight into the mechanisms driving allergic asthma and the allergic response to this highly clinically relevant allergen.

Finally to parallel the OVA and CS co-exposure studies I then combined the CS model with the newly developed HDM model. The results in this model almost completely paralleled those described in the OVA model. CS co-exposure resulted in an increase in airway lymphocytes and macrophages compared to HDM alone, but did not change the steroid sensitivity of the HDM-induced airway influx of eosinophils, lymphocytes or macrophages. CS conferred the addition of a steroid insensitive neutrophil population which was not observed in response to HDM alone. Although the blockade of AHR was not as striking as it was in the OVA model there was still a trend for CS inhibiting the HDM-induced AHR. The fact that the effect of CS was so closely paralleled across two models which utilise entirely different allergens means the data is highly reproducible.

In this thesis I have developed two models driven by different allergens in which CS co-exposure results in a change in the model phenotype. The OVA model features the LAR which is a key clinical feature of allergic asthma. No previous studies describe the effects of CS on allergen-induced LAR in a murine model. The adverse effect of CS co-exposure on

the response of this endpoint to pharmacological intervention provides a very excting opportunity to further understand the adverse effect of smoking on asthma treatment. Possible investigations into this are described below and should be a priority for future work in this area providing the potential for development of more effective therapies. In addition the newly developed HDM model will be a great tool to complement the models utilised in the group; it is driven by a clinically relevant allergen and does not require the use of a non-physiological adjuvant. The combination of CS and HDM described in this thesis provides the opportunity to investigate the effect of CS on the physiological response to HDM exposure on a background of prior sensitisation. In many of the published allergen and CS co-exposure models CS is given during sensitisation and CS is therefore likely to impact upon sensitisation as well as the allergic response to exposure. The model is however in the early stages of its characterization and work should be conducted to further understand the model phenotype before it can be used in drug discovery. This work is also outlined below.

7.2. Further directions

7.2.1. HDM model

In this thesis I developed a model of HDM-driven asthma but much more work could be carried out to fully characterise the model. In the field, much emphasis is placed on the immunological features of asthma models – for example the cytokine and T-helper response profiles are very important in understanding the immunological mechanisms driving the models and how this relates to the clinical phenotype. I would therefore like to perform a thorough characterisation of both the cellular and humoral characteristics of the model. Firstly I would perform fluorescence-activated cell sorting (FACS) analysis on both BAL fluid and lung tissue and specifically assess whether dendritic cells, mast cells and the various T-cell subtypes were found in the lung in this model. I would also like to perform a detailed cytokine analysis in the lungs and BAL fluid, measuring inflammatory mediators such as IL-1β or TNF-α; classical Th2 type asthmatic mediators such as IL-13, IL-5 and IL-4; Th1 mediators such as IFN-γ; Th17 mediators IL-17 and IL-22 and the innate Th2 skewing mediators IL-33, TSLP and IL-25. I would do this by utilising the ELISA or MSD techniques. Alternatively several studies have used allergen recall challenge of splenocytes or lymph node cells to assess the production of mediators in response to HDM on the background of HDM sensitisation (Johnson *et al.* 2004; Phipps *et al.* 2009). It would also be useful to measure pathway activation such as NF-κB as this is widely believed to control production of many inflammatory mediators and has been implicated in asthma (Barnes &

Adcock 1997; Birrell *et al.* 2005). This work would help to further understand the immune processes involved in the model.

The group has multiple knockout mice colonies for immune cells, mediators and signalling pathways. Having characterised further which cell types and mediators are activated/released in the HDM model, testing mice deficient in the relevant cells or genes in the HDM model would help to understand which mediators and cell types are driving the pathological features of this model. While some studies have already utilised these techniques to probe mechanisms driving the responses in published HDM models, it would still be important to perform the described studies in the present model given the differences in sensitisation and challenge regimens. Furthermore because of the temporal overlap between the sensitisation and challenge phases of the topical HDM models, mechanistic investigations tend not to distinguish between sensitisation and allergic response processes, generally investigating the model phenotype as a whole. The newly developed model holds advantage over the solely topical models in that these two processes can be easily distinguished by utilising knockout mice to assess sensitisation processes, or by using pharmacological modulation post sensitisation/during HDM challenge to investigate the allergic response to HDM challenge. It is important to fully understand the model phenotype before utilising it to investigate asthma mechanisms or to test potential new therapies.

With the exception of Kikuchi *et al.* (2006) who showed that the proteolytic activity of Der p 1 is essential for sensitisation and immunoglobulin production, little work has been conducted into the mechanisms driving systemic sensitisation to HDM. I would therefore be interested to further understand the mechanisms driving sensitisation in the present HDM model. Utilising protease inhibitors and heat inactivated HDM I would investigate whether the protease activity in the HDM extract is involved in the sensitisation phase of the present model. As described in previous chapters, several other factors have been implicated in HDM-driven responses including TLR4, Dectin-1 and -2, Syk and TSLP. I would therefore be interested to test the relevant knockouts in the HDM model to profile the role of these factors in the model.

While IgE is the classical allergy-mediating immunoglobulin there is also interest in other Ig subclasses, specifically those of the IgG class (reviewed in (Williams *et al.* 2012)). HDM-directed IgG1 has been detected in several of the published HDM models including a systemically sensitised model (Kikuchi *et al.* 2006) and several of the published topical models (Johnson *et al.* 2004; Cates *et al.* 2004; Phipps *et al.* 2009). I would therefore be very

interested to measure systemic IgG1 levels in the newly developed HDM model; this could be done using an ELISA as described for IgE. In addition it would be useful to determine whether either of these immunoglobulins (IgG or IgE) is driving the response to HDM challenge and to do this I would test B cell, IgE and IgG knockouts in the model. In addition it would be prudent to parallel this with measurement of IgGs in the OVA model. It has been shown in murine OVA models that IgG detection may be delayed compared to IgE (Renz *et al.* 1992) for example only being detected 40 days after sensitisation while IgE was detected after 1 week (Hessel *et al.* 1995), thus it may be necessary to take out measurements to later time points to detect this.

The features of the HDM model measured thus far have been largely acute features: allergic airway inflammation and AHR. I would also be interested in whether any of the more chronic features of asthma are observed in the present model. As such I would like to measure mucus production and goblet cell levels (by mucin assay, MUC5AC expression, or by histology), and collagen deposition and α-SMA levels in lung tissue sections taken from the model using standard histological techniques. If any of these remodelling features were observed this would further support the use of this protocol to model allergic asthma.

Prior to using the model for drug discovery it is vital to show that the model responds to standard asthma treatments. I have already shown that the inflammation and the AHR in the model respond to oral corticosteroids. To support this I would also like to confirm whether these endpoints also respond to topical corticosteroids and a β-agonist (the AHR).

7.2.2. OVA model

I have shown that the OVA model used in-house does not require Alum. In the past a detailed characterisation of the mediators produced in the OVA model (with Alum) has been performed. It would be useful to parallel this work in the OVA model in the absence of Alum. If asthmatic mediators such as IL-4, IL-5 or IL-13 were detected this would give increased confidence to switch to using the adjuvant-free model. Further work would also be required to determine whether the model still responds appropriately to relevant treatments such as steroids and β-agonists when configured without Alum.

7.2.3. CS inhibition of AHR

One of the important research questions remaining from this thesis is to determine what is driving the CS-induced inhibition of the allergen-induced AHR. In order to investigate this it is important to first try to understand what is driving the AHR in the two allergen-driven models.

- First of all it would be interesting to determine which airways are responsible for the AHR in the two allergen-driven models. The AHR in the OVA model was first described using non-invasive lung function, where the upper airways (including the nose) may be involved. AHR was also observed in the isolated trachea thus some of the *in vivo* AHR may be due to changes in the reactivity of the large airways. Further to this however I also demonstrated OVA-induced AHR using invasive lung function, where the upper airways are less likely to be involved. The lower or smaller airways are implicated in the airflow obstruction observed in asthma (Sturton *et al.* 2008). Therefore demonstrating a lower airway component to the *in vivo* AHR would confirm the clinical relevance of this endpoint; I would investigate this using PCLS.
- To parallel the work in the OVA model I would like to further profile the AHR in the HDM model – to date I have only demonstrated this using non-invasive lung function. I would therefore perform resistance and compliance studies, assessment of the isolated trachea and PCLS studies in the HDM model. It is also essential to confirm that the AHR observed in this model is true non-specific AHR by investigating airway reactivity to spasmogens other than 5-HT such as a cholinergic agonist, or AMP.

- Several mediators have been implicated in driving the AHR in asthma; these include eosinophil recruiting mediators IL-5 and eotaxin, and eosinophil granule proteins (Wardlaw *et al.* 1988; Coyle *et al.* 1995; Foster *et al.* 1996; Mattes *et al.* 2002; Shen *et al.* 2003); Th2 mediators IL-4 and IL-13 (Grünig *et al.* 1998; Mattes *et al.* 2001; Taube *et al.* 2002; Brightling *et al.* 2003), and also IL-17 (Barczyk *et al.* 2003). Measuring these mediators (using ELISA) would highlight any mediators which may be important for the AHR. Neutralising antibodies or mice lacking these mediators would then be useful to further pinpoint which mediators are driving the AHR. It would also be interesting to determine whether any of these mediators are inhibited by CS and could therefore provide a possible mechanism as to the inhibitory effect of CS on the allergen-induced AHR. Since cigarette smoke did not inhibit the level of allergen-induced eosinophilia, I do not think eosinophil related mediators such as IL-5 or eotaxin are likely to be involved, but other mediators such as IL-13 or IL-17 may be important.

- The next stage towards understanding what is driving the allergen-induced AHR would be to determine whether any structural changes in the airways are observed in the allergen-driven models. One of the most important structural changes in the airways of asthmatic individuals which is likely to contribute to AHR is an increase in airway smooth muscle mass. I would investigate this by measuring α-SMA levels in the airways using histological samples from the two allergen-driven models. α-SMA has been widely used as a marker of airway smooth muscle, and our group has previously demonstrated changes in this endpoint in an OVA-driven asthma model (Birrell *et al.* 2008*b*).
- I would then determine whether the level of smooth muscle in the allergen-driven models is modulated by cigarette smoke as this could be one mechanism by which CS could attenuate the allergen-induced AHR. To do this I would again measure α-SMA levels in histological samples taken from allergen and CS co-exposed mice.
- As I only investigated the effect of CS on the allergen-induced AHR to 5-HT it is important to back this up with studies using other spasmogens to ensure that this effect is not just specific to 5-HT. A CS-induced inhibition of AHR to other spasmogens would support the hypothesis that a structural change in the airways is involved in the blockade of AHR.
- Another method to investigate the effect of CS on airway smooth muscle levels would be to switch to *in vitro* cell culture studies and look at the effect of CS on the proliferation of ASM cells. In human airway smooth muscle the majority of studies have shown CS to promote ASM proliferation (Fang *et al.* 1997; Lin *et al.* 2005*a*; Zhang *et al.* 2010; Sathish *et al.* 2012) but CS has also been shown to inhibit proliferation in other studies (Yoon *et al.* 2011).

- Another key feature of airway remodelling in asthma is increased deposition of collagen in the airway wall (Davies *et al.* 2003). The elastic properties of the airways may play a role in AHR (Khan *et al.* 2010) and an increase in collagen deposition could diminish AHR by opposing smooth muscle contraction (Bramley *et al.* 1995; Palmans *et al.* 2000). Smoking in asthmatics or smoke exposure in murine models has been shown to induce or potentiate airway remodelling such as increasing levels of airway collagen (Carroll *et al.* 2000; Cisneros-Lira *et al.* 2003; Min *et al.* 2007; Melgert *et al.* 2007; Botelho *et al.* 2011). A CS-induced increase in collagen is therefore another mechanism which could be involved in the CS-inhibited AHR in the present model. Airway collagen levels can be easily measured using histological techniques by staining with picro-sirius red as previously described (Last *et al.* 2004).

- TGF-β is a growth factor which is implicated in remodelling and cell proliferation and this may be involved in CS mediated airway remodelling and collagen deposition (Churg *et al.* 2006; Guo *et al.* 2008; Hizume *et al.* 2012). Furthermore in a murine OVA model CS exposure enhanced both collagen deposition and TGF-β expression (Kim *et al.* 2011) and TGF-β has been shown to inhibit AHR (Hansen *et al.* 2000; Schramm *et al.* 2003). Measuring levels of TGF-β in the two allergen-driven models and the CS co-exposure models may therefore provide interesting data to support the above studies investigating collagen and airway smooth muscle levels.

7.2.4. LAR

The other outstanding research questions from this thesis surround the LAR in the allergen-driven models. One question is why a robust LAR is observed in the OVA model but not in the HDM model despite using almost identical protocols; other features such as AHR and airway inflammation are highly replicated between the two models. Understanding this question may provide clues as to the mechanism driving the LAR in asthmatics, a process which is currently poorly understood, and this could lead to opportunities to develop new therapies.

- There is some evidence that IgG subtypes, specifically IgG1 may be involved in the LAR (Ito *et al.* 1986; Pelikan & Pelikan-Filipek 1986*a*, *b*; Ogurusu *et al.* 1991; Mizutani *et al.* 2012). Measuring IgG and IgE levels in the plasma in both the OVA and the HDM LAR models may highlight different immunoglobulin expression patterns in the two models which could explain the discrepancy in the LAR. IgE and IgG knockout mice could also be utilised to determine whether these mediators are central to the LAR.
- A model has been proposed whereby C3a drives the LAR after allergen challenge by production of IL-1β and neutrophil recruitment into the lungs (Mizutani *et al.* 2009). Increased OVA-specific IgG1 and neutrophilia are observed alongside the LAR after allergen challenge and a C3a antagonist inhibited IgG1 production, neutrophilia and the LAR (Mizutani *et al.* 2012). Furthermore the LAR and IgG1 production are abolished in severe combined immunodeficient mice (Mizutani *et al.* 2012). I would therefore also be interested to determine whether this IgG-C3a-neutrophil axis is involved in the LAR in the present OVA model, and this could be investigated using a C3a antagonist as described (Mizutani *et al.* 2012). Neutrophil depletion, B cell knockouts and anti-IgG antibodies could also be useful to further investigate this.

- In published work our group has previously shown TRPA1 channel activation to be central to the OVA-induced LAR. In addition other groups have shown TRPA1 to be involved in ovalbumin-driven responses including airway inflammation and AHR (Caceres *et al.* 2009). However to my knowledge nobody has investigated whether a role exists for TRPA1 in the response to HDM challenge. It may therefore be interesting to investigate the ability of HDM challenge to induce activation of TRPA1 channels or other airway sensory nerve components. A starting point to investigate this would be to utilise a TRPA1 antagonist or knockout mice as previously described (Caceres *et al.* 2009; Raemdonck *et al.* 2012) in the allergic HDM model.

Another interesting question is why the LAR in the OVA model is dependent on the presence of Alum during sensitisation. Understanding this could be very informative with regards the mechanism of the LAR in allergic asthmatics.

- Firstly sensitisation with Alum has been shown to induce more IgG than Alum-free sensitisation (Conrad *et al.* 2009) so again IgG may be an important factor. I would therefore measure IgG and IgE levels in the absence and presence of Alum in the OVA-LAR model.
- Several research papers have shown that Alum acts to promote allergic sensitisation through activation of the inflammasome and release of IL-1β (Eisenbarth *et al.* 2008; Li *et al.* 2008; Hornung *et al.* 2008; Kool *et al.* 2008*b*, 2011); which may therefore be important for the generation of the LAR in the OVA model. In fact Il-1β has been implicated in the LAR after allergen challenge in guinea pigs (Okada *et al.* 1995). In the OVA model the mice are sensitised systemically, therefore to determine whether the sensitisation protocol induces inflammasome activation and/or IL-1β production I would perform peritoneal lavage on mice following sensitisation with and without Alum and assess inflammasome activation markers in the lavage fluid and lavage cells.
    - I would measure gene expression levels of the different inflammasome complexes (NLRP1, NLRP3 and AIM-2) for example using a Taqman assay on the lavage cells.
    - I would also measure gene expression of Caspase-1 (an important component of the inflammasome) and Caspase-1 activity using a Caspase assay in lavage cells.

- If any of the inflammasome complexes were found to be up regulated and activated in the Alum-treated mice I would then utilise inflammasome complex knockouts to confirm their involvement in the LAR model.

- To profile inflammasome mediators I would measure IL-1β and IL-18 levels in the lavage fluid by ELISA. I would also measure danger signals such as uric acid in the peritoneal lavage fluid
- I would then utilise IL-1β and IL-18 knockout mice and knockouts for their receptors (IL-1R and IL-18R) to determine if these mediators are involved in sensitising the mice to generate an LAR in response to allergen-challenge.

7.2.5. CS and LAR

The final research questions resulting from this thesis concern the effect of CS on the OVA-induced LAR. Understanding how CS enhanced this endpoint and rendered it insensitive to steroid treatment may provide vital clues as the mechanism behind the increased disease severity and worsened treatment outcome in smoking asthmatics.

- First of all it would be really interesting to determine whether the findings described in this thesis are replicated in the clinic. Allergen challenge studies could be performed in smoking and non-smoking asthmatics as described by Meghji *et al.* (2011) and the severity of the LAR and its response to oral steroid treatment could be compared. To my knowledge this data does not exist in the clinic.
- Neutrophil levels were increased in the airways of mice co-exposed to CS, and in these mice steroid treatment failed to reduce the levels of airway neutrophils or the LAR. Other publications have associated pulmonary neutrophils with worsened lung function and a lack of steroid sensitivity (Tanizaki *et al.* 1993; Stănescu *et al.* 1996; Pedersen *et al.* 1996; Green *et al.* 2002; Boulet *et al.* 2006). I would therefore be interested to determine whether the lack of response of the LAR in CS co-exposed mice could be due to the increased levels of airway neutrophils in these mice. I would investigate this using neutrophil depletion using antibodies such as the anti-granulocyte receptor-1 (Gr-1) mAb, RB6-8C5 or the Ly6G-specific mAb, 1A8 (Tateda *et al.* 2001; Daley *et al.* 2008) prior to induction of the LAR.

- One hypothesis regarding the lack of effect of steroid treatment on the LAR in the CS and OVA co-exposed mice surrounds TRPA1. Previous studies within the group have highlighted a role for TRPA1 in the LAR (Raemdonck *et al.* 2012). Although the mechanism hasn't been fully elucidated it is likely that synthesis and release of an endogenous TRPA1 ligand is involved in the generation of the LAR in this model.

Therefore corticosteroids may inhibit the LAR by inhibiting this synthesis. In support of this hypothesis the group also previously showed an IκK-2 inhibitor (which inhibits the activity of NFκB) to be effective in a rodent LAR model (Birrell *et al.* 2005). CS is known to contain TRPA1 activating ligands such as acrolein and crotonaldehyde (Andrè *et al.* 2008; Simon & Liedtke 2008), thus CS exposure may provide exogenous TRPA1 ligands, circumventing the need for TRPA1 ligand synthesis. This may explain the steroid insensitivity of the LAR in CS co-exposed mice. Unfortunately this hypothesis is difficult to test as TRPA1 blockers or knockout mice would be expected to inhibit the allergen-induced LAR itself (Raemdonck *et al.* 2012). One way to investigate this would be to treat the mice with an exogenous TRPA1 ligand such as acrolein in conjunction with the OVA challenge and determine whether this also attenuates the steroid sensitivity of this endpoint.

- Theophylline has been used to restore steroid sensitivity in pulmonary diseases (Cosio *et al.* 2009; Ford *et al.* 2010; To *et al.* 2010; Sun *et al.* 2012) and has shown beneficial effects in asthmatics (Crescioli *et al.* 1991; Spears *et al.* 2009). The effect of theophylline on steroid sensitivity is thought to be through PI3K-δ inhibition (To *et al.* 2010), which lead to the proposal of using PI3K-δ inhibition to restore glucocorticoid responsiveness (Marwick *et al.* 2009, 2010). It would therefore be interesting to determine whether theophylline or a PI3K-δ inhibitor could restore steroid sensitivity to the LAR in this model, as this would support the use of this treatment approach in steroid resistant asthmatics. As described in Chapter 4 I would also be interested to profile other pharmaceutical agents such as monteleukast (a leukotriene antagonist), a long acting β_2-agonist, and a long acting muscarinic antagonist such as tiotropium to investigate which therapies may show efficacy where steroids failed to do so. Given the lack of effect of steroid in the CS-enhanced LAR, it is an exciting model in which to search for compounds which may be effective in treatment-resistant asthma.

Reference list

Aalbers, R., Kauffman, H. F., Vrugt, B., Smith, M., Koëter, G. H., Timens, W. & de Monchy, J. G. (1993*a*) Bronchial lavage and bronchoalveolar lavage in allergen-induced single early and dual asthmatic responders. *The American review of respiratory disease* **147**: 76–81.

Aalbers, R., Kauffman, H. F., Vrugt, B., Koëter, G. H. & de Monchy, J. G. (1993*b*) Allergen-induced recruitment of inflammatory cells in lavage 3 and 24 h after challenge in allergic asthmatic lungs. *Chest* **103**: 1178–1184.

Abdullah, A. S. M. & Husten, C. G. (2004) Promotion of smoking cessation in developing countries: a framework for urgent public health interventions. *Thorax* **59**: 623–630.

Aburuz, S., Heaney, L. G., Millership, J., Gamble, J. & McElnay, J. (2007) A cross-sectional study evaluating the relationship between cortisol suppression and asthma control in patients with difficult asthma. *British journal of clinical pharmacology* **63**: 110–115.

Accordini, S., Janson, C., Svanes, C. & Jarvis, D. (2012) The role of smoking in allergy and asthma: lessons from the ECRHS. *Current allergy and asthma reports* **12**: 185–191.

Acosta-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A. & Sallusto, F. (2007) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nature immunology* **8**: 942–949.

Adam, E., Hansen, K. K., Astudillo Fernandez, O., Astudillo, O. F., Coulon, L., Bex, F., Duhant, X., Jaumotte, E., Hollenberg, M. D. & Jacquet, A. (2006) The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism. *The Journal of biological chemistry* **281**: 6910–6923.

Adams, G. K., 3rd & Lichtenstein, L. (1979) In vitro studies of antigen-induced bronchospasm: effect of antihistamine and SRS-A antagonist on response of sensitized guinea pig and human airways to antigen. *Journal of immunology (Baltimore, Md.: 1950)* **122**: 555–562.

Adcock, I. M., Lane, S. J., Brown, C. R., Peters, M. J., Lee, T. H. & Barnes, P. J. (1995*a*) Differences in binding of glucocorticoid receptor to DNA in steroid-resistant asthma. *Journal of immunology (Baltimore, Md.: 1950)* **154**: 3500–3505.

Adcock, I. M., Lane, S. J., Brown, C. R., Lee, T. H. & Barnes, P. J. (1995*b*) Abnormal glucocorticoid receptor-activator protein 1 interaction in steroid-resistant asthma. *The Journal of experimental medicine* **182**: 1951–1958.

Adcock, I. M., Stevens, D. A. & Barnes, P. J. (1996*a*) Interactions of glucocorticoids and beta 2-agonists. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **9**: 160–168.

Adcock, I. M., Gilbey, T., Gelder, C. M., Chung, K. F. & Barnes, P. J. (1996*b*) Glucocorticoid receptor localization in normal and asthmatic lung. *American journal of respiratory and critical care medicine* **154**: 771–782.

Adenuga, D., Yao, H., March, T. H., Seagrave, J. & Rahman, I. (2009) Histone deacetylase 2 is phosphorylated, ubiquitinated, and degraded by cigarette smoke. *American journal of respiratory cell and molecular biology* **40**: 464–473.

Adler, A., Cieslewicz, G. & Irvin, C. G. (2004) Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. *Journal of Applied Physiology* **97**: 286–292.

Agache, I., Ciobanu, C., Agache, C. & Anghel, M. (2010) Increased serum IL-17 is an independent risk factor for severe asthma. *Respiratory medicine* **104**: 1131–1137.

Ahn, J. H., Kim, C. H., Kim, Y. H., Kim, S. J., Lee, S. Y., Kim, Y. K., Kim, K. H., Moon, H. S., Song, J. S., Park, S. H. & Kwon, S. S. (2007) Inflammatory and remodeling events in asthma with chronic exposure to house dust mites: a murine model. *Journal of Korean medical science* **22**: 1026–1033.

Akbari, O., DeKruyff, R. H. & Umetsu, D. T. (2001) Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nature immunology* **2**: 725–731.

De Alba, J., Raemdonck, K., Dekkak, A., Collins, M., Wong, S., Nials, A. T., Knowles, R. G., Belvisi, M. G. & Birrell, M. A. (2010) House dust mite induces direct airway inflammation in vivo: implications for future disease therapy? *The European Respiratory Journal: Official Journal of the European Society for Clinical Respiratory Physiology* **35**: 1377–1387.

Allakhverdi, Z., Comeau, M. R., Jessup, H. K., Yoon, B.-R. P., Brewer, A., Chartier, S., Paquette, N., Ziegler, S. F., Sarfati, M. & Delespesse, G. (2007*a*) Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *The Journal of experimental medicine* **204**: 253–258.

Allakhverdi, Z., Smith, D. E., Comeau, M. R. & Delespesse, G. (2007*b*) Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *Journal of immunology (Baltimore, Md.: 1950)* **179**: 2051–2054.

Amin, K., Lúdvíksdóttir, D., Janson, C., Nettelbladt, O., Björnsson, E., Roomans, G. M., Boman, G., Sevéus, L. & Venge, P. (2000) Inflammation and Structural Changes in the Airways of Patients with Atopic and Nonatopic Asthma. *American Journal of Respiratory and Critical Care Medicine* **162**: 2295–2301.

Andersson, D. A., Gentry, C., Moss, S. & Bevan, S. (2008) Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **28**: 2485–2494.

Ando, T., Ino, Y., Haida, M., Honma, R., Maeda, H., Yamakawa, H., Iwaki, M. & Okudaira, H. (1991) Isolation of cysteine protease in the crude mite extract, Dermatophagoides farinae. *International archives of allergy and applied immunology* **96**: 199–205.

Andrè, E., Campi, B., Materazzi, S., Trevisani, M., Amadesi, S., Massi, D., Creminon, C., Vaksman, N., Nassini, R., Civelli, M., Baraldi, P. G., Poole, D. P., Bunnett, N. W., Geppetti, P. & Patacchini, R. (2008) Cigarette smoke-induced neurogenic inflammation is mediated by alpha,beta-unsaturated aldehydes and the TRPA1 receptor in rodents. *The Journal of clinical investigation* **118**: 2574–2582.

Angkasekwinai, P., Park, H., Wang, Y.-H., Wang, Y.-H., Chang, S. H., Corry, D. B., Liu, Y.-J., Zhu, Z. & Dong, C. (2007) Interleukin 25 promotes the initiation of proallergic type 2 responses. *The Journal of experimental medicine* **204**: 1509–1517.

Antonicelli, L., Bucca, C., Neri, M., De Benedetto, F., Sabbatani, P., Bonifazi, F., Eichler, H. G., Zhang, Q. & Yin, D. D. (2004) Asthma severity and medical resource utilisation. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **23**: 723–729.

Antoniu, S. A. & Antohe, I. (2013) Evaluation of inhaled tiotropium in asthma, uncontrolled with standard combination therapy. *Expert opinion on pharmacotherapy*.

Apostol, G. G., Jacobs, D. R., Jr, Tsai, A. W., Crow, R. S., Williams, O. D., Townsend, M. C. & Beckett, W. S. (2002) Early life factors contribute to the decrease in lung function between ages 18 and 40: the Coronary Artery Risk Development in Young Adults study. *American journal of respiratory and critical care medicine* **166**: 166–172.

Araujo, B. B., Dolhnikoff, M., Silva, L. F. F., Elliot, J., Lindeman, J. H. N., Ferreira, D. S., Mulder, A., Gomes, H. A. P., Fernezlian, S. M., James, A. & Mauad, T. (2008) Extracellular matrix components and regulators in the airway smooth muscle in asthma. *The European respiratory journal* **32**: 61–69.

Arbes, S. J., Gergen, P. J., Vaughn, B. & Zeldin, D. C. (2007) Asthma Cases Attributable to Atopy: Results from the Third National Health and Nutrition Examination Survey. *The Journal of allergy and clinical immunology* **120**: 1139–1145.

Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J. & Montminy, M. (1994) Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**: 226–229.

Arshad, S. H., Tariq, S. M., Matthews, S. & Hakim, E. (2001) Sensitization to Common Allergens and Its Association With Allergic Disorders at Age 4 Years: A Whole Population Birth Cohort Study. *Pediatrics* **108**: e33–e33.

Asokananthan, N., Graham, P. T., Stewart, D. J., Bakker, A. J., Eidne, K. A., Thompson, P. J. & Stewart, G. A. (2002) House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *Journal of immunology (Baltimore, Md.: 1950)* **169**: 4572–4578.

Atkinson, J. J. & Senior, R. M. (2003) Matrix metalloproteinase-9 in lung remodeling. *American journal of respiratory cell and molecular biology* **28**: 12–24.

Aubier, M., Pieters, W. R., Schlösser, N. J. & Steinmetz, K. O. (1999) Salmeterol/fluticasone propionate (50/500 microg) in combination in a Diskus inhaler (Seretide) is effective and safe in the treatment of steroid-dependent asthma. *Respiratory medicine* **93**: 876–884.

Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A. & Karin, M. (1995) Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science (New York, N.Y.)* **270**: 286–290.

Austin, J. B., Selvaraj, S., Godden, D. & Russell, G. (2005) Deprivation, smoking, and quality of life in asthma. *Archives of disease in childhood* **90**: 253–257.

Avila, P. C. (2007) Does anti-IgE therapy help in asthma? Efficacy and controversies. *Annual review of medicine* **58**: 185–203.

Avital, A., Springer, C., Bar-Yishay, E. & Godfrey, S. (1995) Adenosine, methacholine, and exercise challenges in children with asthma or paediatric chronic obstructive pulmonary disease. *Thorax* **50**: 511–516.

Bacharier, L. B., Strunk, R. C., Mauger, D., White, D., Lemanske, R. F., Jr & Sorkness, C. A. (2004) Classifying asthma severity in children: mismatch between symptoms, medication use, and lung function. *American journal of respiratory and critical care medicine* **170**: 426–432.

Bai, T. R. & Bramley, A. M. (1993) Effect of an inhibitor of nitric oxide synthase on neural relaxation of human bronchi. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **264**: L425–L430.

Bajoriūnienė, I., Malakauskas, K., Lavinskienė, S., Jeroch, J., Gasiūnienė, E., Vitkauskienė, A. & Sakalauskas, R. (2012) Peripheral blood Th17 cells and neutrophils in Dermatophagoides pteronyssinus-induced early- and late-phase asthmatic response. *Medicina (Kaunas, Lithuania)* **48**: 442–451.

Ballantyne, S. J., Barlow, J. L., Jolin, H. E., Nath, P., Williams, A. S., Chung, K. F., Sturton, G., Wong, S. H. & McKenzie, A. N. J. (2007) Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. *The Journal of allergy and clinical immunology* **120**: 1324–1331.

Bamberger, C. M., Bamberger, A. M., de Castro, M. & Chrousos, G. P. (1995) Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. *The Journal of clinical investigation* **95**: 2435–2441.

Bandukwala, H. S., Clay, B. S., Tong, J., Mody, P. D., Cannon, J. L., Shilling, R. A., Verbeek, J. S., Weinstock, J. V., Solway, J. & Sperling, A. I. (2007) Signaling through Fc gamma RIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation. *The Journal of experimental medicine* **204**: 1875–1889.

Banerjee, A., Trivedi, C. M., Damera, G., Jiang, M., Jester, W., Hoshi, T., Epstein, J. A. & Panettieri, R. A., Jr (2012) Trichostatin A abrogates airway constriction, but not inflammation, in murine and human asthma models. *American journal of respiratory cell and molecular biology* **46**: 132–138.

Bannister, A. J., Oehler, T., Wilhelm, D., Angel, P. & Kouzarides, T. (1995) Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro. *Oncogene* **11**: 2509–2514.

Barczyk, A., Pierzchala, W. & Sozañska, E. (2003) Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respiratory medicine* **97**: 726–733.

Barlow, J. L., Bellosi, A., Hardman, C. S., Drynan, L. F., Wong, S. H., Cruickshank, J. P. & McKenzie, A. N. J. (2012) Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *The Journal of allergy and clinical immunology* **129**: 191–198.e1–4.

Barnes, P. J. (1996) Neuroeffector mechanisms: The interface between inflammation and neuronal responses. *Journal of Allergy and Clinical Immunology* **98**: S73–S83.

Barnes, P. J. (2003) Theophylline: new perspectives for an old drug. *American journal of respiratory and critical care medicine* **167**: 813–818.

Barnes, P. J. (2008) Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* **8**: 183–192.

Barnes, P. J. (2010*a*) New therapies for chronic obstructive pulmonary disease. *Medical principles and practice: international journal of the Kuwait University, Health Science Centre* **19**: 330–338.

Barnes, P. J. (2010*b*) New therapies for asthma: is there any progress? *Trends in pharmacological sciences* **31**: 335–343.

Barnes, P. J. & Adcock, I. M. (1997) NF-kappa B: a pivotal role in asthma and a new target for therapy. *Trends in pharmacological sciences* **18**: 46–50.

Barnes, P. J. & Adcock, I. M. (2003) How do corticosteroids work in asthma? *Annals of internal medicine* **139**: 359–370.

Barnes, P. J., Greening, A. P. & Crompton, G. K. (1995) Glucocorticoid resistance in asthma. *American Journal of Respiratory and Critical Care Medicine* **152**: S125–140.

Barrett, E. G., Henson, R. D., Seilkop, S. K., McDonald, J. D. & Reed, M. D. (2006) Effects of hardwood smoke exposure on allergic airway inflammation in mice. *Inhalation toxicology* **18**: 33–43.

Barrett, N. A., Maekawa, A., Rahman, O. M., Austen, K. F. & Kanaoka, Y. (2009) Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *Journal of immunology (Baltimore, Md.: 1950)* **182**: 1119–1128.

Barrios, C., Brawand, P., Berney, M., Brandt, C., Lambert, P. H. & Siegrist, C. A. (1996) Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. *European journal of immunology* **26**: 1489–1496.

Bartemes, K. R., Iijima, K., Kobayashi, T., Kephart, G. M., McKenzie, A. N. & Kita, H. (2012) IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *Journal of immunology (Baltimore, Md.: 1950)* **188**: 1503–1513.

Bascom, R., Wachs, M., Naclerio, R. M., Pipkorn, U., Galli, S. J. & Lichtenstein, L. M. (1988) Basophil influx occurs after nasal antigen challenge: effects of topical corticosteroid pretreatment. *The Journal of allergy and clinical immunology* **81**: 580–589.

Bateman, E. D., Hurd, S. S., Barnes, P. J., Bousquet, J., Drazen, J. M., FitzGerald, M., Gibson, P., Ohta, K., O'Byrne, P., Pedersen, S. E., Pizzichini, E., Sullivan, S. D., Wenzel, S. E. & Zar, H. J. (2008) Global strategy for asthma management and prevention: GINA executive summary. *European Respiratory Journal* **31**: 143–178.

Bates, J., Irvin, C., Brusasco, V., Drazen, J., Fredberg, J., Loring, S., Eidelman, D., Ludwig, M., Macklem, P., Martin, J., Milic-Emili, J., Hantos, Z., Hyatt, R., Lai-Fook, S., Leff, A., Solway, J., Lutchen, K., Suki, B., Mitzner, W., Paré, P., Pride, N. & Sly, P. (2004) The use and misuse of Penh in animal models of lung disease. *American journal of respiratory cell and molecular biology* **31**: 373–374.

Beck, L. & Spiegelberg, H. L. (1989) The polyclonal and antigen-specific IgE and IgG subclass response of mice injected with ovalbumin in alum or complete Freund's adjuvant. *Cellular Immunology* **123**: 1–8.

Bellinghausen, I., Brand, U., Knop, J. & Saloga, J. (2000) Comparison of allergen-stimulated dendritic cells from atopic and nonatopic donors dissecting their effect on autologous naive and memory T helper cells of such donors. *The Journal of allergy and clinical immunology* **105**: 988–996.

Bellinghausen, I., Klostermann, B., Knop, J. & Saloga, J. (2003) Human CD4+CD25+ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. *The Journal of allergy and clinical immunology* **111**: 862–868.

Belvisi, M. G., David Stretton, C., Yacoub, M. & Barnes, P. J. (1992*a*) Nitric oxide is the endogenous neurotransmitter of bronchodilator nerves in humans. *European Journal of Pharmacology* **210**: 221–222.

Belvisi, M. G., Stretton, C. D., Miura, M., Verleden, G. M., Tadjkarimi, S., Yacoub, M. H. & Barnes, P. J. (1992*b*) Inhibitory NANC nerves in human tracheal smooth muscle: a quest for the neurotransmitter. *Journal of applied physiology (Bethesda, Md.: 1985)* **73**: 2505–2510.

Belvisi, M. G., Dubuis, E. & Birrell, M. A. (2011) Transient Receptor Potential A1 Channels. *Chest* **140**: 1040–1047.

Benayoun, L., Druilhe, A., Dombret, M.-C., Aubier, M. & Pretolani, M. (2003) Airway structural alterations selectively associated with severe asthma. *American journal of respiratory and critical care medicine* **167**: 1360–1368.

Bergmann, R. L., Edenharter, G., Bergmann, K. E., Guggenmoos-Holzmann, I., Forster, J., Bauer, C. P., Wahn, V., Zepp, F. & Wahn, U. (1997) Predictability of early atopy by cord blood-IgE and parental history. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **27**: 752–760.

Berkman, N., Avital, A., Breuer, R., Bardach, E., Springer, C. & Godfrey, S. (2005) Exhaled nitric oxide in the diagnosis of asthma: comparison with bronchial provocation tests. *Thorax* **60**: 383–388.

Besnard, A.-G., Guillou, N., Tschopp, J., Erard, F., Couillin, I., Iwakura, Y., Quesniaux, V., Ryffel, B. & Togbe, D. (2011) NLRP3 inflammasome is required in murine asthma in the absence of aluminum adjuvant. *Allergy* **66**: 1047–1057.

Bessac, B. F., Sivula, M., von Hehn, C. A., Escalera, J., Cohn, L. & Jordt, S.-E. (2008) TRPA1 is a major oxidant sensor in murine airway sensory neurons. *The Journal of clinical investigation* **118**: 1899–1910.

De Bie, J. J., Kneepkens, M., Kraneveld, A. D., Jonker, E. H., Henricks, P. A., Nijkamp, F. P. & van Oosterhout, A. J. (2000) Absence of late airway response despite increased airway responsiveness and eosinophilia in a murine model of asthma. *Experimental lung research* **26**: 491–507.

De Bie, J. J., Hessel, E. M., Van Ark, I., Van Esch, B., Hofman, G., Nijkamp, F. P. & Van Oosterhout, A. J. (1996) Effect of dexamethasone and endogenous corticosterone on airway hyperresponsiveness and eosinophilia in the mouse. *British journal of pharmacology* **119**: 1484–1490.

De Bie, J. J., Henricks, P. A., Cruikshank, W. W., Hofman, G., Jonker, E. H., Nijkamp, F. P. & Van Oosterhout, A. J. (1998) Modulation of airway hyperresponsiveness and eosinophilia by selective histamine and 5-HT receptor antagonists in a mouse model of allergic asthma. *British journal of pharmacology* **124**: 857–864.

Van den Biggelaar, A. H., van Ree, R., Rodrigues, L. C., Lell, B., Deelder, A. M., Kremsner, P. G. & Yazdanbakhsh, M. (2000) Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10. *Lancet* **356**: 1723–1727.

Birrell, M. A., Battram, C. H., Woodman, P., McCluskie, K. & Belvisi, M. G. (2003) Dissociation by steroids of eosinophilic inflammation from airway hyperresponsiveness in murine airways. *Respiratory Research* **4**: 3.

Birrell, M. A., Hardaker, E., Wong, S., McCluskie, K., Catley, M., De Alba, J., Newton, R., Haj-Yahia, S., Pun, K. T., Watts, C. J., Shaw, R. J., Savage, T. J. & Belvisi, M. G. (2005) Ikappa-B kinase-2 inhibitor blocks inflammation in human airway smooth muscle and a rat model of asthma. *American journal of respiratory and critical care medicine* **172**: 962–971.

Birrell, M. A., Wong, S., Catley, M. C. & Belvisi, M. G. (2008*a*) Impact of tobacco-smoke on key signaling pathways in the innate immune response in lung macrophages. *Journal of Cellular Physiology* **214**: 27–37.

Birrell, M. A., De Alba, J., Catley, M. C., Hardaker, E., Wong, S., Collins, M., Clarke, D. L., Farrow, S. N., Willson, T. M., Collins, J. L. & Belvisi, M. G. (2008*b*) Liver X Receptor Agonists Increase Airway Reactivity in a Model of Asthma via Increasing Airway Smooth Muscle Growth. *J Immunol* **181**: 4265–4271.

Birrell, M. A., Van Oosterhout, A. J. M. & Belvisi, M. G. (2010) Do the current house dust mite-driven models really mimic allergic asthma? *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **36**: 1220–1221.

Bisgaard, H., Groth, S. & Madsen, F. (1985) Bronchial hyperreactivity to leucotriene D4 and histamine in exogenous asthma. *British medical journal (Clinical research ed.)* **290**: 1468–1471.

Björnsson, E., Plaschke, P., Norrman, E., Janson, C., Lundbäck, B., Rosenhall, A., Lindholm, N., Rosenhall, L., Berglund, E. & Boman, G. (1994) Symptoms related to asthma and chronic bronchitis in three areas of Sweden. *The European respiratory journal* **7**: 2146–2153.

Blyth, D. I., Pedrick, M. S., Savage, T. J., Hessel, E. M. & Fattah, D. (1996) Lung inflammation and epithelial changes in a murine model of atopic asthma. *American Journal of Respiratory Cell and Molecular Biology* **14**: 425–438.

Booij-Noord, H., Orie, N. G. & De Vries, K. (1971) Immediate and late bronchial obstructive reactions to inhalation of house dust and protective effects of disodium cromoglycate and prednisolone. *The Journal of allergy and clinical immunology* **48**: 344–354.

Booij-Noord, H., de Vries, K., Sluiter, H. J. & Orie, N. G. (1972) Late bronchial obstructive reaction to experimental inhalation of house dust extract. *Clinical allergy* **2**: 43–61.

Borish, L., Mascali, J. J. & Rosenwasser, L. J. (1991) IgE-dependent cytokine production by human peripheral blood mononuclear phagocytes. *Journal of immunology (Baltimore, Md.: 1950)* **146**: 63–67.

Botelho, F. M., Llop-Guevara, A., Trimble, N. J., Nikota, J. K., Bauer, C. M. T., Lambert, K. N., Kianpour, S., Jordana, M. & Stämpfli, M. R. (2011) Cigarette smoke differentially affects eosinophilia and remodeling in a model of house dust mite asthma. *American journal of respiratory cell and molecular biology* **45**: 753–760.

Boulet, L. P., Chapman, K. R., Côté, J., Kalra, S., Bhagat, R., Swystun, V. A., Laviolette, M., Cleland, L. D., Deschesnes, F., Su, J. Q., DeVault, A., Fick, R. B., Jr & Cockcroft, D. W. (1997*a*) Inhibitory effects of an anti-IgE antibody E25 on allergen-induced early asthmatic response. *American journal of respiratory and critical care medicine* **155**: 1835–1840.

Boulet, L. P., Laviolette, M., Turcotte, H., Cartier, A., Dugas, M., Malo, J. L. & Boutet, M. (1997*b*) Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. *Chest* **112**: 45–52.

Boulet, L.-P., Phillips, R., O'Byrne, P. & Becker, A. (2002) Evaluation of asthma control by physicians and patients: comparison with current guidelines. *Canadian respiratory journal: journal of the Canadian Thoracic Society* **9**: 417–423.

Boulet, L.-P., Lemière, C., Archambault, F., Carrier, G., Descary, M. C. & Deschesnes, F. (2006) Smoking and asthma: clinical and radiologic features, lung function, and airway inflammation. *Chest* **129**: 661–668.

Bousquet, J., Chanez, P., Lacoste, J. Y., Barnéon, G., Ghavanian, N., Enander, I., Venge, P., Ahlstedt, S., Simony-Lafontaine, J. & Godard, P. (1990) Eosinophilic inflammation in asthma. *The New England journal of medicine* **323**: 1033–1039.

Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M. & Vignola, A. M. (2000) Asthma. From bronchoconstriction to airways inflammation and remodeling. *American journal of respiratory and critical care medicine* **161**: 1720–1745.

Bouzigon, E., Corda, E., Aschard, H., Dizier, M.-H., Boland, A., Bousquet, J., Chateigner, N., Gormand, F., Just, J., Le Moual, N., Scheinmann, P., Siroux, V., Vervloet, D., Zelenika, D., Pin, I., Kauffmann, F., Lathrop, M. & Demenais, F. (2008) Effect of 17q21 variants and smoking exposure in early-onset asthma. *The New England journal of medicine* **359**: 1985–1994.

Bowles, K., Horohov, D., Paulsen, D., Leblanc, C., Littlefield-Chabaud, M., Ahlert, T., Ahlert, K., Pourciau, S. & Penn, A. (2005) Exposure of adult mice to environmental tobacco smoke fails to enhance the immune response to inhaled antigen. *Inhalation toxicology* **17**: 43–51.

Bozza, S., Gaziano, R., Spreca, A., Bacci, A., Montagnoli, C., di Francesco, P. & Romani, L. (2002) Dendritic cells transport conidia and hyphae of Aspergillus fumigatus from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *Journal of immunology (Baltimore, Md.: 1950)* **168**: 1362–1371.

Bradding, P., Feather, I. H., Howarth, P. H., Mueller, R., Roberts, J. A., Britten, K., Bews, J. P., Hunt, T. C., Okayama, Y. & Heusser, C. H. (1992) Interleukin 4 is localized to and released by human mast cells. *The Journal of experimental medicine* **176**: 1381–1386.

Bradding, P., Roberts, J. A., Britten, K. M., Montefort, S., Djukanovic, R., Mueller, R., Heusser, C. H., Howarth, P. H. & Holgate, S. T. (1994) 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. *American journal of respiratory cell and molecular biology* **10**: 471–480.

Bradding, P., Walls, A. F. & Holgate, S. T. (2006) The role of the mast cell in the pathophysiology of asthma. *The Journal of allergy and clinical immunology* **117**: 1277–1284.

Bramley, A. M., Roberts, C. R. & Schellenberg, R. R. (1995) Collagenase increases shortening of human bronchial smooth muscle in vitro. *American journal of respiratory and critical care medicine* **152**: 1513–1517.

Breckler, L. A., Hale, J., Jung, W., Westcott, L., Dunstan, J. A., Thornton, C. A. & Prescott, S. L. (2010) Modulation of in vivo and in vitro cytokine production over the course of pregnancy in allergic and non-allergic mothers. *Pediatric allergy and immunology: official publication of the European Society of Pediatric Allergy and Immunology* **21**: 14–21.

Brightling, C. E., Bradding, P., Symon, F. A., Holgate, S. T., Wardlaw, A. J. & Pavord, I. D. (2002) Mast-cell infiltration of airway smooth muscle in asthma. *The New England journal of medicine* **346**: 1699–1705.

Brightling, C. E., Symon, F. A., Holgate, S. T., Wardlaw, A. J., Pavord, I. D. & Bradding, P. (2003) Interleukin-4 and -13 expression is co-localized to mast cells within the airway smooth muscle in asthma. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **33**: 1711–1716.

Brightling, C. E., Ammit, A. J., Kaur, D., Black, J. L., Wardlaw, A. J., Hughes, J. M. & Bradding, P. (2005) The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *American journal of respiratory and critical care medicine* **171**: 1103–1108.

Ten Brinke, A., Zwinderman, A. H., Sterk, P. J., Rabe, K. F. & Bel, E. H. (2001) Factors associated with persistent airflow limitation in severe asthma. *American journal of respiratory and critical care medicine* **164**: 744–748.

Broekema, M., ten Hacken, N. H. T., Volbeda, F., Lodewijk, M. E., Hylkema, M. N., Postma, D. S. & Timens, W. (2009) Airway epithelial changes in smokers but not in ex-smokers with asthma. *American journal of respiratory and critical care medicine* **180**: 1170–1178.

Broide, D. H., Lotz, M., Cuomo, A. J., Coburn, D. A., Federman, E. C. & Wasserman, S. I. (1992) Cytokines in symptomatic asthma airways. *The Journal of allergy and clinical immunology* **89**: 958–967.

Broide, D. H., Campbell, K., Gifford, T. & Sriramarao, P. (2000) Inhibition of eosinophilic inflammation in allergen-challenged, IL-1 receptor type 1-deficient mice is associated with reduced eosinophil rolling and adhesion on vascular endothelium. *Blood* **95**: 263–269.

Broide, D. H., Stachnick, G., Castaneda, D., Nayar, J. & Sriramarao, P. (2001) Inhibition of eosinophilic inflammation in allergen-challenged TNF receptor p55/p75--and TNF

receptor p55-deficient mice. *American journal of respiratory cell and molecular biology* **24**: 304–311.

Brown, G. P., Iwamoto, G. K., Monick, M. M. & Hunninghake, G. W. (1989) Cigarette smoking decreases interleukin 1 release by human alveolar macrophages. *The American journal of physiology* **256**: C260–264.

Brückner, V., Noster, U., Schulz, K. H. & Stute, J. (1977) [Relationship between the skin test and the specific IgE (radio-allergo-sorbens test) in the diagnosis of inhalative allergies]. *Zeitschrift für Hautkrankheiten* **52**: 109–116.

Bruna, A., Nicolàs, M., Muñoz, A., Kyriakis, J. M. & Caelles, C. (2003) Glucocorticoid receptor-JNK interaction mediates inhibition of the JNK pathway by glucocorticoids. *The EMBO journal* **22**: 6035–6044.

Brunner, T., Heusser, C. H. & Dahinden, C. A. (1993) Human peripheral blood basophils primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *The Journal of experimental medicine* **177**: 605–611.

Brusasco, V., Pellegrino, R., Violante, B. & Crimi, E. (1992) Relationship between quasi-static pulmonary hysteresis and maximal airway narrowing in humans. *Journal of applied physiology (Bethesda, Md.: 1985)* **72**: 2075–2080.

Brusselle, G., Kips, J., Joos, G., Bluethmann, H. & Pauwels, R. (1995) Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *American journal of respiratory cell and molecular biology* **12**: 254–259.

Brusselle, G. G., Kips, J. C., Tavernier, J. H., Van Der Heyden, J. G., Cuvelier, C. A., Pauwels, R. A. & Bluethmann, H. (1994*a*) Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clinical & Experimental Allergy* **24**: 73–80.

Bucchieri, F., Puddicombe, S. M., Lordan, J. L., Richter, A., Buchanan, D., Wilson, S. J., Ward, J., Zummo, G., Howarth, P. H., Djukanović, R., Holgate, S. T. & Davies, D. E. (2002) Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *American journal of respiratory cell and molecular biology* **27**: 179–185.

Bullens, D. M. A., Truyen, E., Coteur, L., Dilissen, E., Hellings, P. W., Dupont, L. J. & Ceuppens, J. L. (2006) IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respiratory research* **7**: 135.

Bumbacea, D., Campbell, D., Nguyen, L., Carr, D., Barnes, P. J., Robinson, D. & Chung, K. F. (2004) Parameters associated with persistent airflow obstruction in chronic severe asthma. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **24**: 122–128.

Burge, P. S., Calverley, P. M. A., Jones, P. W., Spencer, S. & Anderson, J. A. (2003) Prednisolone response in patients with chronic obstructive pulmonary disease: results from the ISOLDE study. *Thorax* **58**: 654–658.

Burke, H., Leonardi-Bee, J., Hashim, A., Pine-Abata, H., Chen, Y., Cook, D. G., Britton, J. R. & McKeever, T. M. (2012) Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. *Pediatrics* **129**: 735–744.

Burnett, D. (1986) Immunoglobulins in the lung. *Thorax* **41**: 337–344.

Burns, A. R., Hosford, S. P., Dunn, L. A., Walker, D. C. & Hogg, J. C. (1989) Respiratory epithelial permeability after cigarette smoke exposure in guinea pigs. *Journal of applied physiology (Bethesda, Md.: 1985)* **66**: 2109–2116.

Burrows, B., Martinez, F. D., Halonen, M., Barbee, R. A. & Cline, M. G. (1989) Association of asthma with serum IgE levels and skin-test reactivity to allergens. *The New England journal of medicine* **320**: 271–277.

Bushell, A. & Wood, K. J. (1999) Permanent survival of organ transplants without immunosuppression: Experimental approaches and possibilities for tolerance induction in clinical transplantation. *Expert reviews in molecular medicine* **1999**: 1–31.

Busse, P. J., Zhang, T. F., Schofield, B., Kilaru, S., Patil, S. & Li, X.-M. (2009) Decrease in airway mucous gene expression caused by treatment with anti-tumor necrosis factor α in a murine model of allergic asthma. *Annals of Allergy, Asthma & Immunology* **103**: 295–303.

Busse, W., Corren, J., Lanier, B. Q., McAlary, M., Fowler-Taylor, A., Cioppa, G. D., van As, A. & Gupta, N. (2001) Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. *The Journal of allergy and clinical immunology* **108**: 184–190.

Busse, W. W., Chervinsky, P., Condemi, J., Lumry, W. R., Petty, T. L., Rennard, S. & Townley, R. G. (1998) Budesonide delivered by Turbuhaler is effective in a dose-dependent fashion when used in the treatment of adult patients with chronic asthma. *The Journal of allergy and clinical immunology* **101**: 457–463.

Caceres, A. I., Brackmann, M., Elia, M. D., Bessac, B. F., del Camino, D., D'Amours, M., Witek, J. S., Fanger, C. M., Chong, J. A., Hayward, N. J., Homer, R. J., Cohn, L., Huang, X., Moran, M. M. & Jordt, S.-E. (2009) A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 9099–9104.

Caelles, C., González-Sancho, J. M. & Muñoz, A. (1997) Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes & development* **11**: 3351–3364.

Carlson, M., Peterson, C. & Venge, P. (1993) The influence of IL-3, IL-5, and GM-CSF on normal human eosinophil and neutrophil C3b-induced degranulation. *Allergy* **48**: 437–442.

Carroll, N., Elliot, J., Morton, A. & James, A. (1993) The structure of large and small airways in nonfatal and fatal asthma. *The American review of respiratory disease* **147**: 405–410.

Carroll, N. G., Perry, S., Karkhanis, A., Harji, S., Butt, J., James, A. L. & Green, F. H. (2000) The airway longitudinal elastic fiber network and mucosal folding in patients with asthma. *American journal of respiratory and critical care medicine* **161**: 244–248.

Cartier, A., Thomson, N. C., Frith, P. A., Roberts, R. & Hargreave, F. E. (1982) Allergen-induced increase in bronchial responsiveness to histamine: relationship to the late

asthmatic response and change in airway caliber. *The Journal of allergy and clinical immunology* **70**: 170–177.

Casale, T. B., Wood, D., Richerson, H. B., Trapp, S., Metzger, W. J., Zavala, D. & Hunninghake, G. W. (1987) Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are associated with methacholine bronchial hyperresponsiveness. *The Journal of clinical investigation* **79**: 1197–1203.

Castle, W., Fuller, R., Hall, J. & Palmer, J. (1993) Serevent nationwide surveillance study: comparison of salmeterol with salbutamol in asthmatic patients who require regular bronchodilator treatment. *BMJ* **306**: 1034–1037.

Castro, M., Mathur, S., Hargreave, F., Boulet, L.-P., Xie, F., Young, J., Wilkins, H. J., Henkel, T. & Nair, P. (2011) Reslizumab for poorly controlled, eosinophilic asthma: a randomized, placebo-controlled study. *American journal of respiratory and critical care medicine* **184**: 1125–1132.

De Castro, M., Elliot, S., Kino, T., Bamberger, C., Karl, M., Webster, E. & Chrousos, G. P. (1996) The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role. *Molecular medicine (Cambridge, Mass.)* **2**: 597–607.

Cates, E. C., Fattouh, R., Wattie, J., Inman, M. D., Goncharova, S., Coyle, A. J., Gutierrez-Ramos, J.-C. & Jordana, M. (2004) Intranasal Exposure of Mice to House Dust Mite Elicits Allergic Airway Inflammation via a GM-CSF-Mediated Mechanism. *J Immunol* **173**: 6384–6392.

Cerveri, I., Cazzoletti, L., Corsico, A. G., Marcon, A., Niniano, R., Grosso, A., Ronzoni, V., Accordini, S., Janson, C., Pin, I., Siroux, V. & de Marco, R. (2012) The impact of cigarette smoking on asthma: a population-based international cohort study. *International archives of allergy and immunology* **158**: 175–183.

Chalmers, G. W., MacLeod, K. J., Thomson, L., Little, S. A., McSharry, C. & Thomson, N. C. (2001) Smoking and airway inflammation in patients with mild asthma. *Chest* **120**: 1917–1922.

Chalmers, G. W., Macleod, K. J., Little, S. A., Thomson, L. J., McSharry, C. P. & Thomson, N. C. (2002) Influence of cigarette smoking on inhaled corticosteroid treatment in mild asthma. *Thorax* **57**: 226 –230.

Chan, M. T., Leung, D. Y., Szefler, S. J. & Spahn, J. D. (1998) Difficult-to-control asthma: clinical characteristics of steroid-insensitive asthma. *The Journal of allergy and clinical immunology* **101**: 594–601.

Chang, W. C., Lee, Y. C., Liu, C. L., Hsu, J. D., Wang, H. C., Chen, C. C. & Wang, C. J. (2001) Increased expression of iNOS and c-fos via regulation of protein tyrosine phosphorylation and MEK1/ERK2 proteins in terminal bronchiole lesions in the lungs of rats exposed to cigarette smoke. *Archives of toxicology* **75**: 28–35.

Chapman, M. D. & Platts-Mills, T. A. (1980) Purification and characterization of the major allergen from Dermatophagoides pteronyssinus-antigen P1. *Journal of Immunology (Baltimore, Md.: 1950)* **125**: 587–592.

Charlesworth, E. N., Hood, A. F., Soter, N. A., Kagey-Sobotka, A., Norman, P. S. & Lichtenstein, L. M. (1989) Cutaneous late-phase response to allergen. Mediator

release and inflammatory cell infiltration. *The Journal of clinical investigation* **83**: 1519–1526.

Chaudhuri, R., Livingston, E., McMahon, A. D., Thomson, L., Borland, W. & Thomson, N. C. (2003) Cigarette Smoking Impairs the Therapeutic Response to Oral Corticosteroids in Chronic Asthma. *Am. J. Respir. Crit. Care Med.* **168**: 1308–1311.

Chaudhuri, R., Livingston, E., McMahon, A. D., Lafferty, J., Fraser, I., Spears, M., McSharry, C. P. & Thomson, N. C. (2006) Effects of Smoking Cessation on Lung Function and Airway Inflammation in Smokers with Asthma. *American Journal of Respiratory and Critical Care Medicine* **174**: 127–133.

Chen, H., Tliba, O., Van Besien, C. R., Panettieri, R. A., Jr & Amrani, Y. (2003) TNF-[alpha] modulates murine tracheal rings responsiveness to G-protein-coupled receptor agonists and KCl. *Journal of applied physiology (Bethesda, Md.: 1985)* **95**: 864–872; discussion 863.

Chen, Q.-W., Edvinsson, L. & Xu, C.-B. (2010) Cigarette smoke extract promotes human vascular smooth muscle cell proliferation and survival through ERK1/2- and NF-κB-dependent pathways. *TheScientificWorldJournal* **10**: 2139–2156.

Chen, Y., Dales, R., Tang, M. & Krewski, D. (2002) Sex-related interactive effect of smoking and household pets on asthma incidence. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **20**: 1162–1166.

Chen, Z.-G., Zhang, T.-T., Li, H.-T., Chen, F.-H., Zou, X.-L., Ji, J.-Z. & Chen, H. (2013) Neutralization of TSLP Inhibits Airway Remodeling in a Murine Model of Allergic Asthma Induced by Chronic Exposure to House Dust Mite. *PLoS ONE* **8**.

Chew, A. D., Hirota, J. A., Ellis, R., Wattie, J., Inman, M. D. & Janssen, L. J. (2008) Effects of allergen on airway narrowing dynamics as assessed by lung-slice technique. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **31**: 532–538.

Chiappara, G., Gagliardo, R., Siena, A., Bonsignore, M. R., Bousquet, J., Bonsignore, G. & Vignola, A. M. (2001) Airway remodelling in the pathogenesis of asthma. *Current opinion in allergy and clinical immunology* **1**: 85–93.

Chiba, Y., Ueno, A., Sakai, H. & Misawa, M. (2004) Hyperresponsiveness of bronchial but not tracheal smooth muscle in a murine model of allergic bronchial asthma. *Inflammation research: official journal of the European Histamine Research Society ... [et al.]* **53**: 636–642.

Chilmonczyk, B. A., Salmun, L. M., Megathlin, K. N., Neveux, L. M., Palomaki, G. E., Knight, G. J., Pulkkinen, A. J. & Haddow, J. E. (1993) Association between exposure to environmental tobacco smoke and exacerbations of asthma in children. *The New England journal of medicine* **328**: 1665–1669.

Cho, J. Y., Miller, M., Baek, K. J., Han, J. W., Nayar, J., Lee, S. Y., McElwain, K., McElwain, S., Friedman, S. & Broide, D. H. (2004) Inhibition of airway remodeling in IL-5-deficient mice. *The Journal of clinical investigation* **113**: 551–560.

Cho, S.-H., Stanciu, L. A., Holgate, S. T. & Johnston, S. L. (2005) Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. *American journal of respiratory and critical care medicine* **171**: 224–230.

Chu, D. K., Llop-Guevara, A., Walker, T. D., Flader, K., Goncharova, S., Boudreau, J. E., Moore, C. L., Seunghyun In, T., Waserman, S., Coyle, A. J., Kolbeck, R., Humbles, A. A. & Jordana, M. (2013) IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization. *The Journal of allergy and clinical immunology* **131**: 187–200.e1–8.

Chua, K. Y., Stewart, G. A., Thomas, W. R., Simpson, R. J., Dilworth, R. J., Plozza, T. M. & Turner, K. J. (1988) Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *The Journal of experimental medicine* **167**: 175–182.

Chung, K. F., Godard, P., Adelroth, E., Ayres, J., Barnes, N., Barnes, P., Bel, E., Burney, P., Chanez, P., Connett, G., Corrigan, C., de Blic, J., Fabbri, L., Holgate, S. T., Ind, P., Joos, G., Kerstjens, H., Leuenberger, P., Lofdahl, C. G., McKenzie, S., Magnussen, H., Postma, D., Saetta, M., Salmeron, S. & Sterk, P. (1999) Difficult/therapy-resistant asthma: the need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. ERS Task Force on Difficult/Therapy-Resistant Asthma. European Respiratory Society. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **13**: 1198–1208.

Chung, K. F., Zhang, J. & Zhong, N. (2011) Outdoor air pollution and respiratory health in Asia. *Respirology (Carlton, Vic.)* **16**: 1023–1026.

Chung, S. W., Wong, P. M., Shen-Ong, G., Ruscetti, S., Ishizaka, T. & Eaves, C. J. (1986) Production of granulocyte-macrophage colony-stimulating factor by Abelson virus-induced tumorigenic mast cell lines. *Blood* **68**: 1074–1081.

Church, D. F. & Pryor, W. A. (1985) Free-radical chemistry of cigarette smoke and its toxicological implications. *Environmental Health Perspectives* **64**: 111–126.

Churg, A., Zay, K., Shay, S., Xie, C., Shapiro, S. D., Hendricks, R. & Wright, J. L. (2002) Acute cigarette smoke-induced connective tissue breakdown requires both neutrophils and macrophage metalloelastase in mice. *American journal of respiratory cell and molecular biology* **27**: 368–374.

Churg, A., Wang, R. D., Tai, H., Wang, X., Xie, C., Dai, J., Shapiro, S. D. & Wright, J. L. (2003) Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release. *American journal of respiratory and critical care medicine* **167**: 1083–1089.

Churg, A., Tai, H., Coulthard, T., Wang, R. & Wright, J. L. (2006) Cigarette smoke drives small airway remodeling by induction of growth factors in the airway wall. *American journal of respiratory and critical care medicine* **174**: 1327–1334.

Cicco, N. A., Lindemann, A., Content, J., Vandenbussche, P., Lübbert, M., Gauss, J., Mertelsmann, R. & Herrmann, F. (1990) Inducible production of interleukin-6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. *Blood* **75**: 2049–2052.

Cieslewicz, G., Tomkinson, A., Adler, A., Duez, C., Schwarze, J., Takeda, K., Larson, K. A., Lee, J. J., Irvin, C. G. & Gelfand, E. W. (1999) The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration. *The Journal of clinical investigation* **104**: 301–308.

Cisneros-Lira, J., Gaxiola, M., Ramos, C., Selman, M. & Pardo, A. (2003) Cigarette smoke exposure potentiates bleomycin-induced lung fibrosis in guinea pigs. *American journal of physiology. Lung cellular and molecular physiology* **285**: L949–956.

Cisternas, M. G., Blanc, P. D., Yen, I. H., Katz, P. P., Earnest, G., Eisner, M. D., Shiboski, S. & Yelin, E. H. (2003) A comprehensive study of the direct and indirect costs of adult asthma. *The Journal of allergy and clinical immunology* **111**: 1212–1218.

Clark, N. A., Demers, P. A., Karr, C. J., Koehoorn, M., Lencar, C., Tamburic, L. & Brauer, M. (2010) Effect of early life exposure to air pollution on development of childhood asthma. *Environmental health perspectives* **118**: 284–290.

Clarke, A. H., Thomas, W. R., Rolland, J. M., Dow, C. & O'Brien, R. M. (1999) Murine allergic respiratory responses to the major house dust mite allergen Der p 1. *International archives of allergy and immunology* **120**: 126–134.

Clatworthy, J., Price, D., Ryan, D., Haughney, J. & Horne, R. (2009) The value of self-report assessment of adherence, rhinitis and smoking in relation to asthma control. *Primary care respiratory journal: journal of the General Practice Airways Group* **18**: 300–305.

Clearie, K. L., McKinlay, L., Williamson, P. A. & Lipworth, B. J. (2012) Fluticasone/Salmeterol combination confers benefits in people with asthma who smoke. *Chest* **141**: 330–338.

Clynes, R. & Ravetch, J. V. (1995) Cytotoxic antibodies trigger inflammation through Fc receptors. *Immunity* **3**: 21–26.

Cockcroft, D. W. (1983) Mechanism of perennial allergic asthma. *Lancet* **2**: 253–256.

Cockcroft, D. W. (2000) Allergen-induced increase in nonallergic airway responsiveness: a citation classic revisited. *Canadian respiratory journal: journal of the Canadian Thoracic Society* **7**: 182–187.

Cockcroft, D. W. & Murdock, K. Y. (1987) Comparative effects of inhaled salbutamol, sodium cromoglycate, and beclomethasone dipropionate on allergen-induced early asthmatic responses, late asthmatic responses, and increased bronchial responsiveness to histamine. *The Journal of allergy and clinical immunology* **79**: 734–740.

Cockcroft, D. W., Ruffin, R. E., Frith, P. A., Cartier, A., Juniper, E. F., Dolovich, J. & Hargreave, F. E. (1979) Determinants of allergen-induced asthma: dose of allergen, circulating IgE antibody concentration, and bronchial responsiveness to inhaled histamine. *The American review of respiratory disease* **120**: 1053–1058.

Cockcroft, D. W., Hargreave, F. E., O'Byrne, P. M. & Boulet, L.-P. (2007) Understanding allergic asthma from allergen inhalation tests. *Canadian respiratory journal: journal of the Canadian Thoracic Society* **14**: 414–418.

Colice, G. L. (2004) Categorizing asthma severity: an overview of national guidelines. *Clinical medicine & research* **2**: 155–163.

Comoy, E. E., Pestel, J., Duez, C., Stewart, G. A., Vendeville, C., Fournier, C., Finkelman, F., Capron, A. & Thyphronitis, G. (1998) The house dust mite allergen, Dermatophagoides pteronyssinus, promotes type 2 responses by modulating the balance between IL-4 and IFN-gamma. *Journal of immunology (Baltimore, Md.: 1950)* **160**: 2456–2462.

Conrad, M. L., Yildirim, A. O., Sonar, S. S., Kiliç, A., Sudowe, S., Lunow, M., Teich, R., Renz, H. & Garn, H. (2009) Comparison of adjuvant and adjuvant-free murine experimental asthma models. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **39**: 1246–1254.

Constant, S. L., Brogdon, J. L., Piggott, D. A., Herrick, C. A., Visintin, I., Ruddle, N. H. & Bottomly, K. (2002) Resident lung antigen-presenting cells have the capacity to promote Th2 T cell differentiation in situ. *The Journal of clinical investigation* **110**: 1441–1448.

Cook, D. G. & Strachan, D. P. (1999) Summary of effects of parental smoking on the respiratory health of children and implications for research. *Thorax* **54**: 357–366.

Corrigan, C. J., Brown, P. H., Barnes, N. C., Szefler, S. J., Tsai, J. J., Frew, A. J. & Kay, A. B. (1991*a*) Glucocorticoid resistance in chronic asthma. Glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics, and inhibition of peripheral blood T cell proliferation by glucocorticoids in vitro. *The American review of respiratory disease* **144**: 1016–1025.

Corrigan, C. J., Brown, P. H., Barnes, N. C., Tsai, J. J., Frew, A. J. & Kay, A. B. (1991*b*) Glucocorticoid resistance in chronic asthma. Peripheral blood T lymphocyte activation and comparison of the T lymphocyte inhibitory effects of glucocorticoids and cyclosporin A. *The American review of respiratory disease* **144**: 1026–1032.

Corry, D. B., Folkesson, H. G., Warnock, M. L., Erle, D. J., Matthay, M. A., Wiener-Kronish, J. P. & Locksley, R. M. (1996) Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *The Journal of Experimental Medicine* **183**: 109–117.

Corry, D. B., Grünig, G., Hadeiba, H., Kurup, V. P., Warnock, M. L., Sheppard, D., Rennick, D. M. & Locksley, R. M. (1998) Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Molecular medicine (Cambridge, Mass.)* **4**: 344–355.

Cosio, B. G., Iglesias, A., Rios, A., Noguera, A., Sala, E., Ito, K., Barnes, P. J. & Agusti, A. (2009) Low-dose theophylline enhances the anti-inflammatory effects of steroids during exacerbations of COPD. *Thorax* **64**: 424–429.

Cottrez, F., Hurst, S. D., Coffman, R. L. & Groux, H. (2000) T regulatory cells 1 inhibit a Th2-specific response in vivo. *Journal of immunology (Baltimore, Md.: 1950)* **165**: 4848–4853.

Coughlan, J. L., Gibson, P. G. & Henry, R. L. (2001) Medical treatment for reflux oesophagitis does not consistently improve asthma control: a systematic review. *Thorax* **56**: 198–204.

Cox, G. (1995) Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *Journal of immunology (Baltimore, Md.: 1950)* **154**: 4719–4725.

Cox, G., Whitehead, L., Dolovich, M., Jordana, M., Gauldie, J. & Newhouse, M. T. (1999) A randomized controlled trial on the effect of inhaled corticosteroids on airways inflammation in adult cigarette smokers. *Chest* **115**: 1271–1277.

Coyle, A. J., Ackerman, S. J., Burch, R., Proud, D. & Irvin, C. G. (1995) Human eosinophil-granule major basic protein and synthetic polycations induce airway hyperresponsiveness in vivo dependent on bradykinin generation. *Journal of Clinical Investigation* **95**: 1735–1740.

Coyle, A. J., Köhler, G., Tsuyuki, S., Brombacher, F. & Kopf, M. (1998) Eosinophils are not required to induce airway hyperresponsiveness after nematode infection. *European journal of immunology* **28**: 2640–2647.

Coyle, A. J., Lloyd, C., Tian, J., Nguyen, T., Erikkson, C., Wang, L., Ottoson, P., Persson, P., Delaney, T., Lehar, S., Lin, S., Poisson, L., Meisel, C., Kamradt, T., Bjerke, T., Levinson, D. & Gutierrez-Ramos, J. C. (1999) Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *The Journal of experimental medicine* **190**: 895–902.

Crescioli, S., Spinazzi, A., Plebani, M., Pozzani, M., Mapp, C. E., Boschetto, P. & Fabbri, L. M. (1991) Theophylline inhibits early and late asthmatic reactions induced by allergens in asthmatic subjects. *Annals of allergy* **66**: 245–251.

Crimi, E., Spanevello, A., Neri, M., Ind, P. W., Rossi, G. A. & Brusasco, V. (1998) Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *American journal of respiratory and critical care medicine* **157**: 4–9.

Crosby, J. R., Cieslewicz, G., Borchers, M., Hines, E., Carrigan, P., Lee, J. J. & Lee, N. A. (2002) Early phase bronchoconstriction in the mouse requires allergen-specific IgG. *Journal of immunology (Baltimore, Md.: 1950)* **168**: 4050–4054.

Cullinan, P., MacNeill, S. J., Harris, J. M., Moffat, S., White, C., Mills, P. & Newman Taylor, A. J. (2004) Early allergen exposure, skin prick responses, and atopic wheeze at age 5 in English children: a cohort study. *Thorax* **59**: 855–861.

Culpitt, S. V., Maziak, W., Loukidis, S., Nightingale, J. A., Matthews, J. L. & Barnes, P. J. (1999) Effect of high dose inhaled steroid on cells, cytokines, and proteases in induced sputum in chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* **160**: 1635–1639.

Culpitt, S. V., Rogers, D. F., Shah, P., De Matos, C., Russell, R. E. K., Donnelly, L. E. & Barnes, P. J. (2003) Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* **167**: 24–31.

Cushley, M. J., Wee, L. H. & Holgate, S. T. (1986) The effect of inhaled 5-hydroxytryptamine (5-HT, serotonin) on airway calibre in man. *British journal of clinical pharmacology* **22**: 487–490.

Cutz, E. (2002) Ultrastructure of airways in children with asthma. E. Cutz, H. Levison, D. M. Cooper. Histopathology 1978; 2; 407-421. *Histopathology* **41**: 19–20, discussion 20–21.

Cyphert, J. M., Kovarova, M., Allen, I. C., Hartney, J. M., Murphy, D. L., Wess, J. & Koller, B. H. (2009) Cooperation between mast cells and neurons is essential for antigen-mediated bronchoconstriction. *Journal of immunology (Baltimore, Md.: 1950)* **182**: 7430–7439.

Dabbagh, K., Dahl, M. E., Stepick-Biek, P. & Lewis, D. B. (2002) Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells. *Journal of immunology (Baltimore, Md.: 1950)* **168**: 4524–4530.

Dahl, R., Lundback, B., Malo, J. L., Mazza, J. A., Nieminen, M. M., Saarelainen, P. & Barnacle, H. (1993) A dose-ranging study of fluticasone propionate in adult patients with moderate asthma. International Study Group. *Chest* **104**: 1352–1358.

Dahlbäck, M., Bergstrand, H. & Sörenby, L. (1984) Bronchial anaphylaxis in actively sensitized Sprague Dawley rats: studies on mediators involved. *Acta pharmacologica et toxicologica* **55**: 6–17.

Dahlen, B., Shute, J. & Howarth, P. (1999) Immunohistochemical localisation of the matrix metalloproteinases MMP-3 and MMP-9 within the airways in asthma. *Thorax* **54**: 590–596.

Dahlén, S. E., Hedqvist, P., Hammarström, S. & Samuelsson, B. (1980) Leukotrienes are potent constrictors of human bronchi. *Nature* **288**: 484–486.

Daley, J. M., Thomay, A. A., Connolly, M. D., Reichner, J. S. & Albina, J. E. (2008) Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *Journal of leukocyte biology* **83**: 64–70.

Damiano, V. V., Tsang, A., Kucich, U., Abrams, W. R., Rosenbloom, J., Kimbel, P., Fallahnejad, M. & Weinbaum, G. (1986) Immunolocalization of elastase in human emphysematous lungs. *The Journal of clinical investigation* **78**: 482–493.

Davies, D. E., Wicks, J., Powell, R. M., Puddicombe, S. M. & Holgate, S. T. (2003) Airway remodeling in asthma: new insights. *The Journal of allergy and clinical immunology* **111**: 215–225; quiz 226.

Davis, B. E., Illamperuma, C., Gauvreau, G. M., Watson, R. M., O'Byrne, P. M., Deschesnes, F., Boulet, L. P. & Cockcroft, D. W. (2009) Single-dose desloratadine and montelukast and allergen-induced late airway responses. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **33**: 1302–1308.

Debarry, J., Garn, H., Hanuszkiewicz, A., Dickgreber, N., Blümer, N., von Mutius, E., Bufe, A., Gatermann, S., Renz, H., Holst, O. & Heine, H. (2007) Acinetobacter lwoffii and Lactococcus lactis strains isolated from farm cowsheds possess strong allergy-protective properties. *The Journal of allergy and clinical immunology* **119**: 1514–1521.

Demehri, S., Morimoto, M., Holtzman, M. J. & Kopan, R. (2009) Skin-derived TSLP triggers progression from epidermal-barrier defects to asthma. *PLoS biology* **7**: e1000067.

Denzel, A., Maus, U. A., Rodriguez Gomez, M., Moll, C., Niedermeier, M., Winter, C., Maus, R., Hollingshead, S., Briles, D. E., Kunz-Schughart, L. A., Talke, Y. & Mack, M. (2008) Basophils enhance immunological memory responses. *Nature immunology* **9**: 733–742.

Denzler, K. L., Farmer, S. C., Crosby, J. R., Borchers, M., Cieslewicz, G., Larson, K. A., Cormier-Regard, S., Lee, N. A. & Lee, J. J. (2000) Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma. *Journal of immunology (Baltimore, Md.: 1950)* **165**: 5509–5517.

Van Der Veen, M. J., Jansen, H. M., Aalberse, R. C. & van der Zee, J. S. (2001) Der p 1 and Der p 2 induce less severe late asthmatic responses than native Dermatophagoides pteronyssinus extract after a similar early asthmatic response. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **31**: 705–714.

Dijkstra, A., Vonk, J. M., Jongepier, H., Koppelman, G. H., Schouten, J. P., ten Hacken, N. H. T., Timens, W. & Postma, D. S. (2006) Lung function decline in asthma: association with inhaled corticosteroids, smoking and sex. *Thorax* **61**: 105–110.

Dilworth, R. J., Chua, K. Y. & Thomas, W. R. (1991) Sequence analysis of cDNA coding for a major house dust mite allergen, Der f I. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **21**: 25–32.

Djukanović, R., Wilson, J. W., Britten, K. M., Wilson, S. J., Walls, A. F., Roche, W. R., Howarth, P. H. & Holgate, S. T. (1992) Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *The American review of respiratory disease* **145**: 669–674.

Djukanović, R., Homeyard, S., Gratziou, C., Madden, J., Walls, A., Montefort, S., Peroni, D., Polosa, R., Holgate, S. & Howarth, P. (1997) The effect of treatment with oral corticosteroids on asthma symptoms and airway inflammation. *American journal of respiratory and critical care medicine* **155**: 826–832.

Djukanović, R., Wilson, S. J., Kraft, M., Jarjour, N. N., Steel, M., Chung, K. F., Bao, W., Fowler-Taylor, A., Matthews, J., Busse, W. W., Holgate, S. T. & Fahy, J. V. (2004) Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma. *American journal of respiratory and critical care medicine* **170**: 583–593.

Dolan, C. M., Fraher, K. E., Bleecker, E. R., Borish, L., Chipps, B., Hayden, M. L., Weiss, S., Zheng, B., Johnson, C. & Wenzel, S. (2004) Design and baseline characteristics of the epidemiology and natural history of asthma: Outcomes and Treatment Regimens (TENOR) study: a large cohort of patients with severe or difficult-to-treat asthma. *Annals of allergy, asthma & immunology: official publication of the American College of Allergy, Asthma, & Immunology* **92**: 32–39.

Dombrowicz, D., Flamand, V., Miyajima, I., Ravetch, J. V., Galli, S. J. & Kinet, J. P. (1997) Absence of Fc epsilonRI alpha chain results in upregulation of Fc gammaRIII-dependent mast cell degranulation and anaphylaxis. Evidence of competition between Fc epsilonRI and Fc gammaRIII for limiting amounts of FcR beta and gamma chains. *The Journal of clinical investigation* **99**: 915–925.

Doz, E., Noulin, N., Boichot, E., Guénon, I., Fick, L., Le Bert, M., Lagente, V., Ryffel, B., Schnyder, B., Quesniaux, V. F. J. & Couillin, I. (2008) Cigarette smoke-induced

pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent. *Journal of immunology (Baltimore, Md.: 1950)* **180**: 1169–1178.

Dubar, V., Gosset, P., Aerts, C., Voisin, C., Wallaert, B. & Tonnel, A. B. (1993) In vitro acute effects of tobacco smoke on tumor necrosis factor alpha and interleukin-6 production by alveolar macrophages. *Experimental lung research* **19**: 345–359.

Dubravec, D. B., Spriggs, D. R., Mannick, J. A. & Rodrick, M. L. (1990) Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 6758–6761.

Dullaers, M., De Bruyne, R., Ramadani, F., Gould, H. J., Gevaert, P. & Lambrecht, B. N. (2012) The who, where, and when of IgE in allergic airway disease. *The Journal of allergy and clinical immunology* **129**: 635–645.

Dunnill, M. S., Massarella, G. R. & Anderson, J. A. (1969) A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* **24**: 176–179.

Duong, M., Gauvreau, G., Watson, R., Obminski, G., Strinich, T., Evans, M., Howie, K., Killian, K. & O'Byrne, P. M. (2007) The effects of inhaled budesonide and formoterol in combination and alone when given directly after allergen challenge. *The Journal of allergy and clinical immunology* **119**: 322–327.

Dupont, L. J., Pype, J. L., Demedts, M. G., Leyn, P. D., Deneffe, G. & Verleden, G. M. (1999) The effects of 5-HT on cholinergic contraction in human airways in vitro. *European Respiratory Journal* **14**: 642–649.

Dupuy, P. M., Shore, S. A., Drazen, J. M., Frostell, C., Hill, W. A. & Zapol, W. M. (1992) Bronchodilator action of inhaled nitric oxide in guinea pigs. *The Journal of clinical investigation* **90**: 421–428.

Dye, J. A. & Adler, K. B. (1994) Effects of cigarette smoke on epithelial cells of the respiratory tract. *Thorax* **49**: 825–834.

Ebeling, C., Lam, T., Gordon, J. R., Hollenberg, M. D. & Vliagoftis, H. (2007) Proteinase-activated receptor-2 promotes allergic sensitization to an inhaled antigen through a TNF-mediated pathway. *Journal of immunology (Baltimore, Md.: 1950)* **179**: 2910–2917.

Eberhardt, W., Schulze, M., Engels, C., Klasmeier, E. & Pfeilschifter, J. (2002) Glucocorticoid-mediated suppression of cytokine-induced matrix metalloproteinase-9 expression in rat mesangial cells: involvement of nuclear factor-kappaB and Ets transcription factors. *Molecular endocrinology (Baltimore, Md.)* **16**: 1752–1766.

Ebina, M., Takahashi, T., Chiba, T. & Motomiya, M. (1993) Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *The American Review of Respiratory Disease* **148**: 720–726.

Ebner, H. & Kraft, D. (1975) [A comparative study of the value of skin tests and radio-allergo-sorbent tests in inhalation allergies (author's transl)]. *Wiener klinische Wochenschrift* **87**: 627–631.

Eden, E., Mitchell, D., Mehlman, B., Khouli, H., Nejat, M., Grieco, M. H. & Turino, G. M. (1997) Atopy, asthma, and emphysema in patients with severe alpha-1-antitrypysin deficiency. *American journal of respiratory and critical care medicine* **156**: 68–74.

Eden, E., Hammel, J., Rouhani, F. N., Brantly, M. L., Barker, A. F., Buist, A. S., Fallat, R. J., Stoller, J. K., Crystal, R. G. & Turino, G. M. (2003) Asthma features in severe alpha1-antitrypsin deficiency: experience of the National Heart, Lung, and Blood Institute Registry. *Chest* **123**: 765–771.

Edwards, A. D., Manickasingham, S. P., Spörri, R., Diebold, S. S., Schulz, O., Sher, A., Kaisho, T., Akira, S. & Reis e Sousa, C. (2002) Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. *Journal of immunology (Baltimore, Md.: 1950)* **169**: 3652–3660.

Eickelberg, O., Roth, M., Lörx, R., Bruce, V., Rüdiger, J., Johnson, M. & Block, L. H. (1999) Ligand-independent activation of the glucocorticoid receptor by beta2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. *The Journal of biological chemistry* **274**: 1005–1010.

Eisenbarth, S. C., Piggott, D. A., Huleatt, J. W., Visintin, I., Herrick, C. A. & Bottomly, K. (2002) Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *The Journal of experimental medicine* **196**: 1645–1651.

Eisenbarth, S. C., Piggott, D. A. & Bottomly, K. (2003) The master regulators of allergic inflammation: dendritic cells in Th2 sensitization. *Current opinion in immunology* **15**: 620–626.

Eisenbarth, S. C., Colegio, O. R., O/'Connor, W., Sutterwala, F. S. & Flavell, R. A. (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* **453**: 1122–1126.

Eiser, N. M., Mills, J., Snashall, P. D. & Guz, A. (1981) The role of histamine receptors in asthma. *Clinical science (London, England: 1979)* **60**: 363–370.

Eisner, M. D. & Iribarren, C. (2007) The influence of cigarette smoking on adult asthma outcomes. *Nicotine & tobacco research: official journal of the Society for Research on Nicotine and Tobacco* **9**: 53–56.

Eisner, M. D., Yelin, E. H., Henke, J., Shiboski, S. C. & Blanc, P. D. (1998) Environmental tobacco smoke and adult asthma. The impact of changing exposure status on health outcomes. *American journal of respiratory and critical care medicine* **158**: 170–175.

Eisner, M. D., Klein, J., Hammond, S. K., Koren, G., Lactao, G. & Iribarren, C. (2005) Directly measured second hand smoke exposure and asthma health outcomes. *Thorax* **60**: 814–821.

Eltom, S., Raemdonck, K., De Alba, J., Belvisi, M. G. & Birrell, M. A. (2010) House Dust Mite Exposure Induces Direct Non-Allergic Inflammation In Rodent Airways: Role Of The Innate Immune System. pp. A2837–A2837. American Thoracic Society.

Eltom, S., Stevenson, C. S., Rastrick, J., Dale, N., Raemdonck, K., Wong, S., Catley, M. C., Belvisi, M. G. & Birrell, M. A. (2011) P2X7 Receptor and Caspase 1 Activation Are

Central to Airway Inflammation Observed after Exposure to Tobacco Smoke. *PLoS ONE* **6**.

Eum, S.-Y., Norel, X., Lefort, J., Labat, C., Vargaftig, B. B. & Brink, C. (1999) Anaphylactic bronchoconstriction in BP2 mice: interactions between serotonin and acetylcholine. *British Journal of Pharmacology* **126**: 312–316.

Evans, C. M., Fryer, A. D., Jacoby, D. B., Gleich, G. J. & Costello, R. W. (1997) Pretreatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal M2 muscarinic receptors in antigen-challenged guinea pigs. *The Journal of clinical investigation* **100**: 2254–2262.

Evans, M. D. & Pryor, W. A. (1994) Cigarette smoking, emphysema, and damage to alpha 1-proteinase inhibitor. *The American journal of physiology* **266**: L593–611.

Fahy, J. V., Kim, K. W., Liu, J. & Boushey, H. A. (1995) Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *The Journal of allergy and clinical immunology* **95**: 843–852.

Fahy, J. V., Fleming, H. E., Wong, H. H., Liu, J. T., Su, J. Q., Reimann, J., Fick, R. B., Jr & Boushey, H. A. (1997) The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *American journal of respiratory and critical care medicine* **155**: 1828–1834.

Fahy, J. V., Cockcroft, D. W., Boulet, L. P., Wong, H. H., Deschesnes, F., Davis, E. E., Ruppel, J., Su, J. Q. & Adelman, D. C. (1999) Effect of aerosolized anti-IgE (E25) on airway responses to inhaled allergen in asthmatic subjects. *American journal of respiratory and critical care medicine* **160**: 1023–1027.

Fallon, P. G., Ballantyne, S. J., Mangan, N. E., Barlow, J. L., Dasvarma, A., Hewett, D. R., McIlgorm, A., Jolin, H. E. & McKenzie, A. N. J. (2006) Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *The Journal of experimental medicine* **203**: 1105–1116.

Fang, Q., Zhao, M. & Ren, G. (1997) [Effects of cigarette smoke extract on proliferation and ET-1 release of airway smooth muscle cells]. *Zhonghua yi xue za zhi* **77**: 201–204.

Fattouh, R., Midence, N. G., Arias, K., Johnson, J. R., Walker, T. D., Goncharova, S., Souza, K. P., Gregory, R. C., Jr, Lonning, S., Gauldie, J. & Jordana, M. (2008) Transforming growth factor-beta regulates house dust mite-induced allergic airway inflammation but not airway remodeling. *American journal of respiratory and critical care medicine* **177**: 593–603.

Fattouh, R., Al-Garawi, A., Fattouh, M., Arias, K., Walker, T. D., Goncharova, S., Coyle, A. J., Humbles, A. A. & Jordana, M. (2010) Eosinophils are Dispensable for Allergic Remodeling and Immunity in a Model of House Dust Mite-Induced Airway Disease. *Am. J. Respir. Crit. Care Med.*: 200905–0736OC.

Fernandez-Rodriguez, S., Ford, W. R., Broadley, K. J. & Kidd, E. J. (2008) Establishing the phenotype in novel acute and chronic murine models of allergic asthma. *International immunopharmacology* **8**: 756–763.

Ferrer, E., Peinado, V. I., Díez, M., Carrasco, J. L., Musri, M. M., Martínez, A., Rodríguez-Roisin, R. & Barberà, J. A. (2009) Effects of cigarette smoke on endothelial function of pulmonary arteries in the guinea pig. *Respiratory research* **10**: 76.

Filley, W. V., Holley, K. E., Kephart, G. M. & Gleich, G. J. (1982) Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet* **2**: 11–16.

Finkelman, F. D., Katona, I. M., Urban, J. F., Jr, Holmes, J., Ohara, J., Tung, A. S., Sample, J. V. & Paul, W. E. (1988) IL-4 is required to generate and sustain in vivo IgE responses. *Journal of immunology (Baltimore, Md.: 1950)* **141**: 2335–2341.

Finkelman, F. D., Katona, I. M., Urban, J. F., Jr & Paul, W. E. (1989) Control of in vivo IgE production in the mouse by interleukin 4. *Ciba Foundation symposium* **147**: 3–17; discussion 17–22.

Finlay, G. A., Russell, K. J., McMahon, K. J., D'arcy, E. M., Masterson, J. B., FitzGerald, M. X. & O'Connor, C. M. (1997*a*) Elevated levels of matrix metalloproteinases in bronchoalveolar lavage fluid of emphysematous patients. *Thorax* **52**: 502–506.

Finlay, G. A., O'Driscoll, L. R., Russell, K. J., D'Arcy, E. M., Masterson, J. B., FitzGerald, M. X. & O'Connor, C. M. (1997*b*) Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *American journal of respiratory and critical care medicine* **156**: 240–247.

Finotto, S., Neurath, M. F., Glickman, J. N., Qin, S., Lehr, H. A., Green, F. H. Y., Ackerman, K., Haley, K., Galle, P. R., Szabo, S. J., Drazen, J. M., De Sanctis, G. T. & Glimcher, L. H. (2002) Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science (New York, N.Y.)* **295**: 336–338.

Fish, J. E., Ankin, M. G., Kelly, J. F. & Peterman, V. I. (1981) Regulation of bronchomotor tone by lung inflation in asthmatic and nonasthmatic subjects. *Journal of applied physiology: respiratory, environmental and exercise physiology* **50**: 1079–1086.

Flach, T. L., Ng, G., Hari, A., Desrosiers, M. D., Zhang, P., Ward, S. M., Seamone, M. E., Vilaysane, A., Mucsi, A. D., Fong, Y., Prenner, E., Ling, C. C., Tschopp, J., Muruve, D. A., Amrein, M. W. & Shi, Y. (2011) Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. *Nature medicine* **17**: 479–487.

Fletcher, C. & Peto, R. (1977) The natural history of chronic airflow obstruction. *British medical journal* **1**: 1645–1648.

Flodin, U., Jönsson, P., Ziegler, J. & Axelson, O. (1995) An epidemiologic study of bronchial asthma and smoking. *Epidemiology (Cambridge, Mass.)* **6**: 503–505.

Flood-Page, P., Swenson, C., Faiferman, I., Matthews, J., Williams, M., Brannick, L., Robinson, D., Wenzel, S., Busse, W., Hansel, T. T. & Barnes, N. C. (2007) A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *American journal of respiratory and critical care medicine* **176**: 1062–1071.

Flores-Romo, L., Shields, J., Humbert, Y., Graber, P., Aubry, J. P., Gauchat, J. F., Ayala, G., Allet, B., Chavez, M. & Bazin, H. (1993) Inhibition of an in vivo antigen-specific IgE response by antibodies to CD23. *Science (New York, N.Y.)* **261**: 1038–1041.

Ford, P. A., Durham, A. L., Russell, R. E. K., Gordon, F., Adcock, I. M. & Barnes, P. J. (2010) Treatment effects of low-dose theophylline combined with an inhaled corticosteroid in COPD. *Chest* **137**: 1338–1344.

Fort, M. M., Cheung, J., Yen, D., Li, J., Zurawski, S. M., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., Muchamuel, T., Hurst, S. D., Zurawski, G., Leach, M. W., Gorman, D. M. & Rennick, D. M. (2001) IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* **15**: 985–995.

Foster, P. S., Hogan, S. P., Ramsay, A. J., Matthaei, K. I. & Young, I. G. (1996) Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *The Journal of experimental medicine* **183**: 195–201.

Franchi, L. & Núñez, G. (2008) The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. *European journal of immunology* **38**: 2085–2089.

Freishtat, R. J., Benton, A. S., Watson, A. M., Wang, Z., Rose, M. C. & Hoffman, E. P. (2009) Delineation of a gene network underlying the pulmonary response to oxidative stress in asthma. *Journal of investigative medicine: the official publication of the American Federation for Clinical Research* **57**: 756–764.

Frigas, E., Loegering, D. A., Solley, G. O., Farrow, G. M. & Gleich, G. J. (1981) Elevated levels of the eosinophil granule major basic protein in the sputum of patients with bronchial asthma. *Mayo Clinic proceedings. Mayo Clinic* **56**: 345–353.

Fulkerson, P. C., Rothenberg, M. E. & Hogan, S. P. (2005) Building a better mouse model: experimental models of chronic asthma. *Clinical & Experimental Allergy* **35**: 1251–1253.

Furmonaviciene, R., Ghaemmaghami, A. M., Boyd, S. E., Jones, N. S., Bailey, K., Willis, A. C., Sewell, H. F., Mitchell, D. A. & Shakib, F. (2007) The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **37**: 231–242.

Gabbrielli, S., Di Lollo, S., Stanflin, N. & Romagnoli, P. (1994) Myofibroblast and elastic and collagen fiber hyperplasia in the bronchial mucosa: a possible basis for the progressive irreversibility of airway obstruction in chronic asthma. *Pathologica* **86**: 157–160.

Gadek, J. E., Fells, G. A., Zimmerman, R. L., Rennard, S. I. & Crystal, R. G. (1981) Antielastases of the human alveolar structures. Implications for the protease-antiprotease theory of emphysema. *The Journal of clinical investigation* **68**: 889–898.

Gaga, M., Papageorgiou, N., Yiourgioti, G., Karydi, P., Liapikou, A., Bitsakou, H., Zervas, E., Koulouris, N. G. & Holgate, S. T. (2005) Risk factors and characteristics associated with severe and difficult to treat asthma phenotype: an analysis of the ENFUMOSA group of patients based on the ECRHS questionnaire. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **35**: 954–959.

Gamble, J., Stevenson, M., McClean, E. & Heaney, L. G. (2009) The prevalence of nonadherence in difficult asthma. *American journal of respiratory and critical care medicine* **180**: 817–822.

Gately, M. K., Renzetti, L. M., Magram, J., Stern, A. S., Adorini, L., Gubler, U. & Presky, D. H. (1998) The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annual review of immunology* **16**: 495–521.

Gauchat, J. F., Henchoz, S., Mazzei, G., Aubry, J. P., Brunner, T., Blasey, H., Life, P., Talabot, D., Flores-Romo, L. & Thompson, J. (1993) Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* **365**: 340–343.

Gauvreau, G. M. & Evans, M. Y. (2007) Allergen inhalation challenge: a human model of asthma exacerbation. *Contributions to microbiology* **14**: 21–32.

Gauvreau, G. M., Watson, R. M. & O'Byrne, P. M. (1999*a*) Protective effects of inhaled PGE2 on allergen-induced airway responses and airway inflammation. *American journal of respiratory and critical care medicine* **159**: 31–36.

Gauvreau, G. M., Watson, R. M. & O'Byrne, P. M. (1999*b*) Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. *American journal of respiratory and critical care medicine* **160**: 640–647.

Gauvreau, G. M., Lee, J. M., Watson, R. M., Irani, A. M., Schwartz, L. B. & O'Byrne, P. M. (2000) Increased numbers of both airway basophils and mast cells in sputum after allergen inhalation challenge of atopic asthmatics. *American journal of respiratory and critical care medicine* **161**: 1473–1478.

Gauvreau, G. M., Inman, M. D., Kelly, M., Watson, R. M., Dorman, S. C. & O'Byrne, P. M. (2002) Increased levels of airway neutrophils reduce the inhibitory effects of inhaled glucocorticosteroids on allergen-induced airway eosinophils. *Canadian respiratory journal: journal of the Canadian Thoracic Society* **9**: 26–32.

Gavett, S. H., Chen, X., Finkelman, F. & Wills-Karp, M. (1994) Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *American journal of respiratory cell and molecular biology* **10**: 587–593.

Gessner, C., Scheibe, R., Wötzel, M., Hammerschmidt, S., Kuhn, H., Engelmann, L., Hoheisel, G., Gillissen, A., Sack, U. & Wirtz, H. (2005) Exhaled breath condensate cytokine patterns in chronic obstructive pulmonary disease. *Respiratory medicine* **99**: 1229–1240.

Ghaemmaghami, A. M., Gough, L., Sewell, H. F. & Shakib, F. (2002) The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **32**: 1468–1475.

Gibson, P. G., Saltos, N. & Borgas, T. (2000) Airway mast cells and eosinophils correlate with clinical severity and airway hyperresponsiveness in corticosteroid-treated asthma. *The Journal of allergy and clinical immunology* **105**: 752–759.

Gilliland, F. D., Berhane, K., McConnell, R., Gauderman, W. J., Vora, H., Rappaport, E. B., Avol, E. & Peters, J. M. (2000) Maternal smoking during pregnancy, environmental tobacco smoke exposure and childhood lung function. *Thorax* **55**: 271–276.

Gilliland, F. D., Li, Y. F. & Peters, J. M. (2001) Effects of maternal smoking during pregnancy and environmental tobacco smoke on asthma and wheezing in children. *American journal of respiratory and critical care medicine* **163**: 429–436.

Ginzberg, H. H., Cherapanov, V., Dong, Q., Cantin, A., McCulloch, C. A., Shannon, P. T. & Downey, G. P. (2001) Neutrophil-mediated epithelial injury during transmigration: role of elastase. *American journal of physiology. Gastrointestinal and liver physiology* **281**: G705–717.

Gleich, G. J. & Adolphson, C. (1993) Bronchial hyperreactivity and eosinophil granule proteins. *Agents and actions. Supplements* **43**: 223–230.

Godard, P., Chanez, P., Siraudin, L., Nicoloyannis, N. & Duru, G. (2002) Costs of asthma are correlated with severity: a 1-yr prospective study. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **19**: 61–67.

Goh, K., Furusawa, S., Kawa, Y., Negishi-Okitsu, S. & Mizoguchi, M. (1989) Production of interleukin-1-alpha and -beta by human peripheral polymorphonuclear neutrophils. *International archives of allergy and applied immunology* **88**: 297–303.

Goleva, E., Li, L.-B. & Leung, D. Y. M. (2009) IFN-gamma reverses IL-2- and IL-4-mediated T-cell steroid resistance. *American journal of respiratory cell and molecular biology* **40**: 223–230.

Gomez, D. E., Alonso, D. F., Yoshiji, H. & Thorgeirsson, U. P. (1997) Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *European journal of cell biology* **74**: 111–122.

Gonzalo, J. A., Lloyd, C. M., Kremer, L., Finger, E., Martinez-A, C., Siegelman, M. H., Cybulsky, M. & Gutierrez-Ramos, J. C. (1996) Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *Journal of Clinical Investigation* **98**: 2332–2345.

Gordon, J. R. & Galli, S. J. (1991) 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. *The Journal of experimental medicine* **174**: 103–107.

Gosens, R., Zaagsma, J., Meurs, H. & Halayko, A. J. (2006) Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respiratory Research* **7**: 73–73.

Goto, N. & Akama, K. (1982) Histopathological studies of reactions in mice injected with aluminum-adsorbed tetanus toxoid. *Microbiology and immunology* **26**: 1121–1132.

Goto, N. & Akama, K. (1984) Local histopathological reactions to aluminum-adsorbed tetanus toxoid. *Die Naturwissenschaften* **71**: 427–428.

Goto, N., Kato, H., Maeyama, J., Shibano, M., Saito, T., Yamaguchi, J. & Yoshihara, S. (1997) Local tissue irritating effects and adjuvant activities of calcium phosphate

and aluminium hydroxide with different physical properties. *Vaccine* **15**: 1364–1371.

Gough, L., Schulz, O., Sewell, H. F. & Shakib, F. (1999) The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *The Journal of experimental medicine* **190**: 1897–1902.

Gowers, A. M., Cullinan, P., Ayres, J. G., Anderson, H. R., Strachan, D. P., Holgate, S. T., Mills, I. C. & Maynard, R. L. (2012) Does outdoor air pollution induce new cases of asthma? Biological plausibility and evidence; a review. *Respirology* **17**: 887–898.

Graffi, S. J., Dekan, G., Stingl, G. & Epstein, M. M. (2002) Systemic administration of antigen-pulsed dendritic cells induces experimental allergic asthma in mice upon aerosol antigen rechallenge. *Clinical immunology (Orlando, Fla.)* **103**: 176–184.

Green, R. H., Brightling, C. E., Woltmann, G., Parker, D., Wardlaw, A. J. & Pavord, I. D. (2002) Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* **57**: 875–879.

Gregory, L. G., Causton, B., Murdoch, J. R., Mathie, S. A., O'Donnell, V., Thomas, C. P., Priest, F. M., Quint, D. J. & Lloyd, C. M. (2009) Inhaled house dust mite induces pulmonary T helper 2 cytokine production. *Clinical & Experimental Allergy* **39**: 1597–1610.

Gregory, L. G., Jones, C. P., Walker, S. A., Sawant, D., Gowers, K. H. C., Campbell, G. A., McKenzie, A. N. J. & Lloyd, C. M. (2013) IL-25 drives remodelling in allergic airways disease induced by house dust mite. *Thorax* **68**: 82–90.

Griffith, S. (1990) A review of the factors associated with patient compliance and the taking of prescribed medicines. *The British journal of general practice: the journal of the Royal College of General Practitioners* **40**: 114–116.

Griffithsjohnson, D. A., Collins, P. D., Rossi, A. G., Jose, P. J. & Williams, T. J. (1993) The Chemokine, Eotaxin, Activates Guinea-Pig Eosinophils in Vitro and Causes Their Accumulation into the Lung in Vivo. *Biochemical and Biophysical Research Communications* **197**: 1167–1172.

Grol, M. H., Gerritsen, J., Vonk, J. M., Schouten, J. P., Koëter, G. H., Rijcken, B. & Postma, D. S. (1999) Risk factors for growth and decline of lung function in asthmatic individuals up to age 42 years. A 30-year follow-up study. *American Journal of Respiratory and Critical Care Medicine* **160**: 1830–1837.

Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J. & Liu, Y. J. (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *The Journal of experimental medicine* **185**: 1101–1111.

Grünig, G., Warnock, M., Wakil, A. E., Venkayya, R., Brombacher, F., Rennick, D. M., Sheppard, D., Mohrs, M., Donaldson, D. D., Locksley, R. M. & Corry, D. B. (1998) Requirement for IL-13 independently of IL-4 in experimental asthma. *Science (New York, N.Y.)* **282**: 2261–2263.

Grun, J. L. & Maurer, P. H. (1989) Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. *Cellular immunology* **121**: 134–145.

Gu, Q. & Lee, L.-Y. (2001) Hypersensitivity of pulmonary C fibre afferents induced by cationic proteins in the rat. *The Journal of Physiology* **537**: 887 –897.

Gueders, M. M., Paulissen, G., Crahay, C., Quesada-Calvo, F., Hacha, J., Van Hove, C., Tournoy, K., Louis, R., Foidart, J.-M., Noël, A. & Cataldo, D. D. (2009) Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflammation research: official journal of the European Histamine Research Society ... [et al.]* **58**: 845–854.

Gundel, R. H., Letts, L. G. & Gleich, G. J. (1991) Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. *The Journal of clinical investigation* **87**: 1470–1473.

Guo, C. B., Liu, M. C., Galli, S. J., Bochner, B. S., Kagey-Sobotka, A. & Lichtenstein, L. M. (1994) Identification of IgE-bearing cells in the late-phase response to antigen in the lung as basophils. *American journal of respiratory cell and molecular biology* **10**: 384–390.

Guo, Z., DU, Y. & Xu, J. (2008) [The effect of cigarette smoke on the expression of transforming growth factor-beta1 mRNA and collagen type III in airways of asthmatic rats]. *Zhonghua jie he he hu xi za zhi = Zhonghua jiehe he huxi zazhi = Chinese journal of tuberculosis and respiratory diseases* **31**: 42–45.

Gustafsson, D., Sjöberg, O. & Foucard, T. (2000) Development of allergies and asthma in infants and young children with atopic dermatitis--a prospective follow-up to 7 years of age. *Allergy* **55**: 240–245.

Haczku, A., Takeda, K., Redai, I., Hamelmann, E., Cieslewicz, G., Joetham, A., Loader, J., Lee, J. J., Irvin, C. & Gelfand, E. W. (1999) Anti-CD86 (B7.2) Treatment Abolishes Allergic Airway Hyperresponsiveness in Mice. *American Journal of Respiratory and Critical Care Medicine* **159**: 1638–1643.

Hagendorens, M. M., Ebo, D. G., Bridts, C. H., Van de Water, L., De Clerck, L. S. & Stevens, W. J. (2004) Prenatal exposure to house dust mite allergen (Der p 1), cord blood T cell phenotype and cytokine production and atopic dermatitis during the first year of life. *Pediatric allergy and immunology: official publication of the European Society of Pediatric Allergy and Immunology* **15**: 308–315.

Hamelmann, E., Vella, A. T., Oshiba, A., Kappler, J. W., Marrack, P. & Gelfand, E. W. (1997*a*) Allergic airway sensitization induces T cell activation but not airway hyperresponsiveness in B cell-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 1350–1355.

Hamelmann, E., Oshiba, A., Loader, J., Larsen, G. L., Gleich, G., Lee, J. & Gelfand, E. W. (1997*b*) Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *American journal of respiratory and critical care medicine* **155**: 819–825.

Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G. L., Irvin, C. G. & Gelfand, E. W. (1997*c*) Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *American journal of respiratory and critical care medicine* **156**: 766–775.

Hamelmann, E., Tadeda, K., Oshiba, A. & Gelfand, E. W. (1999*a*) Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness--a murine model. *Allergy* **54**: 297–305.

Hamelmann, E., Takeda, K., Schwarze, J., Vella, A. T., Irvin, C. G. & Gelfand, E. W. (1999*b*) Development of eosinophilic airway inflammation and airway hyperresponsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *American journal of respiratory cell and molecular biology* **21**: 480–489.

Hamelmann, E., Takeda, K., Haczku, A., Cieslewicz, G., Shultz, L., Hamid, Q., Xing, Z., Gauldie, J. & Gelfand, E. W. (2000) Interleukin (IL)-5 but not immunoglobulin E reconstitutes airway inflammation and airway hyperresponsiveness in IL-4-deficient mice. *American journal of respiratory cell and molecular biology* **23**: 327–334.

Hamid, Q., Azzawi, M., Ying, S., Moqbel, R., Wardlaw, A. J., Corrigan, C. J., Bradley, B., Durham, S. R., Collins, J. V. & Jeffery, P. K. (1991) Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *The Journal of clinical investigation* **87**: 1541–1546.

Hamid, Q. A., Wenzel, S. E., Hauk, P. J., Tsicopoulos, A., Wallaert, B., Lafitte, J. J., Chrousos, G. P., Szefler, S. J. & Leung, D. Y. (1999) Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *American journal of respiratory and critical care medicine* **159**: 1600–1604.

Hamilton, A., Faiferman, I., Stober, P., Watson, R. M. & O'Byrne, P. M. (1998) Pranlukast, a cysteinyl leukotriene receptor antagonist, attenuates allergen-induced early- and late-phase bronchoconstriction and airway hyperresponsiveness in asthmatic subjects. *The Journal of allergy and clinical immunology* **102**: 177–183.

Hamilton, A. L., Watson, R. M., Wyile, G. & O'Byrne, P. M. (1997) Attenuation of early and late phase allergen-induced bronchoconstriction in asthmatic subjects by a 5-lipoxygenase activating protein antagonist, BAYx 1005. *Thorax* **52**: 348–354.

Hammad, H., Charbonnier, A. S., Duez, C., Jacquet, A., Stewart, G. A., Tonnel, A. B. & Pestel, J. (2001) Th2 polarization by Der p 1--pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood* **98**: 1135–1141.

Hammad, H., Lambrecht, B. N., Pochard, P., Gosset, P., Marquillies, P., Tonnel, A.-B. & Pestel, J. (2002) Monocyte-derived dendritic cells induce a house dust mite-specific Th2 allergic inflammation in the lung of humanized SCID mice: involvement of CCR7. *Journal of immunology (Baltimore, Md.: 1950)* **169**: 1524–1534.

Hammad, H., Smits, H. H., Ratajczak, C., Nithiananthan, A., Wierenga, E. A., Stewart, G. A., Jacquet, A., Tonnel, A.-B. & Pestel, J. (2003) Monocyte-derived dendritic cells exposed to Der p 1 allergen enhance the recruitment of Th2 cells: major involvement of the chemokines TARC/CCL17 and MDC/CCL22. *European cytokine network* **14**: 219–228.

Hammad, H., Chieppa, M., Perros, F., Willart, M. A., Germain, R. N. & Lambrecht, B. N. (2009) House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nature medicine* **15**: 410–416.

Hammad, H., Plantinga, M., Deswarte, K., Pouliot, P., Willart, M. A. M., Kool, M., Muskens, F. & Lambrecht, B. N. (2010) Inflammatory dendritic cells--not

basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *The Journal of experimental medicine* **207**: 2097–2111.

Hansen, G., McIntire, J. J., Yeung, V. P., Berry, G., Thorbecke, G. J., Chen, L., DeKruyff, R. H. & Umetsu, D. T. (2000) CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *The Journal of clinical investigation* **105**: 61–70.

Harada, M., Hirota, T., Jodo, A. I., Hitomi, Y., Sakashita, M., Tsunoda, T., Miyagawa, T., Doi, S., Kameda, M., Fujita, K., Miyatake, A., Enomoto, T., Noguchi, E., Masuko, H., Sakamoto, T., Hizawa, N., Suzuki, Y., Yoshihara, S., Adachi, M., Ebisawa, M., Saito, H., Matsumoto, K., Nakajima, T., Mathias, R. A., Rafaels, N., Barnes, K. C., Himes, B. E., Duan, Q. L., Tantisira, K. G., Weiss, S. T., Nakamura, Y., Ziegler, S. F. & Tamari, M. (2011) Thymic stromal lymphopoietin gene promoter polymorphisms are associated with susceptibility to bronchial asthma. *American journal of respiratory cell and molecular biology* **44**: 787–793.

Hardaker, E., Freeman, M., Dale, N., Bahra, P., Raza, F., Banner, K. & Poll, C. (2010) Exposing rodents to a combination of tobacco smoke and lipopolysaccharide results in an exaggerated inflammatory response in the lung. *British Journal of Pharmacology* **160**: 1985–1996.

Harmsen, L., Gottlieb, V., Makowska Rasmussen, L. & Backer, V. (2010) Asthma patients who smoke have signs of chronic airflow limitation before age 45. *The Journal of Asthma: Official Journal of the Association for the Care of Asthma* **47**: 362–366.

Hart, L., Lim, S., Adcock, I., Barnes, P. J. & Chung, K. F. (2000) Effects of inhaled corticosteroid therapy on expression and DNA-binding activity of nuclear factor kappaB in asthma. *American journal of respiratory and critical care medicine* **161**: 224–231.

Hart, L. A., Krishnan, V. L., Adcock, I. M., Barnes, P. J. & Chung, K. F. (1998) Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. *American journal of respiratory and critical care medicine* **158**: 1585–1592.

Hartney, J. M., Coggins, K. G., Tilley, S. L., Jania, L. A., Lovgren, A. K., Audoly, L. P. & Koller, B. H. (2006) Prostaglandin E2 protects lower airways against bronchoconstriction. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **290**: L105–L113.

Hartwig, C., Mazzega, M., Constabel, H., Krishnaswamy, J. K., Gessner, J. E., Braun, A., Tschernig, T. & Behrens, G. M. N. (2010) Fcgamma receptor-mediated antigen uptake by lung DC contributes to allergic airway hyper-responsiveness and inflammation. *European journal of immunology* **40**: 1284–1295.

Hashimoto, T., Akiyama, K., Kobayashi, N. & Mori, A. (2005) Comparison of IL-17 production by helper T cells among atopic and nonatopic asthmatics and control subjects. *International archives of allergy and immunology* **137 Suppl 1**: 51–54.

Hautamaki, R. D., Kobayashi, D. K., Senior, R. M. & Shapiro, S. D. (1997) Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science (New York, N.Y.)* **277**: 2002–2004.

Hayashi, T., Hasegawa, K., Nakai, S., Hamachi, T., Adachi, Y., Yamauchi, Y. & Maeda, K. (2001) Bronchial lesions of the late asthmatic response in BALB/c and C57BL/6 mice. *Journal of comparative pathology* **125**: 208–213.

He, R., Oyoshi, M. K., Jin, H. & Geha, R. S. (2007) Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 15817–15822.

Heaney, L. G. & Robinson, D. S. (2005) Severe asthma treatment: need for characterising patients. *Lancet* **365**: 974–976.

Heaney, L. G., Conway, E., Kelly, C., Johnston, B. T., English, C., Stevenson, M. & Gamble, J. (2003) Predictors of therapy resistant asthma: outcome of a systematic evaluation protocol. *Thorax* **58**: 561–566.

Heck, S., Bender, K., Kullmann, M., Göttlicher, M., Herrlich, P. & Cato, A. C. (1997) I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. *The EMBO journal* **16**: 4698–4707.

De Heer, H. J., Hammad, H., Soullié, T., Hijdra, D., Vos, N., Willart, M. A. M., Hoogsteden, H. C. & Lambrecht, B. N. (2004) Essential Role of Lung Plasmacytoid Dendritic Cells in Preventing Asthmatic Reactions to Harmless Inhaled Antigen. *The Journal of Experimental Medicine* **200**: 89–98.

Heijink, I. H., van Oosterhout, A. & Kapus, A. (2010) Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **36**: 1016–1026.

Hele, D. J., Birrell, M. A., Webber, S. E., Foster, M. L. & Belvisi, M. G. (2001) Mediator involvement in antigen-induced bronchospasm and microvascular leakage in the airways of ovalbumin sensitized Brown Norway rats. *British journal of pharmacology* **132**: 481–488.

Hellings, P. W., Kasran, A., Liu, Z., Vandekerckhove, P., Wuyts, A., Overbergh, L., Mathieu, C. & Ceuppens, J. L. (2003) Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *American journal of respiratory cell and molecular biology* **28**: 42–50.

Henchoz, S., Gauchat, J. F., Aubry, J. P., Graber, P., Pochon, S. & Bonnefoy, J. Y. (1994) Stimulation of human IgE production by a subset of anti-CD21 monoclonal antibodies: requirement of a co-signal to modulate epsilon transcripts. *Immunology* **81**: 285–290.

Henderson, W. R., Jr, Lewis, D. B., Albert, R. K., Zhang, Y., Lamm, W. J., Chiang, G. K., Jones, F., Eriksen, P., Tien, Y. T., Jonas, M. & Chi, E. Y. (1996) The importance of leukotrienes in airway inflammation in a mouse model of asthma. *The Journal of experimental medicine* **184**: 1483–1494.

Henderson, W. R., Jr, Tang, L.-O., Chu, S.-J., Tsao, S.-M., Chiang, G. K. S., Jones, F., Jonas, M., Pae, C., Wang, H. & Chi, E. Y. (2002) A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. *American journal of respiratory and critical care medicine* **165**: 108–116.

Henjakovic, M., Martin, C., Hoymann, H. G., Sewald, K., Ressmeyer, A. R., Dassow, C., Pohlmann, G., Krug, N., Uhlig, S. & Braun, A. (2008) Ex vivo lung function measurements in precision-cut lung slices (PCLS) from chemical allergen-sensitized mice represent a suitable alternative to in vivo studies. *Toxicological sciences: an official journal of the Society of Toxicology* **106**: 444–453.

Henry, P. J. & Goldie, R. G. (1990) Beta 1-adrenoceptors mediate smooth muscle relaxation in mouse isolated trachea. *British journal of pharmacology* **99**: 131–135.

Herbert, C. A., King, C. M., Ring, P. C., Holgate, S. T., Stewart, G. A., Thompson, P. J. & Robinson, C. (1995) Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *American journal of respiratory cell and molecular biology* **12**: 369–378.

Von Hertzen, L. C. & Haahtela, T. (2004) Asthma and atopy – the price of affluence? *Allergy* **59**: 124–137.

Von Hertzen, L. & Haahtela, T. (2006) Disconnection of man and the soil: Reason for the asthma and atopy epidemic? *Journal of Allergy and Clinical Immunology* **117**: 334–344.

Hessel, E. M., Van Oosterhout, A. J., Hofstra, C. L., De Bie, J. J., Garssen, J., Van Loveren, H., Verheyen, A. K., Savelkoul, H. F. & Nijkamp, F. P. (1995) Bronchoconstriction and airway hyperresponsiveness after ovalbumin inhalation in sensitized mice. *European journal of pharmacology* **293**: 401–412.

Hessel, E. M., Van Oosterhout, A. J., Van Ark, I., Van Esch, B., Hofman, G., Van Loveren, H., Savelkoul, H. F. & Nijkamp, F. P. (1997) Development of airway hyperresponsiveness is dependent on interferon-gamma and independent of eosinophil infiltration. *American journal of respiratory cell and molecular biology* **16**: 325–334.

Hessel, E. M., Cruikshank, W. W., Van Ark, I., De Bie, J. J., Van Esch, B., Hofman, G., Nijkamp, F. P., Center, D. M. & Van Oosterhout, A. J. (1998) Involvement of IL-16 in the induction of airway hyper-responsiveness and up-regulation of IgE in a murine model of allergic asthma. *Journal of immunology (Baltimore, Md.: 1950)* **160**: 2998–3005.

Hewitt, C. R., Brown, A. P., Hart, B. J. & Pritchard, D. I. (1995) A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by antiproteases. *The Journal of experimental medicine* **182**: 1537–1544.

Hewitt, C. R., Horton, H., Jones, R. M. & Pritchard, D. I. (1997) Heterogeneous proteolytic specificity and activity of the house dust mite proteinase allergen Der p I. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **27**: 201–207.

Hida, S., Tadachi, M., Saito, T. & Taki, S. (2005) Negative control of basophil expansion by IRF-2 critical for the regulation of Th1/Th2 balance. *Blood* **106**: 2011–2017.

Hiltermann, T. J., Stolk, J., van der Zee, S. C., Brunekreef, B., de Bruijne, C. R., Fischer, P. H., Ameling, C. B., Sterk, P. J., Hiemstra, P. S. & van Bree, L. (1998) Asthma severity and susceptibility to air pollution. *The European respiratory journal* **11**: 686–693.

Hintzen, G., Ohl, L., del Rio, M.-L., Rodriguez-Barbosa, J.-I., Pabst, O., Kocks, J. R., Krege, J., Hardtke, S. & Förster, R. (2006) Induction of tolerance to innocuous inhaled antigen relies on a CCR7-dependent dendritic cell-mediated antigen transport to the bronchial lymph node. *Journal of immunology (Baltimore, Md.: 1950)* **177**: 7346–7354.

Hizume, D. C., Toledo, A. C., Moriya, H. T., Saraiva-Romanholo, B. M., Almeida, F. M., Arantes-Costa, F. M., Vieira, R. P., Dolhnikoff, M., Kasahara, D. I. & Martins, M. A. (2012) Cigarette smoke dissociates inflammation and lung remodeling in OVA-sensitized and challenged mice. *Respiratory physiology & neurobiology* **181**: 167–176.

Ho, L. H., Ohno, T., Oboki, K., Kajiwara, N., Suto, H., Iikura, M., Okayama, Y., Akira, S., Saito, H., Galli, S. J. & Nakae, S. (2007) IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcepsilonRI signals. *Journal of leukocyte biology* **82**: 1481–1490.

Hogan, S. P., Koskinen, A., Matthaei, K. I., Young, I. G. & Foster, P. S. (1998) Interleukin-5-producing CD4+ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice. *American journal of respiratory and critical care medicine* **157**: 210–218.

Holgate, S. T. (2008) Pathogenesis of asthma. *Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology* **38**: 872–897.

Holgate, S. T., Davies, D. E., Lackie, P. M., Wilson, S. J., Puddicombe, S. M. & Lordan, J. L. (2000) Epithelial-mesenchymal interactions in the pathogenesis of asthma. *The Journal of allergy and clinical immunology* **105**: 193–204.

Holgate, S. T., Peters-Golden, M., Panettieri, R. A. & Henderson, W. R., Jr (2003) Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. *The Journal of allergy and clinical immunology* **111**: S18–34; discussion S34–36.

Holgate, S. T., Chuchalin, A. G., Hébert, J., Lötvall, J., Persson, G. B., Chung, K. F., Bousquet, J., Kerstjens, H. A., Fox, H., Thirlwell, J., Cioppa, G. D. & Omalizumab 011 International Study Group (2004) Efficacy and safety of a recombinant anti-immunoglobulin E antibody (omalizumab) in severe allergic asthma. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **34**: 632–638.

Holloway, J. A., Warner, J. O., Vance, G. H., Diaper, N. D., Warner, J. A. & Jones, C. A. (2000) Detection of house-dust-mite allergen in amniotic fluid and umbilical-cord blood. *Lancet* **356**: 1900–1902.

Holt, P. G., Batty, J. E. & Turner, K. J. (1981) Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* **42**: 409–417.

Holt, P. G., Sly, P. D. & Björkstén, B. (1997) Atopic versus infectious diseases in childhood: a question of balance? *Pediatric allergy and immunology: official publication of the European Society of Pediatric Allergy and Immunology* **8**: 53–58.

Hong, C. S. & Park, H. S. (1989) The significance of specific IgE and IgG to dermatophagoides farinae according to the types of asthmatic reaction in house dust asthmatics. *Yonsei medical journal* **30**: 186–192.

Horak, E., Lanigan, A., Roberts, M., Welsh, L., Wilson, J., Carlin, J. B., Olinsky, A. & Robertson, C. F. (2003) Longitudinal study of childhood wheezy bronchitis and asthma: outcome at age 42. *BMJ (Clinical research ed.)* **326**: 422–423.

Hori, S., Nomura, T. & Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science (New York, N.Y.)* **299**: 1057–1061.

Horie, S., Okubo, Y., Hossain, M., Sato, E., Nomura, H., Koyama, S., Suzuki, J., Isobe, M. & Sekiguchi, M. (1997) Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. *Internal medicine (Tokyo, Japan)* **36**: 179–185.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., Fitzgerald, K. A. & Latz, E. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature immunology* **9**: 847–856.

Hoshino, M., Nakamura, Y., Sim, J., Shimojo, J. & Isogai, S. (1998) Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *The Journal of allergy and clinical immunology* **102**: 783–788.

Hossain, S. (1973) Quantitative measurement of bronchial muscle in men with asthma. *The American review of respiratory disease* **107**: 99–109.

Van Hove, C. L., Maes, T., Joos, G. F. & Tournoy, K. G. (2007) Prolonged Inhaled Allergen Exposure Can Induce Persistent Tolerance. *Am. J. Respir. Cell Mol. Biol.* **36**: 573–584.

Van Hove, C. L., Moerloose, K., Maes, T., Joos, G. F. & Tournoy, K. G. (2008) Cigarette smoke enhances Th-2 driven airway inflammation and delays inhalational tolerance. *Respiratory Research* **9**: 42.

Hu, J., Xu, Y., Zhang, Z. & Tian, F. (2007) Effect of cigarette smoke extract on proliferation of rat pulmonary artery smooth muscle cells and the relevant roles of protein kinase C. *Chinese medical journal* **120**: 1523–1528.

Huber, M., Beuscher, H. U., Rohwer, P., Kurrle, R., Röllinghoff, M. & Lohoff, M. (1998) Costimulation via TCR and IL-1 receptor reveals a novel IL-1alpha-mediated autocrine pathway of Th2 cell proliferation. *Journal of immunology (Baltimore, Md.: 1950)* **160**: 4242–4247.

Van der Hulst, A. E., Klip, H. & Brand, P. L. P. (2007) Risk of developing asthma in young children with atopic eczema: a systematic review. *The Journal of allergy and clinical immunology* **120**: 565–569.

Humbles, A. A., Conroy, D. M., Marleau, S., Rankin, S. M., Palframan, R. T., Proudfoot, A. E., Wells, T. N., Li, D., Jeffery, P. K., Griffiths-Johnson, D. A., Williams, T. J. & Jose, P. J. (1997) Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in a guinea pig model in vivo. *The Journal of experimental medicine* **186**: 601–612.

Humbles, A. A., Lloyd, C. M., McMillan, S. J., Friend, D. S., Xanthou, G., McKenna, E. E., Ghiran, S., Gerard, N. P., Yu, C., Orkin, S. H. & Gerard, C. (2004) A Critical Role for Eosinophils in Allergic Airways Remodeling. *Science* **305**: 1776–1779.

Hunninghake, G. W. & Crystal, R. G. (1983) Cigarette smoking and lung destruction. Accumulation of neutrophils in the lungs of cigarette smokers. *The American review of respiratory disease* **128**: 833–838.

Hurst, S. D., Seymour, B. W., Muchamuel, T., Kurup, V. P. & Coffman, R. L. (2001) Modulation of inhaled antigen-induced IgE tolerance by ongoing Th2 responses in the lung. *Journal of immunology (Baltimore, Md.: 1950)* **166**: 4922–4930.

Hutchison, S., Benson, R. A., Gibson, V. B., Pollock, A. H., Garside, P. & Brewer, J. M. (2012) Antigen depot is not required for alum adjuvanticity. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* **26**: 1272–1279.

Hwang, E. S., Szabo, S. J., Schwartzberg, P. L. & Glimcher, L. H. (2005) T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science (New York, N.Y.)* **307**: 430–433.

Idzko, M., Hammad, H., van Nimwegen, M., Kool, M., Willart, M. A. M., Muskens, F., Hoogsteden, H. C., Luttmann, W., Ferrari, D., Di Virgilio, F., Virchow, J. C., Jr & Lambrecht, B. N. (2007) Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nature medicine* **13**: 913–919.

Iikura, M., Suto, H., Kajiwara, N., Oboki, K., Ohno, T., Okayama, Y., Saito, H., Galli, S. J. & Nakae, S. (2007) IL-33 can promote survival, adhesion and cytokine production in human mast cells. *Laboratory investigation; a journal of technical methods and pathology* **87**: 971–978.

Iliopoulos, O., Baroody, F. M., Naclerio, R. M., Bochner, B. S., Kagey-Sobotka, A. & Lichtenstein, L. M. (1992) Histamine-containing cells obtained from the nose hours after antigen challenge have functional and phenotypic characteristics of basophils. *Journal of immunology (Baltimore, Md.: 1950)* **148**: 2223–2228.

Inman, M. D., Watson, R. M., Rerecich, T., Gauvreau, G. M., Lutsky, B. N., Stryszak, P. & O'Byrne, P. M. (2001) Dose-dependent effects of inhaled mometasone furoate on airway function and inflammation after allergen inhalation challenge. *American journal of respiratory and critical care medicine* **164**: 569–574.

Ino, Y., Ando, T., Haida, M., Nakamura, K., Iwaki, M., Okudaira, H. & Miyamoto, T. (1989) Characterization of the proteases in the crude mite extract. *International archives of allergy and applied immunology* **89**: 321–326.

Irusen, E., Matthews, J. G., Takahashi, A., Barnes, P. J., Chung, K. F. & Adcock, I. M. (2002) p38 Mitogen-activated protein kinase-induced glucocorticoid receptor phosphorylation reduces its activity: role in steroid-insensitive asthma. *The Journal of allergy and clinical immunology* **109**: 649–657.

Irwin, R. S., Curley, F. J. & French, C. L. (1993) Difficult-to-control asthma. Contributing factors and outcome of a systematic management protocol. *Chest* **103**: 1662–1669.

Ishikawa, R., Tsujimura, Y., Obata, K., Kawano, Y., Minegishi, Y. & Karasuyama, H. (2010) IgG-mediated systemic anaphylaxis to protein antigen can be induced even under conditions of limited amounts of antibody and antigen. *Biochemical and biophysical research communications* **402**: 742–746.

Ito, K., Kudo, K., Okudaira, H., Yoshinoya, S., Morita, Y., Nakagawa, T., Akiyama, K., Urata, C., Hayakawa, T. & Ohta, K. (1986) IgG1 antibodies to house dust mite (Dermatophagoides farinae) and late asthmatic response. *International archives of allergy and applied immunology* **81**: 69–74.

Ito, K., Barnes, P. J. & Adcock, I. M. (2000) Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. *Molecular and cellular biology* **20**: 6891–6903.

Ito, K., Yamamura, S., Essilfie-Quaye, S., Cosio, B., Ito, M., Barnes, P. J. & Adcock, I. M. (2006) Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. *The Journal of experimental medicine* **203**: 7–13.

Ito, K., Herbert, C., Siegle, J. S., Vuppusetty, C., Hansbro, N., Thomas, P. S., Foster, P. S., Barnes, P. J. & Kumar, R. K. (2008) Steroid-resistant neutrophilic inflammation in a mouse model of an acute exacerbation of asthma. *American journal of respiratory cell and molecular biology* **39**: 543–550.

Ito, T., Wang, Y.-H., Duramad, O., Hori, T., Delespesse, G. J., Watanabe, N., Qin, F. X.-F., Yao, Z., Cao, W. & Liu, Y.-J. (2005) TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *The Journal of experimental medicine* **202**: 1213–1223.

Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F. & Sakaguchi, S. (1999) Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *Journal of immunology (Baltimore, Md.: 1950)* **162**: 5317–5326.

Jacoby, D. B., Gleich, G. J. & Fryer, A. D. (1993) Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor. *Journal of Clinical Investigation* **91**: 1314–1318.

Jacoby, W., Cammarata, P. V., Findlay, S. & Pincus, S. H. (1984) Anaphylaxis in mast cell-deficient mice. *The Journal of investigative dermatology* **83**: 302–304.

Jaffar, Z., Roberts, K., Pandit, A., Linsley, P., Djukanovic, R. & Holgate, S. (1999) B7 Costimulation Is Required for IL-5 and IL-13 Secretion by Bronchial Biopsy Tissue of Atopic Asthmatic Subjects in Response to Allergen Stimulation. *American Journal of Respiratory Cell and Molecular Biology* **20**: 153–162.

Jahnsen, F. L., Moloney, E. D., Hogan, T., Upham, J. W., Burke, C. M. & Holt, P. G. (2001) Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge. *Thorax* **56**: 823–826.

Jakubzick, C., Tacke, F., Llodra, J., van Rooijen, N. & Randolph, G. J. (2006) Modulation of dendritic cell trafficking to and from the airways. *Journal of immunology (Baltimore, Md.: 1950)* **176**: 3578–3584.

Jang, A.-S., Park, J.-S., Lee, J.-H., Park, S.-W., Kim, D.-J., Uh, S.-T., Kim, Y.-H. & Park, C.-S. (2009) The impact of smoking on clinical and therapeutic effects in asthmatics. *Journal of Korean Medical Science* **24**: 209–214.

Jang, S., Morris, S. & Lukacs, N. W. (2013) TSLP Promotes Induction of Th2 Differentiation but Is Not Necessary during Established Allergen-Induced Pulmonary Disease. *PloS one* **8**: e56433.

Janoff, A., Raju, L. & Dearing, R. (1983) Levels of elastase activity in bronchoalveolar lavage fluids of healthy smokers and nonsmokers. *The American review of respiratory disease* **127**: 540–544.

Jarjour, N. N., Calhoun, W. J., Kelly, E. A., Gleich, G. J., Schwartz, L. B. & Busse, W. W. (1997) The immediate and late allergic response to segmental bronchopulmonary provocation in asthma. *American journal of respiratory and critical care medicine* **155**: 1515–1521.

Jarvis, D., Chinn, S., Luczynska, C. & Burney, P. (1999) The association of smoking with sensitization to common environmental allergens: results from the European Community Respiratory Health Survey. *The Journal of allergy and clinical immunology* **104**: 934–940.

Jatakanon, A., Uasuf, C., Maziak, W., Lim, S., Chung, K. F. & Barnes, P. J. (1999) Neutrophilic Inflammation in Severe Persistent Asthma. *Am. J. Respir. Crit. Care Med.* **160**: 1532–1539.

Jember, A. G., Zuberi, R., Liu, F. T. & Croft, M. (2001) Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. *The Journal of experimental medicine* **193**: 387–392.

Jenkins, H. A., Cherniack, R., Szefler, S. J., Covar, R., Gelfand, E. W. & Spahn, J. D. (2003) A comparison of the clinical characteristics of children and adults with severe asthma. *Chest* **124**: 1318–1324.

Jiang, H., Hener, P., Li, J. & Li, M. (2012) Skin thymic stromal lymphopoietin promotes airway sensitization to inhalant house dust mites leading to allergic asthma in mice. *Allergy* **67**: 1078–1082.

Johnson, J. R., Wiley, R. E., Fattouh, R., Swirski, F. K., Gajewska, B. U., Coyle, A. J., Gutierrez-Ramos, J.-C., Ellis, R., Inman, M. D. & Jordana, M. (2004) Continuous Exposure to House Dust Mite Elicits Chronic Airway Inflammation and Structural Remodeling. *Am. J. Respir. Crit. Care Med.* **169**: 378–385.

Johnson, J. R., Roos, A., Berg, T., Nord, M. & Fuxe, J. (2011) Chronic respiratory aeroallergen exposure in mice induces epithelial-mesenchymal transition in the large airways. *PloS one* **6**: e16175.

Johnson, P. R. A., Roth, M., Tamm, M., Hughes, M., Ge, Q., King, G., Burgess, J. K. & Black, J. L. (2001) Airway Smooth Muscle Cell Proliferation Is Increased in Asthma. *American Journal of Respiratory and Critical Care Medicine* **164**: 474–477.

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C., Gebel, S., Ponta, H. & Herrlich, P. (1990) Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* **62**: 1189–1204.

Jones, J. G., Minty, B. D., Lawler, P., Hulands, G., Crawley, J. C. & Veall, N. (1980) Increased alveolar epithelial permeability in cigarette smokers. *Lancet* **1**: 66–68.

Julia, V., Hessel, E. M., Malherbe, L., Glaichenhaus, N., O'Garra, A. & Coffman, R. L. (2002) A restricted subset of dendritic cells captures airborne antigens and remains able to activate specific T cells long after antigen exposure. *Immunity* **16**: 271–283.

Juniper, E. F., Frith, P. A., Dunnett, C., Cockcroft, D. W. & Hargreave, F. E. (1978) Reproducibility and comparison of responses to inhaled histamine and methacholine. *Thorax* **33**: 705–710.

Justice, J. P., Borchers, M. T., Crosby, J. R., Hines, E. M., Shen, H. H., Ochkur, S. I., McGarry, M. P., Lee, N. A. & Lee, J. J. (2003) Ablation of eosinophils leads to a reduction of allergen-induced pulmonary pathology. *American journal of physiology. Lung cellular and molecular physiology* **284**: L169–178.

Kagari, T., Tanaka, D., Doi, H. & Shimozato, T. (2003) Essential role of Fc gamma receptors in anti-type II collagen antibody-induced arthritis. *Journal of immunology (Baltimore, Md.: 1950)* **170**: 4318–4324.

Kagoshima, M., Wilcke, T., Ito, K., Tsaprouni, L., Barnes, P. J., Punchard, N. & Adcock, I. M. (2001) Glucocorticoid-mediated transrepression is regulated by histone acetylation and DNA methylation. *European journal of pharmacology* **429**: 327–334.

Kaiko, G. E. & Foster, P. S. (2011) New insights into the generation of Th2 immunity and potential therapeutic targets for the treatment of asthma. *Current opinion in allergy and clinical immunology* **11**: 39–45.

Kaliński, P., Hilkens, C. M., Snijders, A., Snijdewint, F. G. & Kapsenberg, M. L. (1997) IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *Journal of immunology (Baltimore, Md.: 1950)* **159**: 28–35.

Kam, J. C., Szefler, S. J., Surs, W., Sher, E. R. & Leung, D. Y. (1993) Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cell response to glucocorticoids. *Journal of immunology (Baltimore, Md.: 1950)* **151**: 3460–3466.

Kane, B., Kolsum, U., Southworth, T., Armstrong, J., Woodcock, A. & Singh, D. (2009) The effects of smoking on the lipopolysaccharide response and glucocorticoid sensitivity of alveolar macrophages of patients with asthma. *Chest* **136**: 163–170.

Kanniess, F., Richter, K., Böhme, S., Jörres, R. A. & Magnussen, H. (2001) Effect of Inhaled Ciclesonide on Airway Responsiveness to Inhaled AMP, the Composition of Induced Sputum and Exhaled Nitric Oxide in Patients with Mild Asthma. *Pulmonary Pharmacology & Therapeutics* **14**: 141–147.

Kapoor, R., Menon, C., Hoffstad, O., Bilker, W., Leclerc, P. & Margolis, D. J. (2008) The prevalence of atopic triad in children with physician-confirmed atopic dermatitis. *Journal of the American Academy of Dermatology* **58**: 68–73.

Kapsali, T., Permutt, S., Laube, B., Scichilone, N. & Togias, A. (2000) Potent bronchoprotective effect of deep inspiration and its absence in asthma. *Journal of applied physiology (Bethesda, Md.: 1985)* **89**: 711–720.

Kassel, O., Schmidlin, F., Duvernelle, C., Gasser, B., Massard, G. & Frossard, N. (1999) Human bronchial smooth muscle cells in culture produce stem cell factor. *The European respiratory journal* **13**: 951–954.

Kauffman, H. F., Tamm, M., Timmerman, J. A. B. & Borger, P. (2006) House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells

by protease-dependent and protease-independent mechanisms. *Clinical and molecular allergy: CMA* **4**: 5.

Kaur, D., Saunders, R., Berger, P., Siddiqui, S., Woodman, L., Wardlaw, A., Bradding, P. & Brightling, C. E. (2006) Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. *American journal of respiratory and critical care medicine* **174**: 1179–1188.

Kawaguchi, M., Kokubu, F., Fujita, J., Huang, S.-K. & Hizawa, N. (2009) Role of interleukin-17F in asthma. *Inflammation & allergy drug targets* **8**: 383–389.

Keane-Myers, A., Gause, W. C., Linsley, P. S., Chen, S. J. & Wills-Karp, M. (1997) B7-CD28/CTLA-4 costimulatory pathways are required for the development of T helper cell 2-mediated allergic airway responses to inhaled antigens. *Journal of immunology (Baltimore, Md.: 1950)* **158**: 2042–2049.

Kearley, J., Barker, J. E., Robinson, D. S. & Lloyd, C. M. (2005) Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *The Journal of experimental medicine* **202**: 1539–1547.

Kearley, J., Robinson, D. S. & Lloyd, C. M. (2008) CD4+CD25+ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. *The Journal of allergy and clinical immunology* **122**: 617–624.e6.

Keatings, V. M., Collins, P. D., Scott, D. M. & Barnes, P. J. (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *American journal of respiratory and critical care medicine* **153**: 530–534.

Keatings, V. M., Jatakanon, A., Worsdell, Y. M. & Barnes, P. J. (1997) Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *American journal of respiratory and critical care medicine* **155**: 542–548.

Kelada, S. N. P., Wilson, M. S., Tavarez, U., Kubalanza, K., Borate, B., Whitehead, G. S., Maruoka, S., Roy, M. G., Olive, M., Carpenter, D. E., Brass, D. M., Wynn, T. A., Cook, D. N., Evans, C. M., Schwartz, D. A. & Collins, F. S. (2011) Strain-Dependent Genomic Factors Affect Allergen-Induced Airway Hyperresponsiveness in Mice. *American Journal of Respiratory Cell and Molecular Biology* **45**: 817–824.

Kenyon, N. J., Ward, R. W., McGrew, G. & Last, J. A. (2003) TGF-beta1 causes airway fibrosis and increased collagen I and III mRNA in mice. *Thorax* **58**: 772–777.

Kerstjens, H. A., Overbeek, S. E., Schouten, J. P., Brand, P. L. & Postma, D. S. (1993) Airways hyperresponsiveness, bronchodilator response, allergy and smoking predict improvement in FEV1 during long-term inhaled corticosteroid treatment. Dutch CNSLD Study Group. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **6**: 868–876.

Khan, M. A., Ellis, R., Inman, M. D., Bates, J. H. T., Sanderson, M. J. & Janssen, L. J. (2010) Influence of airway wall stiffness and parenchymal tethering on the dynamics of bronchoconstriction. *American journal of physiology. Lung cellular and molecular physiology* **299**: L98–L108.

Kheradmand, F., Kiss, A., Xu, J., Lee, S.-H., Kolattukudy, P. E. & Corry, D. B. (2002) A Protease-Activated Pathway Underlying Th Cell Type 2 Activation and Allergic Lung Disease. *The Journal of Immunology* **169**: 5904–5911.

Kidney, J. C., Boulet, L. P., Hargreave, F. E., Deschesnes, F., Swystun, V. A., O'Byrne, P. M., Choudry, N., Morris, M. M., Jennings, B., Andersson, N., Andreasson, A. & Cockcroft, D. W. (1997) Evaluation of single-dose inhaled corticosteroid activity with an allergen challenge model. *The Journal of allergy and clinical immunology* **100**: 65–70.

Kikuchi, Y., Takai, T., Kuhara, T., Ota, M., Kato, T., Hatanaka, H., Ichikawa, S., Tokura, T., Akiba, H., Mitsuishi, K., Ikeda, S., Okumura, K. & Ogawa, H. (2006) Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. *Journal of immunology (Baltimore, Md.: 1950)* **177**: 1609–1617.

Kim, D. C., Hsu, F. I., Barrett, N. A., Friend, D. S., Grenningloh, R., Ho, I.-C., Al-Garawi, A., Lora, J. M., Lam, B. K., Austen, K. F. & Kanaoka, Y. (2006) Cysteinyl leukotrienes regulate Th2 cell-dependent pulmonary inflammation. *Journal of immunology (Baltimore, Md.: 1950)* **176**: 4440–4448.

Kim, D. Y., Kwon, E. Y., Hong, G. U., Lee, Y. S., Lee, S.-H. & Ro, J. Y. (2011) Cigarette smoke exacerbates mouse allergic asthma through Smad proteins expressed in mast cells. *Respiratory research* **12**: 49.

Kimura, I., Tanizaki, Y., Saito, K., Takahashi, K., Ueda, N. & Sato, S. (1975) Appearance of basophils in the sputum of patients with bronchial asthma. *Clinical allergy* **5**: 95–98.

Kimura, K., Inoue, H., Ichinose, M., Miura, M., Katsumata, U., Takahashi, T. & Takishima, T. (1992) Bradykinin causes airway hyperresponsiveness and enhances maximal airway narrowing. Role of microvascular leakage and airway edema. *The American review of respiratory disease* **146**: 1301–1305.

King, C., Brennan, S., Thompson, P. J. & Stewart, G. A. (1998) Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *Journal of immunology (Baltimore, Md.: 1950)* **161**: 3645–3651.

Kitz, R., Ahrens, P. & Zielen, S. (2000) Immunoglobulin levels in bronchoalveolar lavage fluid of children with chronic chest disease. *Pediatric pulmonology* **29**: 443–451.

Kobayashi, T., Miura, T., Haba, T., Sato, M., Serizawa, I., Nagai, H. & Ishizaka, K. (2000) An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *Journal of immunology (Baltimore, Md.: 1950)* **164**: 3855–3861.

Kogevinas, M., Antó, J. M., Tobias, A., Alonso, J., Soriano, J., Almar, E., Muniozguren, N., Payo, F., Pereira, A. & Sunyer, J. (1998) Respiratory symptoms, lung function and use of health services among unemployed young adults in Spain. Spanish Group of the European Community Respiratory Health Survey. *The European respiratory journal* **11**: 1363–1368.

Koh, Y. Y., Dupuis, R., Pollice, M., Albertine, K. H., Fish, J. E. & Peters, S. P. (1993) Neutrophils recruited to the lungs of humans by segmental antigen challenge display a reduced chemotactic response to leukotriene B4. *American journal of respiratory cell and molecular biology* **8**: 493–499.

Kolbe, J., Fergusson, W., Vamos, M. & Garrett, J. (2002) Case-control study of severe life threatening asthma (SLTA) in adults: psychological factors. *Thorax* **57**: 317–322.

Kondo, Y., Yoshimoto, T., Yasuda, K., Futatsugi-Yumikura, S., Morimoto, M., Hayashi, N., Hoshino, T., Fujimoto, J. & Nakanishi, K. (2008) Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *International immunology* **20**: 791–800.

Kool, M., Soullié, T., van Nimwegen, M., Willart, M. A. M., Muskens, F., Jung, S., Hoogsteden, H. C., Hammad, H. & Lambrecht, B. N. (2008*a*) Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *The Journal of Experimental Medicine* **205**: 869–882.

Kool, M., Pétrilli, V., Smedt, T. D., Rolaz, A., Hammad, H., Nimwegen, M. van, Bergen, I. M., Castillo, R., Lambrecht, B. N. & Tschopp, J. (2008*b*) Cutting Edge: Alum Adjuvant Stimulates Inflammatory Dendritic Cells through Activation of the NALP3 Inflammasome. *The Journal of Immunology* **181**: 3755–3759.

Kool, M., Willart, M. A. M., van Nimwegen, M., Bergen, I., Pouliot, P., Virchow, J. C., Rogers, N., Osorio, F., Reis e Sousa, C., Reis E Sousa, C., Hammad, H. & Lambrecht, B. N. (2011) An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity* **34**: 527–540.

Kool, M., Fierens, K. & Lambrecht, B. N. (2012) Alum adjuvant: some of the tricks of the oldest adjuvant. *Journal of medical microbiology* **61**: 927–934.

Korsgren, M., Erjefält, J. S., Korsgren, O., Sundler, F. & Persson, C. G. (1997) Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *The Journal of experimental medicine* **185**: 885–892.

Kouzaki, H., Iijima, K., Kobayashi, T., O'Grady, S. M. & Kita, H. (2011) The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *Journal of immunology (Baltimore, Md.: 1950)* **186**: 4375–4387.

Kudo, M., Melton, A. C., Chen, C., Engler, M. B., Huang, K. E., Ren, X., Wang, Y., Bernstein, X., Li, J. T., Atabai, K., Huang, X. & Sheppard, D. (2012) IL-17A produced by αβ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nature medicine* **18**: 547–554.

Kuehr, J., Frischer, T., Karmaus, W., Meinert, R., Barth, R. & Urbanek, R. (1992) Clinical atopy and associated factors in primary-school pupils. *Allergy* **47**: 650–655.

Kumar, R., Curtis, L. M., Khiani, S., Moy, J., Shalowitz, M. U., Sharp, L., Durazo-Arvizu, R. A., Shannon, J. J. & Weiss, K. B. (2008) A community-based study of tobacco smoke exposure among inner-city children with asthma in Chicago. *The Journal of allergy and clinical immunology* **122**: 754–759.e1.

Kumar, R. K., Herbert, C. & Kasper, M. (2004) Reversibility of airway inflammation and remodelling following cessation of antigenic challenge in a model of chronic asthma. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **34**: 1796–1802.

Kupczyk, M. & Wenzel, S. (2012) U.S. and European severe asthma cohorts: what can they teach us about severe asthma? *Journal of internal medicine* **272**: 121–132.

Kurashima, K., Fujimura, M., Myou, S., Ishiura, Y., Onai, N. & Matsushima, K. (2006) Asthma severity is associated with an increase in both blood CXCR3+ and CCR4+ T cells. *Respirology (Carlton, Vic.)* **11**: 152–157.

Kuroda, E., Ishii, K. J., Uematsu, S., Ohata, K., Coban, C., Akira, S., Aritake, K., Urade, Y. & Morimoto, Y. (2011) Silica crystals and aluminum salts regulate the production of prostaglandin in macrophages via NALP3 inflammasome-independent mechanisms. *Immunity* **34**: 514–526.

Kurowska-Stolarska, M., Kewin, P., Murphy, G., Russo, R. C., Stolarski, B., Garcia, C. C., Komai-Koma, M., Pitman, N., Li, Y., Niedbala, W., McKenzie, A. N. J., Teixeira, M. M., Liew, F. Y. & Xu, D. (2008) IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *Journal of immunology (Baltimore, Md.: 1950)* **181**: 4780–4790.

Kuschner, W. G., D'Alessandro, A., Wong, H. & Blanc, P. D. (1996) Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **9**: 1989–1994.

Kuwano, K., Bosken, C. H., Paré, P. D., Bai, T. R., Wiggs, B. R. & Hogg, J. C. (1993) Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *The American review of respiratory disease* **148**: 1220–1225.

Laan, M., Cui, Z. H., Hoshino, H., Lötvall, J., Sjöstrand, M., Gruenert, D. C., Skoogh, B. E. & Lindén, A. (1999) Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *Journal of immunology (Baltimore, Md.: 1950)* **162**: 2347–2352.

Laan, M., Lötvall, J., Chung, K. F. & Lindén, A. (2001) IL-17-induced cytokine release in human bronchial epithelial cells in vitro: role of mitogen-activated protein (MAP) kinases. *British journal of pharmacology* **133**: 200–206.

Laforest, L., Van Ganse, E., Devouassoux, G., Bousquet, J., Chretin, S., Bauguil, G., Pacheco, Y. & Chamba, G. (2006) Influence of patients' characteristics and disease management on asthma control. *The Journal of allergy and clinical immunology* **117**: 1404–1410.

Laitinen, L. A., Laitinen, A. & Haahtela, T. (1992) A comparative study of the effects of an inhaled corticosteroid, budesonide, and a beta 2-agonist, terbutaline, on airway inflammation in newly diagnosed asthma: a randomized, double-blind, parallel-group controlled trial. *The Journal of allergy and clinical immunology* **90**: 32–42.

Laitinen, L. A., Laitinen, A., Haahtela, T., Vilkka, V., Spur, B. W. & Lee, T. H. (1993) Leukotriene E4 and granulocytic infiltration into asthmatic airways. *Lancet* **341**: 989–990.

Lalloo, U. G., Malolepszy, J., Kozma, D., Krofta, K., Ankerst, J., Johansen, B. & Thomson, N. C. (2003) Budesonide and formoterol in a single inhaler improves asthma control compared with increasing the dose of corticosteroid in adults with mild-to-moderate asthma. *Chest* **123**: 1480–1487.

Lambert, R. K., Wiggs, B. R., Kuwano, K., Hogg, J. C. & Paré, P. D. (1993) Functional significance of increased airway smooth muscle in asthma and COPD. *Journal of applied physiology (Bethesda, Md.: 1985)* **74**: 2771–2781.

Lambrecht, B. N., Salomon, B., Klatzmann, D. & Pauwels, R. A. (1998) Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. *Journal of immunology (Baltimore, Md.: 1950)* **160**: 4090–4097.

Lambrecht, B. N., De Veerman, M., Coyle, A. J., Gutierrez-Ramos, J. C., Thielemans, K. & Pauwels, R. A. (2000) Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *The Journal of clinical investigation* **106**: 551–559.

Lanckacker, E. A., Tournoy, K. G., Hammad, H., Holtappels, G., Lambrecht, B. N., Joos, G. F. & Maes, T. (2012) Short cigarette smoke exposure facilitates sensitization and asthma development in mice. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology*.

Lange, P., Parner, J., Vestbo, J., Schnohr, P. & Jensen, G. (1998) A 15-year follow-up study of ventilatory function in adults with asthma. *The New England journal of medicine* **339**: 1194–1200.

Lange, P., Scharling, H., Ulrik, C. S. & Vestbo, J. (2006) Inhaled corticosteroids and decline of lung function in community residents with asthma. *Thorax* **61**: 100–104.

Larsen, G. L., Renz, H., Loader, J. E., Bradley, K. L. & Gelfand, E. W. (1992) Airway response to electrical field stimulation in sensitized inbred mice. Passive transfer of increased responsiveness with peribronchial lymph nodes. *The Journal of clinical investigation* **89**: 747–752.

Larsson-Callerfelt, A.-K., Dahlén, S.-E., Kühl, A.-R., Lex, D., Uhlig, S. & Martin, C. (2013) Modulation of antigen-induced responses by serotonin and prostaglandin E2 via EP1 and EP4 receptors in the peripheral rat lung. *European Journal of Pharmacology* **699**: 141–149.

Lasa, M., Brook, M., Saklatvala, J. & Clark, A. R. (2001) Dexamethasone destabilizes cyclooxygenase 2 mRNA by inhibiting mitogen-activated protein kinase p38. *Molecular and cellular biology* **21**: 771–780.

Lasa, M., Abraham, S. M., Boucheron, C., Saklatvala, J. & Clark, A. R. (2002) Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. *Molecular and cellular biology* **22**: 7802–7811.

Last, J. A., Ward, R., Temple, L., Pinkerton, K. E. & Kenyon, N. J. (2004) Ovalbumin-induced airway inflammation and fibrosis in mice also exposed to ultrafine particles. *Inhalation toxicology* **16**: 93–102.

Lau, S., Illi, S., Platts-Mills, T. A. E., Riposo, D., Nickel, R., Grüber, C., Niggemann, B. & Wahn, U. (2005) Longitudinal study on the relationship between cat allergen and endotoxin exposure, sensitization, cat-specific IgG and development of asthma in childhood--report of the German Multicentre Allergy Study (MAS 90). *Allergy* **60**: 766–773.

Lazarus, S. C., Chinchilli, V. M., Rollings, N. J., Boushey, H. A., Cherniack, R., Craig, T. J., Deykin, A., DiMango, E., Fish, J. E., Ford, J. G., Israel, E., Kiley, J., Kraft, M., Lemanske, R. F., Leone, F. T., Martin, R. J., Pesola, G. R., Peters, S. P., Sorkness, C. A., Szefler, S. J., Wechsler, M. E., Fahy, J. V. & for the National Heart Lung and Blood Institute's Asthma Clinical Research Network (2007) Smoking Affects Response to Inhaled Corticosteroids or Leukotriene Receptor Antagonists in Asthma. *Am. J. Respir. Crit. Care Med.* **175**: 783–790.

Leckie, M. J., ten Brinke, A., Khan, J., Diamant, Z., O'Connor, B. J., Walls, C. M., Mathur, A. K., Cowley, H. C., Chung, K. F., Djukanovic, R., Hansel, T. T., Holgate, S. T., Sterk, P. J. & Barnes, P. J. (2000) Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* **356**: 2144–2148.

Leclerc, O., Lagente, V., Planquois, J.-M., Berthelier, C., Artola, M., Eichholtz, T., Bertrand, C. P. & Schmidlin, F. (2006) Involvement of MMP-12 and phosphodiesterase type 4 in cigarette smoke-induced inflammation in mice. *The European respiratory journal* **27**: 1102–1109.

Lee, E. B., Kim, K. W., Hong, J. Y., Jee, H. M., Sohn, M. H. & Kim, K.-E. (2010) Increased serum thymic stromal lymphopoietin in children with atopic dermatitis. *Pediatric allergy and immunology: official publication of the European Society of Pediatric Allergy and Immunology* **21**: e457–460.

Lee, G. R. & Flavell, R. A. (2004) Transgenic mice which overproduce Th2 cytokines develop spontaneous atopic dermatitis and asthma. *International immunology* **16**: 1155–1160.

Lee, J. J., Dimina, D., Macias, M. P., Ochkur, S. I., McGarry, M. P., O'Neill, K. R., Protheroe, C., Pero, R., Nguyen, T., Cormier, S. A., Lenkiewicz, E., Colbert, D., Rinaldi, L., Ackerman, S. J., Irvin, C. G. & Lee, N. A. (2004*a*) Defining a link with asthma in mice congenitally deficient in eosinophils. *Science (New York, N.Y.)* **305**: 1773–1776.

Lee, J.-H., Yu, H.-H., Wang, L.-C., Yang, Y.-H., Lin, Y.-T. & Chiang, B.-L. (2007) The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. *Clinical and experimental immunology* **148**: 53–63.

Lee, Y. C., Lee, K. S., Park, S. J., Park, H. S., Lim, J. S., Park, K.-H., Im, M.-J., Choi, I.-W., Lee, H.-K. & Kim, U.-H. (2004*b*) Blockade of airway hyperresponsiveness and inflammation in a murine model of asthma by a prodrug of cysteine, L-2-oxothiazolidine-4-carboxylic acid. *The FASEB Journal*.

Lefort, J., Nahori, M. A., Ruffie, C., Vargaftig, B. B. & Pretolani, M. (1996) In vivo neutralization of eosinophil-derived major basic protein inhibits antigen-induced bronchial hyperreactivity in sensitized guinea pigs. *The Journal of clinical investigation* **97**: 1117–1121.

Leigh, R., Vethanayagam, D., Yoshida, M., Watson, R. M., Rerecich, T., Inman, M. D. & O'Byrne, P. M. (2002) Effects of montelukast and budesonide on airway responses and airway inflammation in asthma. *American journal of respiratory and critical care medicine* **166**: 1212–1217.

Leung, D. Y., Martin, R. J., Szefler, S. J., Sher, E. R., Ying, S., Kay, A. B. & Hamid, Q. (1995) Dysregulation of interleukin 4, interleukin 5, and interferon gamma gene

expression in steroid-resistant asthma. *The Journal of experimental medicine* **181**: 33–40.

Leung, D. Y., Hamid, Q., Vottero, A., Szefler, S. J., Surs, W., Minshall, E., Chrousos, G. P. & Klemm, D. J. (1997) Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *The Journal of experimental medicine* **186**: 1567–1574.

Leung, D. Y. M., Szefler, S. J. & Associate Editors of the JACI (2010) In utero smoke (IUS) exposure has been associated with increased prevalence of asthma and reduced lung function in healthy children. *The Journal of allergy and clinical immunology* **126**: 481–482.

Leuppi, J. D., Steurer-Stey, C., Peter, M., Chhajed, P. N., Wildhaber, J. H. & Spertini, F. (2006) Asthma control in Switzerland: a general practitioner based survey. *Current medical research and opinion* **22**: 2159–2166.

Leyva-Castillo, J. M., Hener, P., Jiang, H. & Li, M. (2013) TSLP produced by keratinocytes promotes allergen sensitization through skin and thereby triggers atopic march in mice. *The Journal of investigative dermatology* **133**: 154–163.

Li, H., Willingham, S. B., Ting, J. P.-Y. & Re, F. (2008) Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. *Journal of immunology (Baltimore, Md.: 1950)* **181**: 17–21.

Li, X. Y., Donaldson, K., Rahman, I. & MacNee, W. (1994) An investigation of the role of glutathione in increased epithelial permeability induced by cigarette smoke in vivo and in vitro. *American journal of respiratory and critical care medicine* **149**: 1518–1525.

Liberati, T. A., Randle, M. R. & Toth, L. A. (2010) In vitro lung slices: a powerful approach for assessment of lung pathophysiology. *Expert review of molecular diagnostics* **10**: 501–508.

Liew, F. Y. (2002) T(H)1 and T(H)2 cells: a historical perspective. *Nature reviews. Immunology* **2**: 55–60.

Lin, J., Xu, Y., Zhang, Z., Ni, W. & Chen, S. (2005*a*) Effect of cigarette smoke extract on the role of protein kinase C in the proliferation of passively sensitized human airway smooth muscle cells. *Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban* **25**: 269–273.

Lin, W., Truong, N., Grossman, W. J., Haribhai, D., Williams, C. B., Wang, J., Martín, M. G. & Chatila, T. A. (2005*b*) Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice. *The Journal of allergy and clinical immunology* **116**: 1106–1115.

Lindén, A. (2001) Role of interleukin-17 and the neutrophil in asthma. *International archives of allergy and immunology* **126**: 179–184.

Ling, E. M., Smith, T., Nguyen, X. D., Pridgeon, C., Dallman, M., Arbery, J., Carr, V. A. & Robinson, D. S. (2004) Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* **363**: 608–615.

Liu, L., Yang, J. & Huang, Y. (2006) Human airway smooth muscle cells express eotaxin in response to signaling following mast cell contact. *Respiration; international review of thoracic diseases* **73**: 227–235.

Liu, Y., Zhang, S., Li, D. & Jiang, S. (2013) Efficacy of anti-interleukin-5 therapy with mepolizumab in patients with asthma: a meta-analysis of randomized placebo-controlled trials. *PloS one* **8**: e59872.

Liu, Y. J. (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* **106**: 259–262.

Liu, Y.-J. (2006) Thymic stromal lymphopoietin: master switch for allergic inflammation. *The Journal of experimental medicine* **203**: 269–273.

Livingston, E., Darroch, C. E., Chaudhuri, R., McPhee, I., McMahon, A. D., Mackenzie, S. J. & Thomson, N. C. (2004) Glucocorticoid receptor alpha:beta ratio in blood mononuclear cells is reduced in cigarette smokers. *The Journal of allergy and clinical immunology* **114**: 1475–1478.

Lomask, M. (2006) Further exploration of the Penh parameter. *Experimental and Toxicologic Pathology* **57**: 13–20.

Lopez, A. F., Sanderson, C. J., Gamble, J. R., Campbell, H. D., Young, I. G. & Vadas, M. A. (1988) Recombinant human interleukin 5 is a selective activator of human eosinophil function. *The Journal of experimental medicine* **167**: 219–224.

Louis, R., Lau, L. C. K., Bron, A. O., Roldaan, A. C., Radermecker, M. & Djukanovic, R. (2000) The Relationship between Airways Inflammation and Asthma Severity. *Am. J. Respir. Crit. Care Med.* **161**: 9–16.

Lu, Y., Sjöstrand, M., Malmhäll, C., Rådinger, M., Jeurink, P., Lötvall, J. & Bossios, A. (2010) New production of eosinophils and the corresponding TH1/TH2 balance in the lungs after allergen exposure in BALB/c and C57BL/6 mice. *Scandinavian journal of immunology* **71**: 176–185.

Lúdvíksdóttir, D., Björnsson, E., Janson, C. & Boman, G. (1996) Habitual coughing and its associations with asthma, anxiety, and gastroesophageal reflux. *Chest* **109**: 1262–1268.

Lundblad, L. K. A., Irvin, C. G., Hantos, Z., Sly, P., Mitzner, W. & Bates, J. H. T. (2007) Penh is not a measure of airway resistance! *European Respiratory Journal* **30**: 805–805.

Lynch, D. A., Newell, J. D., Tschomper, B. A., Cink, T. M., Newman, L. S. & Bethel, R. (1993) Uncomplicated asthma in adults: comparison of CT appearance of the lungs in asthmatic and healthy subjects. *Radiology* **188**: 829–833.

Ma, X., Cheng, Z., Kong, H., Wang, Y., Unruh, H., Stephens, N. L. & Laviolette, M. (2002) Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol Physiol* **283**: L1181–1189.

Mackay, D., Haw, S., Ayres, J. G., Fischbacher, C. & Pell, J. P. (2010) Smoke-free legislation and hospitalizations for childhood asthma. *The New England journal of medicine* **363**: 1139–1145.

Macklem, P. T. (1996) A theoretical analysis of the effect of airway smooth muscle load on airway narrowing. *American journal of respiratory and critical care medicine* **153**: 83–89.

MacNee, W., Wiggs, B., Belzberg, A. S. & Hogg, J. C. (1989) The effect of cigarette smoking on neutrophil kinetics in human lungs. *The New England journal of medicine* **321**: 924–928.

Magnusson, C. G. (1986) Maternal smoking influences cord serum IgE and IgD levels and increases the risk for subsequent infant allergy. *The Journal of allergy and clinical immunology* **78**: 898–904.

Mak, J. C., Nishikawa, M., Shirasaki, H., Miyayasu, K. & Barnes, P. J. (1995) Protective effects of a glucocorticoid on downregulation of pulmonary beta 2-adrenergic receptors in vivo. *The Journal of clinical investigation* **96**: 99–106.

Mak, J. C. W., Hisada, T., Salmon, M., Barnes, P. J. & Chung, K. F. (2002) Glucocorticoids reverse IL-1beta-induced impairment of beta-adrenoceptor-mediated relaxation and up-regulation of G-protein-coupled receptor kinases. *British journal of pharmacology* **135**: 987–996.

Mäkelä, M. J., Kanehiro, A., Borish, L., Dakhama, A., Loader, J., Joetham, A., Xing, Z., Jordana, M., Larsen, G. L. & Gelfand, E. W. (2000) IL-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 6007–6012.

Mannhalter, J. W., Neychev, H. O., Zlabinger, G. J., Ahmad, R. & Eibl, M. M. (1985) Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation. *Clinical and Experimental Immunology* **61**: 143–151.

Mannino, D. M., Moorman, J. E., Kingsley, B., Rose, D. & Repace, J. (2001) Health effects related to environmental tobacco smoke exposure in children in the United States: data from the Third National Health and Nutrition Examination Survey. *Archives of pediatrics & adolescent medicine* **155**: 36–41.

Martin, T. R., Takeishi, T., Katz, H. R., Austen, K. F., Drazen, J. M. & Galli, S. J. (1993) Mast cell activation enhances airway responsiveness to methacholine in the mouse. *The Journal of clinical investigation* **91**: 1176–1182.

Martinez, F. D. (1994) Role of viral infections in the inception of asthma and allergies during childhood: could they be protective? *Thorax* **49**: 1189–1191.

Martinez, F. D. & Holt, P. G. (1999) Role of microbial burden in aetiology of allergy and asthma. *Lancet* **354 Suppl 2**: SII12–15.

Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A. & Tschopp, J. (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**: 237–241.

Marwick, J. A., Kirkham, P. A., Stevenson, C. S., Danahay, H., Giddings, J., Butler, K., Donaldson, K., MacNee, W. & Rahman, I. (2004) Cigarette Smoke Alters Chromatin Remodeling and Induces Proinflammatory Genes in Rat Lungs. *American Journal of Respiratory Cell and Molecular Biology* **31**: 633–642.

Marwick, J. A., Caramori, G., Stevenson, C. S., Casolari, P., Jazrawi, E., Barnes, P. J., Ito, K., Adcock, I. M., Kirkham, P. A. & Papi, A. (2009) Inhibition of PI3Kδ Restores Glucocorticoid Function in Smoking-induced Airway Inflammation in Mice. *American Journal of Respiratory and Critical Care Medicine* **179**: 542–548.

Marwick, J. A., Stevenson, C. S., Chung, K. F., Adcock, I. M. & Kirkham, P. A. (2010) Cigarette Smoke Exposure Alters mSin3a and Mi-2alpha/beta Expression; implications in the control of pro-inflammatory gene transcription and glucocorticoid function. *Journal of inflammation (London, England)* **7**: 33.

Masoli, M., Fabian, D., Holt, S. & Beasley, R. (2004) The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* **59**: 469–478.

Masubuchi, T., Koyama, S., Sato, E., Takamizawa, A., Kubo, K., Sekiguchi, M., Nagai, S. & Izumi, T. (1998) Smoke extract stimulates lung epithelial cells to release neutrophil and monocyte chemotactic activity. *The American journal of pathology* **153**: 1903–1912.

Matricardi, P. M., Rosmini, F., Ferrigno, L., Nisini, R., Rapicetta, M., Chionne, P., Stroffolini, T., Pasquini, P. & D'Amelio, R. (1997) Cross sectional retrospective study of prevalence of atopy among Italian military students with antibodies against hepatitis A virus. *BMJ (Clinical research ed.)* **314**: 999–1003.

Mattes, J., Yang, M., Siqueira, A., Clark, K., MacKenzie, J., McKenzie, A. N., Webb, D. C., Matthaei, K. I. & Foster, P. S. (2001) IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. *Journal of immunology (Baltimore, Md.: 1950)* **167**: 1683–1692.

Mattes, J., Yang, M., Mahalingam, S., Kuehr, J., Webb, D. C., Simson, L., Hogan, S. P., Koskinen, A., McKenzie, A. N. J., Dent, L. A., Rothenberg, M. E., Matthaei, K. I., Young, I. G. & Foster, P. S. (2002) Intrinsic Defect in T Cell Production of Interleukin (IL)-13 in the Absence of Both IL-5 and Eotaxin Precludes the Development of Eosinophilia and Airways Hyperreactivity in Experimental Asthma. *The Journal of Experimental Medicine* **195**: 1433–1444.

Maurer, D., Fiebiger, S., Ebner, C., Reininger, B., Fischer, G. F., Wichlas, S., Jouvin, M. H., Schmitt-Egenolf, M., Kraft, D., Kinet, J. P. & Stingl, G. (1996) Peripheral blood dendritic cells express Fc epsilon RI as a complex composed of Fc epsilon RI alpha- and Fc epsilon RI gamma-chains and can use this receptor for IgE-mediated allergen presentation. *Journal of immunology (Baltimore, Md.: 1950)* **157**: 607–616.

Mautino, G., Henriquet, C., Jaffuel, D., Bousquet, J. & Capony, F. (1999) Tissue inhibitor of metalloproteinase-1 levels in bronchoalveolar lavage fluid from asthmatic subjects. *American journal of respiratory and critical care medicine* **160**: 324–330.

Mayr, S. I., Zuberi, R. I., Zhang, M., de Sousa-Hitzler, J., Ngo, K., Kuwabara, Y., Yu, L., Fung-Leung, W.-P. & Liu, F.-T. (2002) IgE-dependent mast cell activation potentiates airway responses in murine asthma models. *Journal of immunology (Baltimore, Md.: 1950)* **169**: 2061–2068.

McConnell, R., Berhane, K., Yao, L., Jerrett, M., Lurmann, F., Gilliland, F., Künzli, N., Gauderman, J., Avol, E., Thomas, D. & Peters, J. (2006) Traffic, Susceptibility, and Childhood Asthma. *Environmental Health Perspectives* **114**: 766–772.

McCrea, K. A., Ensor, J. E., Nall, K., Bleecker, E. R. & Hasday, J. D. (1994) Altered cytokine regulation in the lungs of cigarette smokers. *American journal of respiratory and critical care medicine* **150**: 696–703.

McCreanor, J., Cullinan, P., Nieuwenhuijsen, M. J., Stewart-Evans, J., Malliarou, E., Jarup, L., Harrington, R., Svartengren, M., Han, I.-K., Ohman-Strickland, P., Chung, K. F. & Zhang, J. (2007) Respiratory effects of exposure to diesel traffic in persons with asthma. *The New England journal of medicine* **357**: 2348–2358.

McKee, A. S., Munks, M. W., MacLeod, M. K. L., Fleenor, C. J., Van Rooijen, N., Kappler, J. W. & Marrack, P. (2009) Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *Journal of immunology (Baltimore, Md.: 1950)* **183**: 4403–4414.

McKenzie, G. J., Fallon, P. G., Emson, C. L., Grencis, R. K. & McKenzie, A. N. (1999) Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. *The Journal of experimental medicine* **189**: 1565–1572.

McKinley, L., Alcorn, J. F., Peterson, A., Dupont, R. B., Kapadia, S., Logar, A., Henry, A., Irvin, C. G., Piganelli, J. D., Ray, A. & Kolls, J. K. (2008) TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *Journal of immunology (Baltimore, Md.: 1950)* **181**: 4089–4097.

McMenamin, C., Pimm, C., McKersey, M. & Holt, P. G. (1994) Regulation of IgE responses to inhaled antigen in mice by antigen-specific gamma delta T cells. *Science (New York, N.Y.)* **265**: 1869–1871.

McMillan, S. J. & Lloyd, C. M. (2004) Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **34**: 497–507.

McMillan, S. J., Xanthou, G. & Lloyd, C. M. (2005) Therapeutic administration of Budesonide ameliorates allergen-induced airway remodelling. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **35**: 388–396.

McParland, B. E., Tait, R. R., Paré, P. D. & Seow, C. Y. (2005) The role of airway smooth muscle during an attack of asthma simulated in vitro. *American journal of respiratory cell and molecular biology* **33**: 500–504.

McSharry, C. P., McKay, I. C., Chaudhuri, R., Livingston, E., Fraser, I. & Thomson, N. C. (2005) Short and long-term effects of cigarette smoking independently influence exhaled nitric oxide concentration in asthma. *The Journal of allergy and clinical immunology* **116**: 88–93.

McVicker, C. G., Leung, S.-Y., Kanabar, V., Moir, L. M., Mahn, K., Chung, K. F. & Hirst, S. J. (2007) Repeated allergen inhalation induces cytoskeletal remodeling in smooth muscle from rat bronchioles. *American journal of respiratory cell and molecular biology* **36**: 721–727.

Meghji, Z., Dua, B., Watson, R. M., Gauvreau, G. M. & O'Byrne, P. M. (2011) Allergen inhalation challenge in smoking compared with non-smoking asthmatic subjects. *Clinical & Experimental Allergy* **41**: 1084–1090.

Mehlhop, P. D., van de Rijn, M., Goldberg, A. B., Brewer, J. P., Kurup, V. P., Martin, T. R. & Oettgen, H. C. (1997) Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 1344–1349.

Melewicz, F. M., Kline, L. E., Cohen, A. B. & Spiegelberg, H. L. (1982) Characterization of Fc receptors for IgE on human alveolar macrophages. *Clinical and experimental immunology* **49**: 364–370.

Melgert, B. N., Postma, D. S., Geerlings, M., Luinge, M. A., Klok, P. A., van der Strate, B. W. A., Kerstjens, H. A. M., Timens, W. & Hylkema, M. N. (2004) Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. *American Journal of Respiratory Cell and Molecular Biology* **30**: 880–885.

Melgert, B. N., Timens, W., Kerstjens, H. A., Geerlings, M., Luinge, M. A., Schouten, J. P., Postma, D. S. & Hylkema, M. N. (2007) Effects of 4 months of smoking in mice with ovalbumin-induced airway inflammation. *Clinical & Experimental Allergy* **37**: 1798–1808.

Menon, P., Rando, R. J., Stankus, R. P., Salvaggio, J. E. & Lehrer, S. B. (1992) Passive cigarette smoke-challenge studies: increase in bronchial hyperreactivity. *The Journal of allergy and clinical immunology* **89**: 560–566.

Meyer, C. H., Bond, J. F., Chen, M. S. & Kasaian, M. T. (1994) Comparison of the levels of the major allergens Der p I and Der p II in standardized extracts of the house dust mite, Dermatophagoides pteronyssinus. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **24**: 1041–1048.

Milgrom, H., Fick, R. B., Jr, Su, J. Q., Reimann, J. D., Bush, R. K., Watrous, M. L. & Metzger, W. J. (1999) Treatment of allergic asthma with monoclonal anti-IgE antibody. rhuMAb-E25 Study Group. *The New England journal of medicine* **341**: 1966–1973.

Milgrom, H., Berger, W., Nayak, A., Gupta, N., Pollard, S., McAlary, M., Taylor, A. F. & Rohane, P. (2001) Treatment of childhood asthma with anti-immunoglobulin E antibody (omalizumab). *Pediatrics* **108**: E36.

Miller, R. L., Chew, G. L., Bell, C. A., Biedermann, S. A., Aggarwal, M., Kinney, P. L., Tsai, W. Y., Whyatt, R. M., Perera, F. P. & Ford, J. G. (2001) Prenatal exposure, maternal sensitization, and sensitization in utero to indoor allergens in an inner-city cohort. *American journal of respiratory and critical care medicine* **164**: 995–1001.

Millett, C., Lee, J. T., Laverty, A. A., Glantz, S. A. & Majeed, A. (2013) Hospital Admissions for Childhood Asthma After Smoke-Free Legislation in England. *Pediatrics*.

Min, B., Prout, M., Hu-Li, J., Zhu, J., Jankovic, D., Morgan, E. S., Urban, J. F., Dvorak, A. M., Finkelman, F. D., LeGros, G. & Paul, W. E. (2004) Basophils Produce IL-4 and Accumulate in Tissues after Infection with a Th2-inducing Parasite. *The Journal of Experimental Medicine* **200**: 507–517.

Min, M. G., Song, D. J., Miller, M., Cho, J. Y., McElwain, S., Ferguson, P. & Broide, D. H. (2007) Coexposure to environmental tobacco smoke increases levels of allergen-

induced airway remodeling in mice. *Journal of immunology (Baltimore, Md.: 1950)* **178**: 5321–5328.

Minette, P. A., Lammers, J. W., Dixon, C. M., McCusker, M. T. & Barnes, P. J. (1989) A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. *Journal of Applied Physiology* **67**: 2461–2465.

Miranda, C., Busacker, A., Balzar, S., Trudeau, J. & Wenzel, S. E. (2004) Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. *The Journal of allergy and clinical immunology* **113**: 101–108.

Miyajima, I., Dombrowicz, D., Martin, T. R., Ravetch, J. V., Kinet, J. P. & Galli, S. J. (1997) Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc gammaRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *The Journal of clinical investigation* **99**: 901–914.

Mizutani, N., Nabe, T. & Yoshino, S. (2009) Complement C3a regulates late asthmatic response and airway hyperresponsiveness in mice. *Journal of immunology (Baltimore, Md.: 1950)* **183**: 4039–4046.

Mizutani, N., Goshima, H., Nabe, T. & Yoshino, S. (2012) Establishment and characterization of a murine model for allergic asthma using allergen-specific IgE monoclonal antibody to study pathological roles of IgE. *Immunology Letters* **141**: 235–245.

Moerloose, K. B., Pauwels, R. A. & Joos, G. F. (2005) Short-Term Cigarette Smoke Exposure Enhances Allergic Airway Inflammation in Mice. *Am. J. Respir. Crit. Care Med.* **172**: 168–172.

Moerloose, K. B., Robays, L. J., Maes, T., Brusselle, G. G., Tournoy, K. G. & Joos, G. F. (2006) Cigarette smoke exposure facilitates allergic sensitization in mice. *Respiratory Research* **7**: 49–49.

Mohan, G., Harrison, B. D., Badminton, R. M., Mildenhall, S. & Wareham, N. J. (1996) A confidential enquiry into deaths caused by asthma in an English health region: implications for general practice. *The British journal of general practice: the journal of the Royal College of General Practitioners* **46**: 529–532.

Moir, L. M., Leung, S.-Y., Eynott, P. R., McVicker, C. G., Ward, J. P. T., Chung, K. F. & Hirst, S. J. (2003) Repeated allergen inhalation induces phenotypic modulation of smooth muscle in bronchioles of sensitized rats. *American journal of physiology. Lung cellular and molecular physiology* **284**: L148–159.

Molet, S., Hamid, Q., Davoine, F., Nutku, E., Taha, R., Pagé, N., Olivenstein, R., Elias, J. & Chakir, J. (2001) IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *The Journal of allergy and clinical immunology* **108**: 430–438.

De Monchy, J. G., Kauffman, H. F., Venge, P., Koëter, G. H., Jansen, H. M., Sluiter, H. J. & De Vries, K. (1985) Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *The American review of respiratory disease* **131**: 373–376.

Monteseirín, J., Bonilla, I., Camacho, M. J., Chacón, P., Vega, A., Chaparro, A., Conde, J. & Sobrino, F. (2003) Specific allergens enhance elastase release in stimulated

neutrophils from asthmatic patients. *International archives of allergy and immunology* **131**: 174–181.

Moon, H., Choi, H. H., Lee, J. Y., Moon, H. J., Sim, S. S. & Kim, C. J. (2008) Quercetin inhalation inhibits the asthmatic responses by exposure to aerosolized-ovalbumin in conscious guinea-pigs. *Archives of pharmacal research* **31**: 771–778.

Moore, K. W., O'Garra, A., Malefyt, R. W., Vieira, P. & Mosmann, T. R. (1993) Interleukin-10. *Annual Review of Immunology* **11**: 165–190.

Moore, W. C. & Peters, S. P. (2006) Severe asthma: an overview. *The Journal of allergy and clinical immunology* **117**: 487–494; quiz 495.

Mori, L., Kleimberg, J., Mancini, C., Bellini, A., Marini, M. & Mattoli, S. (1995) Bronchial epithelial cells of atopic patients with asthma lack the ability to inactivate allergens. *Biochemical and biophysical research communications* **217**: 817–824.

Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J.-I., Ohtani, M., Fujii, H. & Koyasu, S. (2010) Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* **463**: 540–544.

Morokata, T., Ishikawa, J., Ida, K. & Yamada, T. (1999) C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses. *Immunology* **98**: 345–351.

Morokata, T., Ishikawa, J. & Yamada, T. (2000) Antigen dose defines T helper 1 and T helper 2 responses in the lungs of C57BL/6 and BALB/c mice independently of splenic responses. *Immunology letters* **72**: 119–126.

Mosmann, T. R. & Coffman, R. L. (1989) TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties. *Annual Review of Immunology* **7**: 145–173.

Mosmann, T. R. & Sad, S. (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology Today* **17**: 138–146.

Motojima, S., Frigas, E., Loegering, D. A. & Gleich, G. J. (1989) Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro. *The American review of respiratory disease* **139**: 801–805.

Mukai, K., Matsuoka, K., Taya, C., Suzuki, H., Yokozeki, H., Nishioka, K., Hirokawa, K., Etori, M., Yamashita, M., Kubota, T., Minegishi, Y., Yonekawa, H. & Karasuyama, H. (2005) Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* **23**: 191–202.

Muller, B. A., Leick, C. A., Smith, R. M., Suelzer, M. T. & Richerson, H. B. (1993) Comparisons of specific and nonspecific bronchoprovocation in subjects with asthma, rhinitis, and healthy subjects. *The Journal of allergy and clinical immunology* **91**: 758–772.

Munakata, M., Masaki, Y., Sakuma, I., Ukita, H., Otsuka, Y., Homma, Y. & Kawakami, Y. (1990) Pharmacological differentiation of epithelium-derived relaxing factor from nitric oxide. *Journal of applied physiology (Bethesda, Md.: 1985)* **69**: 665–670.

Munks, M. W., McKee, A. S., Macleod, M. K., Powell, R. L., Degen, J. L., Reisdorph, N. A., Kappler, J. W. & Marrack, P. (2010) Aluminum adjuvants elicit fibrin-dependent extracellular traps in vivo. *Blood* **116**: 5191–5199.

Mushaben, E. M., Brandt, E. B., Hershey, G. K. K. & Le Cras, T. D. (2013) Differential effects of rapamycin and dexamethasone in mouse models of established allergic asthma. *PloS one* **8**: e54426.

Mustafa, S. J., Nadeem, A., Fan, M., Zhong, H., Belardinelli, L. & Zeng, D. (2007) Effect of a Specific and Selective A2B Adenosine Receptor Antagonist on Adenosine Agonist AMP and Allergen-Induced Airway Responsiveness and Cellular Influx in a Mouse Model of Asthma. *Journal of Pharmacology and Experimental Therapeutics* **320**: 1246–1251.

Von Mutius, E. & Vercelli, D. (2010) Farm living: effects on childhood asthma and allergy. *Nature reviews. Immunology* **10**: 861–868.

Von Mutius, E., Fritzsch, C., Weiland, S. K., Röll, G. & Magnussen, H. (1992) Prevalence of asthma and allergic disorders among children in united Germany: a descriptive comparison. *BMJ (Clinical research ed.)* **305**: 1395–1399.

Von Mutius, E., Martinez, F. D., Fritzsch, C., Nicolai, T., Roell, G. & Thiemann, H. H. (1994) Prevalence of asthma and atopy in two areas of West and East Germany. *American journal of respiratory and critical care medicine* **149**: 358–364.

Myers, A. C. & Undem, B. J. (1995) Antigen depolarizes guinea pig bronchial parasympathetic ganglion neurons by activation of histamine H1 receptors. *The American journal of physiology* **268**: L879–884.

Myers, A. C., Undem, B. J. & Weinreich, D. (1991) Influence of antigen on membrane properties of guinea pig bronchial ganglion neurons. *Journal of applied physiology (Bethesda, Md.: 1985)* **71**: 970–976.

Nabe, T., Yamamoto, M., Suga, M. & Kohno, S. (2004) Intratracheal dosing with disodium cromoglycate inhibits late asthmatic response by attenuating eicosanoid production in guinea pigs. *European journal of pharmacology* **497**: 97–104.

Nabe, T., Zindl, C. L., Jung, Y. W., Stephens, R., Sakamoto, A., Kohno, S., Atkinson, T. P. & Chaplin, D. D. (2005) Induction of a late asthmatic response associated with airway inflammation in mice. *European journal of pharmacology* **521**: 144–155.

Nabe, T., Hosokawa, F., Matsuya, K., Morishita, T., Ikedo, A., Fujii, M., Mizutani, N., Yoshino, S. & Chaplin, D. D. (2011) Important role of neutrophils in the late asthmatic response in mice. *Life sciences* **88**: 1127–1135.

Nabe, T., Matsuya, K., Akamizu, K., Fujita, M., Nakagawa, T., Shioe, M., Kida, H., Takiguchi, A., Wakamori, H., Fujii, M., Ishihara, K., Akiba, S., Mizutani, N., Yoshino, S. & Chaplin, D. D. (2013) Roles of basophils and mast cells infiltrating the lung by multiple antigen challenges in asthmatic responses of mice. *British journal of pharmacology*.

Naclerio, R. M., Proud, D., Togias, A. G., Adkinson, N. F., Jr, Meyers, D. A., Kagey-Sobotka, A., Plaut, M., Norman, P. S. & Lichtenstein, L. M. (1985) Inflammatory mediators in late antigen-induced rhinitis. *The New England journal of medicine* **313**: 65–70.

Nadel, J. A. & Tierney, D. F. (1961) Effect of a previous deep inspiration on airway resistance in man. *Journal of applied physiology* **16**: 717–719.

Nagai, H., Yamaguchi, S., Maeda, Y. & Tanaka, H. (1996) Role of mast cells, eosinophils and IL-5 in the development of airway hyperresponsiveness in sensitized mice. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **26**: 642–647.

Nakae, S., Komiyama, Y., Nambu, A., Sudo, K., Iwase, M., Homma, I., Sekikawa, K., Asano, M. & Iwakura, Y. (2002) Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* **17**: 375–387.

Nakae, S., Suto, H., Berry, G. J. & Galli, S. J. (2007) Mast cell-derived TNF can promote Th17 cell-dependent neutrophil recruitment in ovalbumin-challenged OTII mice. *Blood* **109**: 3640–3648.

Nakamura, Y., Miyata, M., Ohba, T., Ando, T., Hatsushika, K., Suenaga, F., Shimokawa, N., Ohnuma, Y., Katoh, R., Ogawa, H. & Nakao, A. (2008) Cigarette smoke extract induces thymic stromal lymphopoietin expression, leading to T(H)2-type immune responses and airway inflammation. *The Journal of allergy and clinical immunology* **122**: 1208–1214.

Nambu, M., Shintaku, N. & Ohta, S. (2003) Relationship between cord blood level of IgE specific for Dermatophagoides pteronyssinus and allergic manifestations in infancy. *Biology of the neonate* **83**: 102–106.

Nathan, A. T., Peterson, E. A., Chakir, J. & Wills-Karp, M. (2009) Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. *The Journal of allergy and clinical immunology* **123**: 612–618.

Neill, D. R., Wong, S. H., Bellosi, A., Flynn, R. J., Daly, M., Langford, T. K. A., Bucks, C., Kane, C. M., Fallon, P. G., Pannell, R., Jolin, H. E. & McKenzie, A. N. J. (2010) Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**: 1367–1370.

Nelde, A., Teufel, M., Hahn, C., Duschl, A., Sebald, W., Bröcker, E. B. & Grunewald, S. M. (2001) The impact of the route and frequency of antigen exposure on the IgE response in allergy. *International archives of allergy and immunology* **124**: 461–469.

Nelson, H. S., Weiss, S. T., Bleecker, E. R., Yancey, S. W. & Dorinsky, P. M. (2006) The Salmeterol Multicenter Asthma Research Trial: a comparison of usual pharmacotherapy for asthma or usual pharmacotherapy plus salmeterol. *Chest* **129**: 15–26.

Nguyen, K. D., Vanichsarn, C. & Nadeau, K. C. (2010) TSLP directly impairs pulmonary Treg function: association with aberrant tolerogenic immunity in asthmatic airway. *Allergy, asthma, and clinical immunology: official journal of the Canadian Society of Allergy and Clinical Immunology* **6**: 4.

Niewoehner, D. E., Kleinerman, J. & Rice, D. B. (1974) Pathologic Changes in the Peripheral Airways of Young Cigarette Smokers. *New England Journal of Medicine* **291**: 755–758.

Nightingale, J. A., Maggs, R., Cullinan, P., Donnelly, L. E., Rogers, D. F., Kinnersley, R., Chung, K. F., Barnes, P. J., Ashmore, M. & Newman-Taylor, A. (2000) Airway inflammation after controlled exposure to diesel exhaust particulates. *American journal of respiratory and critical care medicine* **162**: 161–166.

Nocker, R. E., Out, T. A., Weller, F. R., Mul, E. P., Jansen, H. M. & van der Zee, J. S. (1999) Influx of neutrophils into the airway lumen at 4 h after segmental allergen challenge in asthma. *International archives of allergy and immunology* **119**: 45–53.

Nogami, H., Honjo, S. & Iwanaga, T. (2012) [The effect of inhaled anticholinergic drugs (tiotropium bromide) on asthma patients with persistent obstructive ventilatory impairment]. *Arerugī = [Allergy]* **61**: 1675–1682.

Nonaka, M., Nonaka, R., Woolley, K., Adelroth, E., Miura, K., Okhawara, Y., Glibetic, M., Nakano, K., O'Byrne, P. & Dolovich, J. (1995) Distinct immunohistochemical localization of IL-4 in human inflamed airway tissues. IL-4 is localized to eosinophils in vivo and is released by peripheral blood eosinophils. *Journal of immunology (Baltimore, Md.: 1950)* **155**: 3234–3244.

Novak, N., Allam, J.-P., Hagemann, T., Jenneck, C., Laffer, S., Valenta, R., Kochan, J. & Bieber, T. (2004) Characterization of FcepsilonRI-bearing CD123 blood dendritic cell antigen-2 plasmacytoid dendritic cells in atopic dermatitis. *The Journal of allergy and clinical immunology* **114**: 364–370.

O'Byrne, P. M., Dolovich, J. & Hargreave, F. E. (1987) Late asthmatic responses. *The American review of respiratory disease* **136**: 740–751.

O'Byrne, P. M., Lamm, C. J., Busse, W. W., Tan, W. C. & Pedersen, S. (2009) The effects of inhaled budesonide on lung function in smokers and nonsmokers with mild persistent asthma. *Chest* **136**: 1514–1520.

O'Connor, C. M. & FitzGerald, M. X. (1994) Matrix metalloproteases and lung disease. *Thorax* **49**: 602–609.

O'Reilly, R., Ullmann, N., Irving, S., Bossley, C. J., Sonnappa, S., Zhu, J., Oates, T., Banya, W., Jeffery, P. K., Bush, A. & Saglani, S. (2012) Increased airway smooth muscle in preschool wheezers who have asthma at school age. *The Journal of allergy and clinical immunology*.

Oakley, R. H., Sar, M. & Cidlowski, J. A. (1996) The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. *The Journal of biological chemistry* **271**: 9550–9559.

Obata, K., Mukai, K., Tsujimura, Y., Ishiwata, K., Kawano, Y., Minegishi, Y., Watanabe, N. & Karasuyama, H. (2007) Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* **110**: 913–920.

Oboki, K., Ohno, T., Kajiwara, N., Arae, K., Morita, H., Ishii, A., Nambu, A., Abe, T., Kiyonari, H., Matsumoto, K., Sudo, K., Okumura, K., Saito, H. & Nakae, S. (2010) IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 18581–18586.

Oettgen, H. C., Martin, T. R., Wynshaw-Boris, A., Deng, C., Drazen, J. M. & Leder, P. (1994) Active anaphylaxis in IgE-deficient mice. *Nature* **370**: 367–370.

Ogirala, R. G., Aldrich, T. K., Prezant, D. J., Sinnett, M. J., Enden, J. B. & Williams, M. H., Jr (1991) High-dose intramuscular triamcinolone in severe, chronic, life-threatening asthma. *The New England journal of medicine* **324**: 585–589.

Ogurusu, K., Takahashi, K., Tada, S., Soda, R., Nanba, K., Takata, M., Nanba, Y., Kanehiro, A., Tanimoto, Y. & Kimura, G. (1991) [Studies on IgG subclass antibodies in adult asthma. 1. Serum antigen specific IgG subclass antibodies in asthmatics with late asthmatic response]. *Arerugī = [Allergy]* **40**: 506–515.

Oh, K., Shen, T., Le Gros, G. & Min, B. (2007) Induction of Th2 type immunity in a mouse system reveals a novel immunoregulatory role of basophils. *Blood* **109**: 2921–2927.

Okada, S., Inoue, H., Yamauchi, K., Iijima, H., Ohkawara, Y., Takishima, T. & Shirato, K. (1995) Potential role of interleukin-1 in allergen-induced late asthmatic reactions in guinea pigs: suppressive effect of interleukin-1 receptor antagonist on late asthmatic reaction. *The Journal of allergy and clinical immunology* **95**: 1236–1245.

Okayama, Y., Petit-Frére, C., Kassel, O., Semper, A., Quint, D., Tunon-de-Lara, M. J., Bradding, P., Holgate, S. T. & Church, M. K. (1995) IgE-dependent expression of mRNA for IL-4 and IL-5 in human lung mast cells. *Journal of immunology (Baltimore, Md.: 1950)* **155**: 1796–1808.

Okayama, Y., Tkaczyk, C., Metcalfe, D. D. & Gilfillan, A. M. (2003) Comparison of Fc epsilon RI- and Fc gamma RI-mediated degranulation and TNF-alpha synthesis in human mast cells: selective utilization of phosphatidylinositol-3-kinase for Fc gamma RI-induced degranulation. *European journal of immunology* **33**: 1450–1459.

Olivera, D., Knall, C., Boggs, S. & Seagrave, J. (2010) Cytoskeletal modulation and tyrosine phosphorylation of tight junction proteins are associated with mainstream cigarette smoke-induced permeability of airway epithelium. *Experimental and toxicologic pathology: official journal of the Gesellschaft für Toxikologische Pathologie* **62**: 133–143.

Olivera, D. S., Boggs, S. E., Beenhouwer, C., Aden, J. & Knall, C. (2007) Cellular mechanisms of mainstream cigarette smoke-induced lung epithelial tight junction permeability changes in vitro. *Inhalation toxicology* **19**: 13–22.

Olweus, J., BitMansour, A., Warnke, R., Thompson, P. A., Carballido, J., Picker, L. J. & Lund-Johansen, F. (1997) Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 12551–12556.

Omori, M. & Ziegler, S. (2007) Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. *Journal of immunology (Baltimore, Md.: 1950)* **178**: 1396–1404.

Oshiba, A., Hamelmann, E., Takeda, K., Bradley, K. L., Loader, J. E., Larsen, G. L. & Gelfand, E. W. (1996) Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *The Journal of clinical investigation* **97**: 1398–1408.

D' Ostiani, C. F., Del Sero, G., Bacci, A., Montagnoli, C., Spreca, A., Mencacci, A., Ricciardi-Castagnoli, P. & Romani, L. (2000) Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans. Implications for initiation of T

helper cell immunity in vitro and in vivo. *The Journal of experimental medicine* **191**: 1661–1674.

Ostroukhova, M., Seguin-Devaux, C., Oriss, T. B., Dixon-McCarthy, B., Yang, L., Ameredes, B. T., Corcoran, T. E. & Ray, A. (2004) Tolerance induced by inhaled antigen involves CD4+ T cells expressing membrane-bound TGF-β and FOXP3. *Journal of Clinical Investigation* **114**: 28–38.

Out, T. A., van de Graaf, E. A., van den Berg, N. J. & Jansen, H. M. (1991) IgG subclasses in bronchoalveolar lavage fluid from patients with asthma. *Scandinavian journal of immunology* **33**: 719–727.

Ouyang, W., Kolls, J. K. & Zheng, Y. (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* **28**: 454–467.

Paganin, F., Trussard, V., Seneterre, E., Chanez, P., Giron, J., Godard, P., Sénac, J. P., Michel, F. B. & Bousquet, J. (1992) Chest radiography and high resolution computed tomography of the lungs in asthma. *The American review of respiratory disease* **146**: 1084–1087.

Paggiaro, P. L., Dente, F. L., Morelli, M. C., Bancalari, L., Di Franco, A., Giannini, D., Vagaggini, B., Bacci, E., Fabbri, L. M. & Giuntini, C. (1994) Postallergen inhaled budesonide reduces late asthmatic response and inhibits the associated increase of airway responsiveness to methacholine in asthmatics. *American journal of respiratory and critical care medicine* **149**: 1447–1451.

Palframan, R. T., Collins, P. D., Severs, N. J., Rothery, S., Williams, T. J. & Rankin, S. M. (1998) Mechanisms of acute eosinophil mobilization from the bone marrow stimulated by interleukin 5: the role of specific adhesion molecules and phosphatidylinositol 3-kinase. *The Journal of experimental medicine* **188**: 1621–1632.

Palmans, E., Kips, J. C. & Pauwels, R. A. (2000) Prolonged allergen exposure induces structural airway changes in sensitized rats. *American journal of respiratory and critical care medicine* **161**: 627–635.

Palmer, C. N. A., Irvine, A. D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S. P., Goudie, D. R., Sandilands, A., Campbell, L. E., Smith, F. J. D., O'Regan, G. M., Watson, R. M., Cecil, J. E., Bale, S. J., Compton, J. G., DiGiovanna, J. J., Fleckman, P., Lewis-Jones, S., Arseculeratne, G., Sergeant, A., Munro, C. S., El Houate, B., McElreavey, K., Halkjaer, L. B., Bisgaard, H., Mukhopadhyay, S. & McLean, W. H. I. (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nature genetics* **38**: 441–446.

Palmer, L. J., Rye, P. J., Gibson, N. A., Burton, P. R., Landau, L. I. & Lesouëf, P. N. (2001) Airway responsiveness in early infancy predicts asthma, lung function, and respiratory symptoms by school age. *American Journal of Respiratory and Critical Care Medicine* **163**: 37–42.

Pang, L. & Knox, A. J. (2001) Regulation of TNF-alpha-induced eotaxin release from cultured human airway smooth muscle cells by beta2-agonists and corticosteroids. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* **15**: 261–269.

Partridge, M. R., van der Molen, T., Myrseth, S.-E. & Busse, W. W. (2006) Attitudes and actions of asthma patients on regular maintenance therapy: the INSPIRE study. *BMC Pulmonary Medicine* **6**: 13.

Pascual, G. & Glass, C. K. (2006) Nuclear receptors versus inflammation: mechanisms of transrepression. *Trends in endocrinology and metabolism: TEM* **17**: 321–327.

Patel, H. J., Barnes, P. J., Takahashi, T., Tadjkarimi, S., Yacoub, M. H. & Belvisi, M. G. (1995) Evidence for prejunctional muscarinic autoreceptors in human and guinea pig trachea. *American journal of respiratory and critical care medicine* **152**: 872–878.

Patel, M. M., Quinn, J. W., Jung, K. H., Hoepner, L., Diaz, D., Perzanowski, M., Rundle, A., Kinney, P. L., Perera, F. P. & Miller, R. L. (2011) Traffic density and stationary sources of air pollution associated with wheeze, asthma, and immunoglobulin E from birth to age 5 years among New York City children. *Environmental research* **111**: 1222–1229.

Patel, S. N., Tsai, C.-L., Boudreaux, E. D., Kilgannon, J. H., Sullivan, A. F., Blumenthal, D. & Camargo, C. A., Jr (2009) Multicenter study of cigarette smoking among patients presenting to the emergency department with acute asthma. *Annals of allergy, asthma & immunology: official publication of the American College of Allergy, Asthma, & Immunology* **103**: 121–127.

Pauwels, R. A., Löfdahl, C. G., Laitinen, L. A., Schouten, J. P., Postma, D. S., Pride, N. B. & Ohlsson, S. V. (1999) Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pulmonary disease who continue smoking. European Respiratory Society Study on Chronic Obstructive Pulmonary Disease. *The New England journal of medicine* **340**: 1948–1953.

Pavord, I. D., Brightling, C. E., Woltmann, G. & Wardlaw, A. J. (1999) Non-eosinophilic corticosteroid unresponsive asthma. *Lancet* **353**: 2213–2214.

Pearce, N., Pekkanen, J. & Beasley, R. (1999) How much asthma is really attributable to atopy? *Thorax* **54**: 268–272.

Peat, J. K., Woolcock, A. J. & Cullen, K. (1987) Rate of decline of lung function in subjects with asthma. *European journal of respiratory diseases* **70**: 171–179.

Pedersen, B., Dahl, R., Larsen, B. B. & Venge, P. (1993) The effect of salmeterol on the early- and late-phase reaction to bronchial allergen and postchallenge variation in bronchial reactivity, blood eosinophils, serum eosinophil cationic protein, and serum eosinophil protein X. *Allergy* **48**: 377–382.

Pedersen, B., Dahl, R., Karlström, R., Peterson, C. G. & Venge, P. (1996) Eosinophil and neutrophil activity in asthma in a one-year trial with inhaled budesonide. The impact of smoking. *American journal of respiratory and critical care medicine* **153**: 1519–1529.

Pedersen, S. E., Bateman, E. D., Bousquet, J., Busse, W. W., Yoxall, S. & Clark, T. J. (2007) Determinants of response to fluticasone propionate and salmeterol/fluticasone propionate combination in the Gaining Optimal Asthma controL study. *The Journal of allergy and clinical immunology* **120**: 1036–1042.

Peebles, R. S., Jr, Hamilton, R. G., Lichtenstein, L. M., Schlosberg, M., Liu, M. C., Proud, D. & Togias, A. (2001) Antigen-specific IgE and IgA antibodies in bronchoalveolar

lavage fluid are associated with stronger antigen-induced late phase reactions. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **31**: 239–248.

Peebles, R. S., Jr, Hashimoto, K., Morrow, J. D., Dworski, R., Collins, R. D., Hashimoto, Y., Christman, J. W., Kang, K.-H., Jarzecka, K., Furlong, J., Mitchell, D. B., Talati, M., Graham, B. S. & Sheller, J. R. (2002) Selective cyclooxygenase-1 and -2 inhibitors each increase allergic inflammation and airway hyperresponsiveness in mice. *American journal of respiratory and critical care medicine* **165**: 1154–1160.

Pelikan, Z. & Pelikan-Filipek, M. (1986*a*) The late asthmatic response to allergen challenge--Part I. *Annals of allergy* **56**: 414–420.

Pelikan, Z. & Pelikan-Filipek, M. (1986*b*) The late asthmatic response to allergen challenge--Part II. *Annals of allergy* **56**: 421–435.

Pera, T., Gosens, R., Lesterhuis, A. H., Sami, R., Toorn, M. van der, Zaagsma, J. & Meurs, H. (2010) Cigarette smoke and lipopolysaccharide induce a proliferative airway smooth muscle phenotype. *Respiratory research* **11**: 48.

Perera, F. P., Tang, D., Tu, Y.-H., Cruz, L. A., Borjas, M., Bernert, T. & Whyatt, R. M. (2004) Biomarkers in maternal and newborn blood indicate heightened fetal susceptibility to procarcinogenic DNA damage. *Environmental health perspectives* **112**: 1133–1136.

Perrigoue, J. G., Saenz, S. A., Siracusa, M. C., Allenspach, E. J., Taylor, B. C., Giacomin, P. R., Nair, M. G., Du, Y., Zaph, C., Rooijen, N. van, Comeau, M. R., Pearce, E. J., Laufer, T. M. & Artis, D. (2009) MHC class II–dependent basophil–CD4+ T cell interactions promote TH2 cytokine–dependent immunity. *Nature Immunology* **10**: 697–705.

Peters, J. L., Suglia, S. F., Platts-Mills, T. A. E., Hosen, J., Gold, D. R. & Wright, R. J. (2009) Relationships among Prenatal Aeroallergen Exposure, Maternal and Cord Blood Immunoglobulin E: Project ACCESS. *The Journal of allergy and clinical immunology* **123**: 1041–1046.

Peters, J. L., Cohen, S., Staudenmayer, J., Hosen, J., Platts-Mills, T. A. E. & Wright, R. J. (2012) Prenatal negative life events increases cord blood IgE: interactions with dust mite allergen and maternal atopy. *Allergy* **67**: 545–551.

Peters, M., Kauth, M., Schwarze, J., Körner-Rettberg, C., Riedler, J., Nowak, D., Braun-Fahrländer, C., Mutius, E. von, Bufe, A. & Holst, O. (2006) Inhalation of stable dust extract prevents allergen induced airway inflammation and hyperresponsiveness. *Thorax* **61**: 134–139.

Phelan, P. D., Robertson, C. F. & Olinsky, A. (2002) The Melbourne Asthma Study: 1964-1999. *The Journal of allergy and clinical immunology* **109**: 189–194.

Phillips, C., Coward, W. R., Pritchard, D. I. & Hewitt, C. R. A. (2003) Basophils express a type 2 cytokine profile on exposure to proteases from helminths and house dust mites. *Journal of leukocyte biology* **73**: 165–171.

Phipps, S., Lam, C. E., Kaiko, G. E., Foo, S. Y., Collison, A., Mattes, J., Barry, J., Davidson, S., Oreo, K., Smith, L., Mansell, A., Matthaei, K. I. & Foster, P. S. (2009) Toll/IL-1 signaling is critical for house dust mite-specific helper T cell type 2 and type 17

[corrected] responses. *American Journal of Respiratory and Critical Care Medicine* **179**: 883–893.

Piggott, D. A., Eisenbarth, S. C., Xu, L., Constant, S. L., Huleatt, J. W., Herrick, C. A. & Bottomly, K. (2005) MyD88-dependent induction of allergic Th2 responses to intranasal antigen. *The Journal of clinical investigation* **115**: 459–467.

Piipari, R., Jaakkola, J. J. K., Jaakkola, N. & Jaakkola, M. S. (2004) Smoking and asthma in adults. *European Respiratory Journal* **24**: 734 –739.

Piitulainen, E. & Sveger, T. (2002) Respiratory symptoms and lung function in young adults with severe alpha(1)-antitrypsin deficiency (PiZZ). *Thorax* **57**: 705–708.

Plaschke, P. P., Janson, C., Norrman, E., Björnsson, E., Ellbjär, S. & Järvholm, B. (2000) Onset and remission of allergic rhinitis and asthma and the relationship with atopic sensitization and smoking. *American journal of respiratory and critical care medicine* **162**: 920–924.

Platts-Mills, T., Vaughan, J., Squillace, S., Woodfolk, J. & Sporik, R. (2001) Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. *Lancet* **357**: 752–756.

Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J., Nordan, R. P. & Paul, W. E. (1989) Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores. *Nature* **339**: 64–67.

Pliss, L. B., Ingenito, E. P. & Ingram, R. H., Jr (1989) Responsiveness, inflammation, and effects of deep breaths on obstruction in mild asthma. *Journal of applied physiology (Bethesda, Md.: 1985)* **66**: 2298–2304.

Polosa, R. & Thomson, N. C. (2013) Smoking and asthma: dangerous liaisons. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **41**: 716–726.

Polosa, R., Knoke, J. D., Russo, C., Piccillo, G., Caponnetto, P., Sarvà, M., Proietti, L. & Al-Delaimy, W. K. (2008) Cigarette smoking is associated with a greater risk of incident asthma in allergic rhinitis. *The Journal of allergy and clinical immunology* **121**: 1428–1434.

Polosa, R., Russo, C., Caponnetto, P., Bertino, G., Sarvà, M., Antic, T., Mancuso, S. & Al-Delaimy, W. K. (2011) Greater severity of new onset asthma in allergic subjects who smoke: a 10-year longitudinal study. *Respiratory research* **12**: 16.

Possa, S. S., Charafeddine, H. T., Righetti, R. F., Silva, P. A. da, Almeida-Reis, R., Saraiva-Romanholo, B. M., Perini, A., Prado, C. M., Leick-Maldonado, E. A., Martins, M. A. & Tibério, I. de F. L. C. (2012) Rho-kinase inhibition attenuates airway responsiveness, inflammation, matrix remodeling, and oxidative stress activation induced by chronic inflammation. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **303**: L939–L952.

Post, S., Nawijn, M. C., Hackett, T. L., Baranowska, M., Gras, R., van Oosterhout, A. J. M. & Heijink, I. H. (2012) The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax* **67**: 488–495.

Van der Pouw Kraan, T. C., Boeije, L. C., de Groot, E. R., Stapel, S. O., Snijders, A., Kapsenberg, M. L., van der Zee, J. S. & Aarden, L. A. (1997) Reduced production of IL-12 and IL-12-dependent IFN-gamma release in patients with allergic asthma. *Journal of immunology (Baltimore, Md.: 1950)* **158**: 5560–5565.

Prause, O., Bozinovski, S., Anderson, G. P. & Lindén, A. (2004) Increased matrix metalloproteinase-9 concentration and activity after stimulation with interleukin-17 in mouse airways. *Thorax* **59**: 313–317.

Préfontaine, D., Lajoie-Kadoch, S., Foley, S., Audusseau, S., Olivenstein, R., Halayko, A. J., Lemière, C., Martin, J. G. & Hamid, Q. (2009) Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *Journal of immunology (Baltimore, Md.: 1950)* **183**: 5094–5103.

Préfontaine, D., Nadigel, J., Chouiali, F., Audusseau, S., Semlali, A., Chakir, J., Martin, J. G. & Hamid, Q. (2010) Increased IL-33 expression by epithelial cells in bronchial asthma. *The Journal of allergy and clinical immunology* **125**: 752–754.

Prescott, S. L., Macaubas, C., Holt, B. J., Smallacombe, T. B., Loh, R., Sly, P. D. & Holt, P. G. (1998*a*) Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *Journal of immunology (Baltimore, Md.: 1950)* **160**: 4730–4737.

Prescott, S. L., Macaubas, C., Smallacombe, T., Holt, B. J., Sly, P. D., Loh, R. & Holt, P. G. (1998*b*) Reciprocal age-related patterns of allergen-specific T-cell immunity in normal vs. atopic infants. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **28 Suppl 5**: 39–44; discussion 50–51.

Price, A. E., Liang, H.-E., Sullivan, B. M., Reinhardt, R. L., Eisley, C. J., Erle, D. J. & Locksley, R. M. (2010) Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 11489–11494.

Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society (2000) *American journal of respiratory and critical care medicine* **162**: 2341–2351.

Proud, D., Bailey, G. S., Naclerio, R. M., Reynolds, C. J., Cruz, A. A., Eggleston, P. A., Lichtenstein, L. M. & Togias, A. G. (1992) Tryptase and histamine as markers to evaluate mast cell activation during the responses to nasal challenge with allergen, cold, dry air, and hyperosmolar solutions. *The Journal of allergy and clinical immunology* **89**: 1098–1110.

Pryor, W. A. & Stone, K. (1993) Oxidants in Cigarette Smoke Radicals, Hydrogen Peroxide, Peroxynitrate, and Peroxynitritea. *Annals of the New York Academy of Sciences* **686**: 12–27.

Pulendran, B. (2004) Modulating TH1/TH2 responses with microbes, dendritic cells, and pathogen recognition receptors. *Immunologic research* **29**: 187–196.

Pulendran, B., Smith, J. L., Caspary, G., Brasel, K., Pettit, D., Maraskovsky, E. & Maliszewski, C. R. (1999) Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 1036–1041.

Rabe, K. F., Vermeire, P. A., Soriano, J. B. & Maier, W. C. (2000) Clinical management of asthma in 1999: the Asthma Insights and Reality in Europe (AIRE) study. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **16**: 802–807.

Rabe, K. F., Adachi, M., Lai, C. K. W., Soriano, J. B., Vermeire, P. A., Weiss, K. B. & Weiss, S. T. (2004) Worldwide severity and control of asthma in children and adults: the global asthma insights and reality surveys. *The Journal of allergy and clinical immunology* **114**: 40–47.

Raemdonck, K., de Alba, J., Birrell, M. A., Grace, M., Maher, S. A., Irvin, C. G., Fozard, J. R., O'Byrne, P. M. & Belvisi, M. G. (2012) A role for sensory nerves in the late asthmatic response. *Thorax* **67**: 19–25.

Rahman, I., Biswas, S. K. & Kode, A. (2006) Oxidant and antioxidant balance in the airways and airway diseases. *European Journal of Pharmacology* **533**: 222–239.

Rankin, J. A., Hitchcock, M., Merrill, W., Bach, M. K., Brashler, J. R. & Askenase, P. W. (1982) IgE-dependent release of leukotriene C4 from alveolar macrophages. *Nature* **297**: 329–331.

Rankin, J. A., Hitchcock, M., Merrill, W. W., Huang, S. S., Brashler, J. R., Bach, M. K. & Askenase, P. W. (1984) IgE immune complexes induce immediate and prolonged release of leukotriene C4 (LTC4) from rat alveolar macrophages. *Journal of immunology (Baltimore, Md.: 1950)* **132**: 1993–1999.

Rastrick, J. M. D., Stevenson, C. S., Eltom, S., Grace, M., Davies, M., Kilty, I., Evans, S. M., Pasparakis, M., Catley, M. C., Lawrence, T., Adcock, I. M., Belvisi, M. G. & Birrell, M. A. (2013) Cigarette Smoke Induced Airway Inflammation Is Independent of NF-?B Signalling. *PLoS ONE* **8**.

Ray, A. & Prefontaine, K. E. (1994) Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 752–756.

Rea, H. H., Scragg, R., Jackson, R., Beaglehole, R., Fenwick, J. & Sutherland, D. C. (1986) A case-control study of deaths from asthma. *Thorax* **41**: 833–839.

Rédier, H., Chanez, P., De Vos, C., Rifaï, N., Clauzel, A. M., Michel, F. B. & Godard, P. (1992) Inhibitory effect of cetirizine on the bronchial eosinophil recruitment induced by allergen inhalation challenge in allergic patients with asthma. *The Journal of allergy and clinical immunology* **90**: 215–224.

Redington, A. E. & Howarth, P. H. (1997) Airway wall remodelling in asthma. *Thorax* **52**: 310–312.

Reider, N., Reider, D., Ebner, S., Holzmann, S., Herold, M., Fritsch, P. & Romani, N. (2002) Dendritic cells contribute to the development of atopy by an insufficiency in IL-12 production. *The Journal of allergy and clinical immunology* **109**: 89–95.

Van Rensen, E. L. J., Sont, J. K., Evertse, C. E., Willems, L. N. A., Mauad, T., Hiemstra, P. S., Sterk, P. J. & AMPUL Study Group (2005) Bronchial CD8 cell infiltrate and lung function decline in asthma. *American journal of respiratory and critical care medicine* **172**: 837–841.

Renz, H. & Herz, U. (2002) The bidirectional capacity of bacterial antigens to modulate allergy and asthma. *The European respiratory journal* **19**: 158–171.

Renz, H., Smith, H. R., Henson, J. E., Ray, B. S., Irvin, C. G. & Gelfand, E. W. (1992) Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *The Journal of allergy and clinical immunology* **89**: 1127–1138.

Renz, H., Bradley, K., Saloga, J., Loader, J., Larsen, G. L. & Gelfand, E. W. (1993) T cells expressing specific V beta elements regulate immunoglobulin E production and airways responsiveness in vivo. *The Journal of experimental medicine* **177**: 1175–1180.

Ricci, G., Patrizi, A., Baldi, E., Menna, G., Tabanelli, M. & Masi, M. (2006) Long-term follow-up of atopic dermatitis: retrospective analysis of related risk factors and association with concomitant allergic diseases. *Journal of the American Academy of Dermatology* **55**: 765–771.

Riedler, J., Braun-Fahrländer, C., Eder, W., Schreuer, M., Waser, M., Maisch, S., Carr, D., Schierl, R., Nowak, D. & von Mutius, E. (2001) Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *The Lancet* **358**: 1129–1133.

Van Rijt, L. S., Jung, S., Kleinjan, A., Vos, N., Willart, M., Duez, C., Hoogsteden, H. C. & Lambrecht, B. N. (2005) In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *The Journal of experimental medicine* **201**: 981–991.

Van Rijt, L. S., Vos, N., Willart, M., Muskens, F., Tak, P. P., van der Horst, C., Hoogsteden, H. C. & Lambrecht, B. N. (2011) Persistent activation of dendritic cells after resolution of allergic airway inflammation breaks tolerance to inhaled allergens in mice. *American journal of respiratory and critical care medicine* **184**: 303–311.

Robbins, C. S., Pouladi, M. A., Fattouh, R., Dawe, D. E., Vujicic, N., Richards, C. D., Jordana, M., Inman, M. D. & Stampfli, M. R. (2005) Mainstream Cigarette Smoke Exposure Attenuates Airway Immune Inflammatory Responses to Surrogate and Common Environmental Allergens in Mice, Despite Evidence of Increased Systemic Sensitization. *J Immunol* **175**: 2834–2842.

Robertson, D. G., Kerigan, A. T., Hargreave, F. E., Chalmers, R. & Dolovich, J. (1974) Late asthmatic responses induced by ragweed pollen allergen. *The Journal of allergy and clinical immunology* **54**: 244–254.

Robinson, D., Hamid, Q., Bentley, A., Ying, S., Kay, A. B. & Durham, S. R. (1993*a*) Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *Journal of Allergy and Clinical Immunology* **92**: 313–324.

Robinson, D., Hamid, Q., Ying, S., Bentley, A., Assoufi, B., Durham, S. & Kay, A. B. (1993*b*) Prednisolone treatment in asthma is associated with modulation of bronchoalveolar lavage cell interleukin-4, interleukin-5, and interferon-gamma cytokine gene expression. *The American review of respiratory disease* **148**: 401–406.

Robinson, D. S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A. M., Corrigan, C., Durham, S. R. & Kay, A. B. (1992) Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *The New England Journal of Medicine* **326**: 298–304.

Robinson, D. S., Campbell, D. A., Durham, S. R., Pfeffer, J., Barnes, P. J. & Chung, K. F. (2003) Systematic assessment of difficult-to-treat asthma. *European Respiratory Journal* **22**: 478–483.

Rosenwasser, L. J. (2011) Mechanisms of IgE Inflammation. *Current allergy and asthma reports* **11**: 178–183.

Rossi, G. A., Crimi, E., Lantero, S., Gianiorio, P., Oddera, S., Crimi, P. & Brusasco, V. (1991) Late-phase asthmatic reaction to inhaled allergen is associated with early recruitment of eosinophils in the airways. *The American review of respiratory disease* **144**: 379–383.

Roth, M. D., Arora, A., Barsky, S. H., Kleerup, E. C., Simmons, M. & Tashkin, D. P. (1998) Airway inflammation in young marijuana and tobacco smokers. *American journal of respiratory and critical care medicine* **157**: 928–937.

Rothenberg, M. E., MacLean, J. A., Pearlman, E., Luster, A. D. & Leder, P. (1997) Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *The Journal of experimental medicine* **185**: 785–790.

Ruck, L. M., Rizzo, C. A., Anthes, J. C., Eckel, S., Egan, R. W., Cuss, F. M. & Hey, J. A. (2001) Synergistic antiallergic activity of combined histamine H1- and cysteinyl leukotriene1-receptor blockade in human bronchus. *Life sciences* **68**: 2825–2834.

Rumold, R., Jyrala, M. & Diaz-Sanchez, D. (2001) Secondhand smoke induces allergic sensitization in mice. *Journal of immunology (Baltimore, Md.: 1950)* **167**: 4765–4770.

Rusznak, C., Sapsford, R. J., Devalia, J. L., Justin John, R., Hewitt, E. L., Lamont, A. G., Wood, A. J., Shah, S. S., Davies, R. J. & Lozewicz, S. (1999) Cigarette smoke potentiates house dust mite allergen-induced increase in the permeability of human bronchial epithelial cells in vitro. *American journal of respiratory cell and molecular biology* **20**: 1238–1250.

Saenz, S. A., Siracusa, M. C., Perrigoue, J. G., Spencer, S. P., Urban, J. F., Jr, Tocker, J. E., Budelsky, A. L., Kleinschek, M. A., Kastelein, R. A., Kambayashi, T., Bhandoola, A. & Artis, D. (2010) IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* **464**: 1362–1366.

Saetta, M., Di Stefano, A., Rosina, C., Thiene, G. & Fabbri, L. M. (1991) Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. *The American review of respiratory disease* **143**: 138–143.

Saetta, M., Di Stefano, A., Maestrelli, P., Ferraresso, A., Drigo, R., Potena, A., Ciaccia, A. & Fabbri, L. M. (1993) Activated T-lymphocytes and macrophages in bronchial mucosa of subjects with chronic bronchitis. *The American review of respiratory disease* **147**: 301–306.

Saglani, S., Payne, D. N., Zhu, J., Wang, Z., Nicholson, A. G., Bush, A. & Jeffery, P. K. (2007) Early detection of airway wall remodeling and eosinophilic inflammation in

preschool wheezers. *American journal of respiratory and critical care medicine* **176**: 858–864.

Sakai, K., Yokoyama, A., Kohno, N. & Hiwada, K. (1999) Effect of different sensitizing doses of antigen in a murine model of atopic asthma. *Clinical and Experimental Immunology* **118**: 9–15.

Sakai, K., Yokoyama, A., Kohno, N., Hamada, H. & Hiwada, K. (2001) Prolonged antigen exposure ameliorates airway inflammation but not remodeling in a mouse model of bronchial asthma. *International archives of allergy and immunology* **126**: 126–134.

Salam, M. T., Islam, T. & Gilliland, F. D. (2008) Recent evidence for adverse effects of residential proximity to traffic sources on asthma. *Current opinion in pulmonary medicine* **14**: 3–8.

Salek-Ardakani, S., Song, J., Halteman, B. S., Jember, A. G.-H., Akiba, H., Yagita, H. & Croft, M. (2003) OX40 (CD134) controls memory T helper 2 cells that drive lung inflammation. *The Journal of experimental medicine* **198**: 315–324.

Saloga, J., Renz, H., Lack, G., Bradley, K. L., Greenstein, J. L., Larsen, G. & Gelfand, E. W. (1993) Development and transfer of immediate cutaneous hypersensitivity in mice exposed to aerosolized antigen. *The Journal of clinical investigation* **91**: 133–140.

Sanderson, M. J. (2011) Exploring lung physiology in health and disease with lung slices. *Pulmonary pharmacology & therapeutics* **24**: 452–465.

Sandford, A., Weir, T. & Paré, P. (1996) The genetics of asthma. *American journal of respiratory and critical care medicine* **153**: 1749–1765.

Sandford, A. J., Chagani, T., Weir, T. D., Connett, J. E., Anthonisen, N. R. & Paré, P. D. (2001) Susceptibility genes for rapid decline of lung function in the lung health study. *American journal of respiratory and critical care medicine* **163**: 469–473.

Sathish, V., Vanoosten, S. K., Miller, B. S., Aravamudan, B., Thompspon, M. A., Pabelick, C. M., Vassallo, R. & Prakash, Y. S. (2012) Brain-Derived Neurotrophic Factor in Cigarette Smoke-Induced Airway Hyperreactivity. *American journal of respiratory cell and molecular biology*.

Sauty, A., Mauel, J., Philippeaux, M. M. & Leuenberger, P. (1994) Cytostatic activity of alveolar macrophages from smokers and nonsmokers: role of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha. *American journal of respiratory cell and molecular biology* **11**: 631–637.

Scheinman, R. I., Cogswell, P. C., Lofquist, A. K. & Baldwin, A. S., Jr (1995) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science (New York, N.Y.)* **270**: 283–286.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., Gorman, D. M., Bazan, J. F. & Kastelein, R. A. (2005) IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23**: 479–490.

Schnyder-Candrian, S., Togbe, D., Couillin, I., Mercier, I., Brombacher, F., Quesniaux, V., Fossiez, F., Ryffel, B. & Schnyder, B. (2006) Interleukin-17 is a negative regulator

of established allergic asthma. *The Journal of experimental medicine* **203**: 2715–2725.

Schönberger, H. J., Dompeling, E., Knottnerus, J. A., Kuiper, S., van Weel, C. & Schayck, C. P. (2005) Prenatal exposure to mite and pet allergens and total serum IgE at birth in high-risk children. *Pediatric allergy and immunology: official publication of the European Society of Pediatric Allergy and Immunology* **16**: 27–31.

Schram-Bijkerk, D., Doekes, G., Boeve, M., Douwes, J., Riedler, J., Ublagger, E., von Mutius, E., Budde, J., Pershagen, G., van Hage, M., Wickman, M., Braun-Fahrländer, C., Waser, M. & Brunekreef, B. (2006) Nonlinear relations between house dust mite allergen levels and mite sensitization in farm and nonfarm children. *Allergy* **61**: 640–647.

Schramm, C., Herz, U., Podlech, J., Protschka, M., Finotto, S., Reddehase, M. J., Köhler, H., Galle, P. R., Lohse, A. W. & Blessing, M. (2003) TGF-beta regulates airway responses via T cells. *Journal of immunology (Baltimore, Md.: 1950)* **170**: 1313–1319.

Schramm, C. M., Puddington, L., Wu, C., Guernsey, L., Gharaee-Kermani, M., Phan, S. H. & Thrall, R. S. (2004) Chronic Inhaled Ovalbumin Exposure Induces Antigen-Dependent but Not Antigen-Specific Inhalational Tolerance in a Murine Model of Allergic Airway Disease. *The American Journal of Pathology* **164**: 295–304.

Schroeder, J. T., MacGlashan, D. W., Jr, Kagey-Sobotka, A., White, J. M. & Lichtenstein, L. M. (1994) IgE-dependent IL-4 secretion by human basophils. The relationship between cytokine production and histamine release in mixed leukocyte cultures. *Journal of immunology (Baltimore, Md.: 1950)* **153**: 1808–1817.

Schüle, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M. & Evans, R. M. (1990) Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* **62**: 1217–1226.

Schulz, O., Laing, P., Sewell, H. F. & Shakib, F. (1995) Der p I, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23). *European journal of immunology* **25**: 3191–3194.

Schulz, O., Sutton, B. J., Beavil, R. L., Shi, J., Sewell, H. F., Gould, H. J., Laing, P. & Shakib, F. (1997) Cleavage of the low-affinity receptor for human IgE (CD23) by a mite cysteine protease: nature of the cleaved fragment in relation to the structure and function of CD23. *European journal of immunology* **27**: 584–588.

Schulz, O., Sewell, H. F. & Shakib, F. (1998) Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *The Journal of experimental medicine* **187**: 271–275.

Schwartz, H. J., Lowell, F. C. & Melby, J. C. (1968) Steroid resistance in bronchial asthma. *Annals of internal medicine* **69**: 493–499.

Seder, R. A., Paul, W. E., Dvorak, A. M., Sharkis, S. J., Kagey-Sobotka, A., Niv, Y., Finkelman, F. D., Barbieri, S. A., Galli, S. J. & Plaut, M. (1991) Mouse splenic and bone marrow cell populations that express high-affinity Fc epsilon receptors and produce interleukin 4 are highly enriched in basophils. *Proceedings of the National Academy of Sciences* **88**: 2835–2839.

Sehmi, R., Wood, L. J., Watson, R., Foley, R., Hamid, Q., O'Byrne, P. M. & Denburg, J. A. (1997) Allergen-induced increases in IL-5 receptor alpha-subunit expression on bone marrow-derived CD34+ cells from asthmatic subjects. A novel marker of progenitor cell commitment towards eosinophilic differentiation. *The Journal of clinical investigation* **100**: 2466–2475.

Serra-Batlles, J., Plaza, V., Morejón, E., Comella, A. & Brugués, J. (1998) Costs of asthma according to the degree of severity. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **12**: 1322–1326.

Seubert, A., Monaci, E., Pizza, M., O'Hagan, D. T. & Wack, A. (2008) The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. *Journal of immunology (Baltimore, Md.: 1950)* **180**: 5402–5412.

Seymour, B. W., Pinkerton, K. E., Friebertshauser, K. E., Coffman, R. L. & Gershwin, L. J. (1997) Second-hand smoke is an adjuvant for T helper-2 responses in a murine model of allergy. *Journal of immunology (Baltimore, Md.: 1950)* **159**: 6169–6175.

Shahana, S., Björnsson, E., Lúdvíksdóttir, D., Janson, C., Nettelbladt, O., Venge, P. & Roomans, G. M. (2005) Ultrastructure of bronchial biopsies from patients with allergic and non-allergic asthma. *Respiratory medicine* **99**: 429–443.

Shaheen, S. O., Aaby, P., Hall, A. J., Barker, D. J., Heyes, C. B., Shiell, A. W. & Goudiaby, A. (1996) Measles and atopy in Guinea-Bissau. *Lancet* **347**: 1792–1796.

Shakib, F., Schulz, O. & Sewell, H. (1998) A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunology today* **19**: 313–316.

Sharkhuu, T., Matthaei, K. I., Forbes, E., Mahalingam, S., Hogan, S. P., Hansbro, P. M. & Foster, P. S. (2006) Mechanism of interleukin-25 (IL-17E)-induced pulmonary inflammation and airways hyper-reactivity. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **36**: 1575–1583.

Sharp, F. A., Ruane, D., Claass, B., Creagh, E., Harris, J., Malyala, P., Singh, M., O'Hagan, D. T., Pétrilli, V., Tschopp, J., O'Neill, L. A. J. & Lavelle, E. C. (2009) Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 870–875.

Shavit, O., Swern, A., Dong, Q., Newcomb, K., Sazonov Kocevar, V. & Taylor, S. D. (2007) Impact of smoking on asthma symptoms, healthcare resource use, and quality of life outcomes in adults with persistent asthma. *Quality of life research: an international journal of quality of life aspects of treatment, care and rehabilitation* **16**: 1555–1565.

Shaw, D. E., Berry, M. A., Hargadon, B., McKenna, S., Shelley, M. J., Green, R. H., Brightling, C. E., Wardlaw, A. J. & Pavord, I. D. (2007) Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest* **132**: 1871–1875.

Sheller, J. R., Mitchell, D., Meyrick, B., Oates, J. & Breyer, R. (2000) EP2 receptor mediates bronchodilation by PGE2 in mice. *J Appl Physiol* **88**: 2214–2218.

Shen, H. & Wang, S. (2005) [Effects of budesonide on airway inflammation and airway remodeling in the ovalbumin sensitized and challenged mice]. *Zhonghua jie he he hu xi za zhi = Zhonghua jiehe he huxi zazhi = Chinese journal of tuberculosis and respiratory diseases* **28**: 154–159.

Shen, H., O'Byrne, P. M., Ellis, R., Wattie, J., Tang, C. & Inman, M. D. (2002) The effects of intranasal budesonide on allergen-induced production of interleukin-5 and eotaxin, airways, blood, and bone marrow eosinophilia, and eosinophil progenitor expansion in sensitized mice. *American journal of respiratory and critical care medicine* **166**: 146–153.

Shen, H. H., Ochkur, S. I., McGarry, M. P., Crosby, J. R., Hines, E. M., Borchers, M. T., Wang, H., Biechelle, T. L., O'Neill, K. R., Ansay, T. L., Colbert, D. C., Cormier, S. A., Justice, J. P., Lee, N. A. & Lee, J. J. (2003) A causative relationship exists between eosinophils and the development of allergic pulmonary pathologies in the mouse. *Journal of immunology (Baltimore, Md.: 1950)* **170**: 3296–3305.

Sher, A., Coffman, R. L., Hieny, S. & Cheever, A. W. (1990) Ablation of eosinophil and IgE responses with anti-IL-5 or anti-IL-4 antibodies fails to affect immunity against Schistosoma mansoni in the mouse. *Journal of immunology (Baltimore, Md.: 1950)* **145**: 3911–3916.

Sher, E. R., Leung, D. Y., Surs, W., Kam, J. C., Zieg, G., Kamada, A. K. & Szefler, S. J. (1994) Steroid-resistant asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. *The Journal of clinical investigation* **93**: 33–39.

Sherr, E., Macy, E., Kimata, H., Gilly, M. & Saxon, A. (1989) Binding the low affinity Fc epsilon R on B cells suppresses ongoing human IgE synthesis. *Journal of immunology (Baltimore, Md.: 1950)* **142**: 481–489.

Shi, H. Z., Xiao, C. Q., Zhong, D., Qin, S. M., Liu, Y., Liang, G. R., Xu, H., Chen, Y. Q., Long, X. M. & Xie, Z. F. (1998) Effect of inhaled interleukin-5 on airway hyperreactivity and eosinophilia in asthmatics. *American journal of respiratory and critical care medicine* **157**: 204–209.

Shi, L., Leu, S.-W., Xu, F., Zhou, X., Yin, H., Cai, L. & Zhang, L. (2008) Local blockade of TSLP receptor alleviated allergic disease by regulating airway dendritic cells. *Clinical immunology (Orlando, Fla.)* **129**: 202–210.

Shi, Y., Evans, J. E. & Rock, K. L. (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**: 516–521.

Shikotra, A., Choy, D. F., Ohri, C. M., Doran, E., Butler, C., Hargadon, B., Shelley, M., Abbas, A. R., Austin, C. D., Jackman, J., Wu, L. C., Heaney, L. G., Arron, J. R. & Bradding, P. (2012) Increased expression of immunoreactive thymic stromal lymphopoietin in patients with severe asthma. *The Journal of allergy and clinical immunology* **129**: 104–111.e1–9.

Shirakawa, T., Enomoto, T., Shimazu, S. & Hopkin, J. M. (1997) The inverse association between tuberculin responses and atopic disorder. *Science (New York, N.Y.)* **275**: 77–79.

Siddiqui, S., Mistry, V., Doe, C., Roach, K., Morgan, A., Wardlaw, A., Pavord, I., Bradding, P. & Brightling, C. (2008) Airway hyperresponsiveness is dissociated from airway

wall structural remodeling. *The Journal of allergy and clinical immunology* **122**: 335–341, 341.e1–3.

Sigsgaard, T., Brandslund, I., Omland, O., Hjort, C., Lund, E. D., Pedersen, O. F. & Miller, M. R. (2000) S and Z alpha1-antitrypsin alleles are risk factors for bronchial hyperresponsiveness in young farmers: an example of gene/environment interaction. *The European respiratory journal* **16**: 50–55.

Simon, S. A. & Liedtke, W. (2008) How irritating: the role of TRPA1 in sensing cigarette smoke and aerogenic oxidants in the airways. *The Journal of clinical investigation* **118**: 2383–2386.

Singh, D., Richards, D., Knowles, R. G., Schwartz, S., Woodcock, A., Langley, S. & O'Connor, B. J. (2007) Selective inducible nitric oxide synthase inhibition has no effect on allergen challenge in asthma. *American journal of respiratory and critical care medicine* **176**: 988–993.

Singh, D., Fairwood, J., Murdoch, R., Weeks, A., Russell, P., Roy, K., Langley, S. & Woodcock, A. (2008) The reproducibility of adenosine monophosphate bronchial challenges in mild, steroid-naive asthmatics. *British journal of clinical pharmacology* **66**: 261–265.

Siroux, V., Pin, I., Oryszczyn, M., Le Moual, N. & Kauffmann, F. (2000) Relationships of active smoking to asthma and asthma severity in the EGEA study. Epidemiological study on the Genetics and Environment of Asthma. *European Respiratory Journal* **15**: 470 –477.

Skorge, T. D., Eagan, T. M. L., Eide, G. E., Gulsvik, A. & Bakke, P. S. (2005) The adult incidence of asthma and respiratory symptoms by passive smoking in uterus or in childhood. *American journal of respiratory and critical care medicine* **172**: 61–66.

Slats, A. M., Janssen, K., van Schadewijk, A., van der Plas, D. T., Schot, R., van den Aardweg, J. G., de Jongste, J. C., Hiemstra, P. S., Mauad, T., Rabe, K. F. & Sterk, P. J. (2008) Expression of smooth muscle and extracellular matrix proteins in relation to airway function in asthma. *The Journal of allergy and clinical immunology* **121**: 1196–1202.

Sly, P. D. (2011) The early origins of asthma: who is really at risk? *Current opinion in allergy and clinical immunology* **11**: 24–28.

Sly, P. D., Turner, D. J., Collins, R. A. & Hantos, Z. (2005) Penh Is Not a Validated Technique for Measuring Airway Function in Mice. *American Journal of Respiratory and Critical Care Medicine* **172**: 256–256.

Smith, J. M., Disney, M. E., Williams, J. D. & Goels, Z. A. (1969) Clinical Significance of Skin Reactions to Mite Extracts in Children with Asthma. *British Medical Journal* **2**: 723 –726.

Smith, K. G. C. & Clatworthy, M. R. (2010) FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications. *Nature reviews. Immunology* **10**: 328–343.

Smith, L. J., Greenberger, P. A., Patterson, R., Krell, R. D. & Bernstein, P. R. (1985) The effect of inhaled leukotriene D4 in humans. *The American review of respiratory disease* **131**: 368–372.

Snapper, C. M., Finkelman, F. D. & Paul, W. E. (1988) Differential regulation of IgG1 and IgE synthesis by interleukin 4. *The Journal of experimental medicine* **167**: 183–196.

Sokol, C. L., Barton, G. M., Farr, A. G. & Medzhitov, R. (2008) A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nature immunology* **9**: 310–318.

Sokol, C. L., Chu, N.-Q., Yu, S., Nish, S. A., Laufer, T. M. & Medzhitov, R. (2009) Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nature Immunology* **10**: 713–720.

Soliman, D. M. & Twigg, H. L., 3rd (1992) Cigarette smoking decreases bioactive interleukin-6 secretion by alveolar macrophages. *The American journal of physiology* **263**: L471–478.

Song, D. J., Min, M. G., Miller, M., Cho, J. Y. & Broide, D. H. (2009) Environmental tobacco smoke exposure does not prevent corticosteroids reducing inflammation, remodeling, and airway hyperreactivity in mice exposed to allergen. *Am J Physiol Lung Cell Mol Physiol* **297**: L380–387.

Sont, J. K., Booms, P., Bel, E. H., Vandenbroucke, J. P. & Sterk, P. J. (1995) The severity of breathlessness during challenges with inhaled methacholine and hypertonic saline in atopic asthmatic subjects. The relationship with deep breath-induced bronchodilation. *American journal of respiratory and critical care medicine* **152**: 38–44.

Soumelis, V., Reche, P. A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., Smith, K., Gorman, D., Zurawski, S., Abrams, J., Menon, S., McClanahan, T., de Waal-Malefyt Rd, R., Bazan, F., Kastelein, R. A. & Liu, Y.-J. (2002) Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nature immunology* **3**: 673–680.

Southam, D. S., Ellis, R., Wattie, J. & Inman, M. D. (2007) Components of airway hyperresponsiveness and their associations with inflammation and remodeling in mice. *The Journal of Allergy and Clinical Immunology* **119**: 848–854.

Souwer, Y., Szegedi, K., Kapsenberg, M. L. & de Jong, E. C. (2010) IL-17 and IL-22 in atopic allergic disease. *Current opinion in immunology* **22**: 821–826.

Spears, M., Donnelly, I., Jolly, L., Brannigan, M., Ito, K., McSharry, C., Lafferty, J., Chaudhuri, R., Braganza, G., Adcock, I. M., Barnes, P. J., Wood, S. & Thomson, N. C. (2009) Effect of low-dose theophylline plus beclometasone on lung function in smokers with asthma: a pilot study. *European Respiratory Journal* **33**: 1010–1017.

Spears, M., Weir, C. J., Smith, A. D., McSharry, C., Chaudhuri, R., Johnson, M., Cameron, E. & Thomson, N. C. (2011) Bronchial nitric oxide flux (J′aw) is sensitive to oral corticosteroids in smokers with asthma. *Respiratory Medicine* **105**: 1823–1830.

Spencer, S., Calverley, P. M. A., Burge, P. S. & Jones, P. W. (2004) Impact of preventing exacerbations on deterioration of health status in COPD. *European Respiratory Journal* **23**: 698–702.

Spergel, J. M. (2010) From atopic dermatitis to asthma: the atopic march. *Annals of Allergy, Asthma & Immunology* **105**: 99–106.

Spergel, J. M., Mizoguchi, E., Brewer, J. P., Martin, T. R., Bhan, A. K. & Geha, R. S. (1998) Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *The Journal of clinical investigation* **101**: 1614–1622.

Spicuzza, L., Belvisi, M. G., Birrell, M. A., Barnes, P. J., Hele, D. J. & Giembycz, M. A. (2001) Evidence that the anti-spasmogenic effect of the beta-adrenoceptor agonist, isoprenaline, on guinea-pig trachealis is not mediated by cyclic AMP-dependent protein kinase. *British journal of pharmacology* **133**: 1201–1212.

Stämpfli, M. R., Ritz, S. A., Neigh, G. S., Sime, P. J., Lei, X. F., Xing, Z., Croitoru, K. & Jordana, M. (1998) Adenoviral infection inhibits allergic airways inflammation in mice. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **28**: 1581–1590.

Stănescu, D., Sanna, A., Veriter, C., Kostianev, S., Calcagni, P. G., Fabbri, L. M. & Maestrelli, P. (1996) Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* **51**: 267–271.

Stankus, R. P., Menon, P. K., Rando, R. J., Glindmeyer, H., Salvaggio, J. E. & Lehrer, S. B. (1988) Cigarette smoke-sensitive asthma: challenge studies. *The Journal of allergy and clinical immunology* **82**: 331–338.

Stavenow, L., Falke, P. & Berglund, A. (1983) Effects of different injurious stimuli on cell death, proliferation, and collagen secretion by rabbit aortic smooth muscle cells and human umbilical vein endothelial cells in culture. *Medical biology* **61**: 214–218.

Stevens, E., Cullinan, P. & Colvile, R. (2004) Urban air pollution and children's asthma: What do parents and health professionals think? *Pediatric Pulmonology* **37**: 530–536.

Stevens, W. J. & van Bever, H. P. (1989) Frequency and intensity of late asthmatic reactions after bronchial allergen challenge in asthma and rhinitis. *Allergy* **44**: 471–476.

Stevenson, C. S. & Belvisi, M. G. (2008) Preclinical animal models of asthma and chronic obstructive pulmonary disease. *Expert Review of Respiratory Medicine* **2**: 631–643.

Stewart, G. A., Lake, F. R. & Thompson, P. J. (1991) Faecally derived hydrolytic enzymes from Dermatophagoides pteronyssinus: physicochemical characterisation of potential allergens. *International archives of allergy and applied immunology* **95**: 248–256.

St-Laurent, J., Bergeron, C., Pagé, N., Couture, C., Laviolette, M. & Boulet, L.-P. (2008) Influence of smoking on airway inflammation and remodelling in asthma. *Clinical & Experimental Allergy* **38**: 1582–1589.

Stock, P., Lombardi, V., Kohlrautz, V. & Akbari, O. (2009) Induction of airway hyperreactivity by IL-25 is dependent on a subset of invariant NKT cells expressing IL-17RB. *Journal of immunology (Baltimore, Md.: 1950)* **182**: 5116–5122.

Stockley, R. A., Mannino, D. & Barnes, P. J. (2009) Burden and pathogenesis of chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* **6**: 524–526.

Stolarski, B., Kurowska-Stolarska, M., Kewin, P., Xu, D. & Liew, F. Y. (2010) IL-33 exacerbates eosinophil-mediated airway inflammation. *Journal of immunology (Baltimore, Md.: 1950)* **185**: 3472–3480.

Strachan, D. P. (1989) Hay fever, hygiene, and household size. *BMJ (Clinical research ed.)* **299**: 1259–1260.

Strachan, D. P. & Carey, I. M. (1995) Home environment and severe asthma in adolescence: a population based case-control study. *BMJ (Clinical research ed.)* **311**: 1053–1056.

Strait, R. T., Morris, S. C. & Finkelman, F. D. (2006) IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and Fc gamma RIIb cross-linking. *The Journal of clinical investigation* **116**: 833–841.

Strickland, I., Kisich, K., Hauk, P. J., Vottero, A., Chrousos, G. P., Klemm, D. J. & Leung, D. Y. (2001) High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids. *The Journal of experimental medicine* **193**: 585–593.

Strieter, R. M., Kasahara, K., Allen, R. M., Standiford, T. J., Rolfe, M. W., Becker, F. S., Chensue, S. W. & Kunkel, S. L. (1992) Cytokine-induced neutrophil-derived interleukin-8. *The American journal of pathology* **141**: 397–407.

Sturton, G., Persson, C. & Barnes, P. J. (2008) Small airways: an important but neglected target in the treatment of obstructive airway diseases. *Trends in pharmacological sciences* **29**: 340–345.

Sukkar, M. B., Hughes, J. M., Armour, C. L. & Johnson, P. R. (2001) Tumour necrosis factor-alpha potentiates contraction of human bronchus in vitro. *Respirology (Carlton, Vic.)* **6**: 199–203.

Sun, G., Stacey, M. A., Schmidt, M., Mori, L. & Mattoli, S. (2001) Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *Journal of immunology (Baltimore, Md.: 1950)* **167**: 1014–1021.

Sun, X., Li, Q., Gong, Y., Ren, L., Wan, H. & Deng, W. (2012) Low-dose theophylline restores corticosteroid responsiveness in rats with smoke-induced airway inflammation. *Canadian journal of physiology and pharmacology* **90**: 895–902.

Sur, S., Crotty, T. B., Kephart, G. M., Hyma, B. A., Colby, T. V., Reed, C. E., Hunt, L. W. & Gleich, G. J. (1993) Sudden-onset fatal asthma. A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? *The American review of respiratory disease* **148**: 713–719.

Sutcliffe, A., Kaur, D., Page, S., Woodman, L., Armour, C. L., Baraket, M., Bradding, P., Hughes, J. M. & Brightling, C. E. (2006) Mast cell migration to Th2 stimulated airway smooth muscle from asthmatics. *Thorax* **61**: 657–662.

Swedin, L., Neimert-Andersson, T., Hjoberg, J., Jonasson, S., van Hage, M., Adner, M., Ryrfeldt, A. & Dahlén, S.-E. (2009) Dissociation of airway inflammation and hyperresponsiveness by cyclooxygenase inhibition in allergen challenged mice. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **34**: 200–208.

Swedin, L., Ellis, R., Kemi, C., Ryrfeldt, A., Inman, M., Dahlén, S.-E. & Adner, M. (2010*a*) Comparison of aerosol and intranasal challenge in a mouse model of allergic airway inflammation and hyperresponsiveness. *International archives of allergy and immunology* **153**: 249–258.

Swedin, L., Ellis, R., Neimert-Andersson, T., Ryrfeldt, Å., Nilsson, G., Inman, M., Dahlén, S.-E. & Adner, M. (2010*b*) Prostaglandin modulation of airway inflammation and hyperresponsiveness in mice sensitized without adjuvant. *Prostaglandins & Other Lipid Mediators* **92**: 44–53.

Swirski, F. K., Sajic, D., Robbins, C. S., Gajewska, B. U., Jordana, M. & Stampfli, M. R. (2002) Chronic Exposure to Innocuous Antigen in Sensitized Mice Leads to Suppressed Airway Eosinophilia That Is Reversed by Granulocyte Macrophage Colony-Stimulating Factor. *J Immunol* **169**: 3499–3506.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. & Glimcher, L. H. (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**: 655–669.

Takahashi, T., Ward, J. K., Tadjkarimi, S., Yacoub, M. H., Barnes, P. J. & Belvisi, M. G. (1995) 5-Hydroxytryptamine facilitates cholinergic bronchoconstriction in human and guinea pig airways. *American journal of respiratory and critical care medicine* **152**: 377–380.

Takai, T., Ono, M., Hikida, M., Ohmori, H. & Ravetch, J. V. (1996) Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature* **379**: 346–349.

Takamoto, M. & Sugane, K. (1995) Synergism of IL-3, IL-5, and GM-CSF on eosinophil differentiation and its application for an assay of murine IL-5 as an eosinophil differentiation factor. *Immunology letters* **45**: 43–46.

Takeda, K., Haczku, A., Lee, J. J., Irvin, C. G. & Gelfand, E. W. (2001) Strain dependence of airway hyperresponsiveness reflects differences in eosinophil localization in the lung. *American journal of physiology. Lung cellular and molecular physiology* **281**: L394–402.

Takhar, P., Corrigan, C. J., Smurthwaite, L., O'Connor, B. J., Durham, S. R., Lee, T. H. & Gould, H. J. (2007) Class switch recombination to IgE in the bronchial mucosa of atopic and nonatopic patients with asthma. *The Journal of allergy and clinical immunology* **119**: 213–218.

Tamimi, A., Serdarevic, D. & Hanania, N. A. (2012) The effects of cigarette smoke on airway inflammation in asthma and COPD: therapeutic implications. *Respiratory medicine* **106**: 319–328.

Tanaka, H., Masuda, T., Tokuoka, S., Komai, M., Nagao, K., Takahashi, Y. & Nagai, H. (2001) The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflammation research: official journal of the European Histamine Research Society ... [et al.]* **50**: 616–624.

Tanizaki, Y., Kitani, H., Okazaki, M., Mifune, T., Mitsunobu, F. & Kimura, I. (1993) Effects of long-term glucocorticoid therapy on bronchoalveolar cells in adult patients with bronchial asthma. *The Journal of asthma: official journal of the Association for the Care of Asthma* **30**: 309–318.

Tateda, K., Moore, T. A., Deng, J. C., Newstead, M. W., Zeng, X., Matsukawa, A., Swanson, M. S., Yamaguchi, K. & Standiford, T. J. (2001) Early recruitment of neutrophils determines subsequent T1/T2 host responses in a murine model of Legionella pneumophila pneumonia. *Journal of immunology (Baltimore, Md.: 1950)* **166**: 3355–3361.

Taube, C., Duez, C., Cui, Z.-H., Takeda, K., Rha, Y.-H., Park, J.-W., Balhorn, A., Donaldson, D. D., Dakhama, A. & Gelfand, E. W. (2002) The role of IL-13 in established allergic airway disease. *Journal of immunology (Baltimore, Md.: 1950)* **169**: 6482–6489.

Taylor, J. D. (2010) COPD and the response of the lung to tobacco smoke exposure. *Pulmonary Pharmacology & Therapeutics* **23**: 376–383.

Teeter, J. G. & Bleecker, E. R. (1998) Relationship between airway obstruction and respiratory symptoms in adult asthmatics. *Chest* **113**: 272–277.

Temelkovski, J., Hogan, S. P., Shepherd, D. P., Foster, P. S. & Kumar, R. K. (1998) An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* **53**: 849–856.

Thatcher, T. H., Benson, R. P., Phipps, R. P. & Sime, P. J. (2008) High-dose but not low-dose mainstream cigarette smoke suppresses allergic airway inflammation by inhibiting T cell function. *Am J Physiol Lung Cell Mol Physiol* **295**: L412–421.

The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic severe asthma. European Network for Understanding Mechanisms of Severe Asthma (2003) *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **22**: 470–477.

Thomas, W. R., Smith, W.-A., Hales, B. J., Mills, K. L. & O'Brien, R. M. (2002) Characterization and immunobiology of house dust mite allergens. *International archives of allergy and immunology* **129**: 1–18.

Thompson, H. L., Metcalfe, D. D. & Kinet, J. P. (1990) Early expression of high-affinity receptor for immunoglobulin E (Fc epsilon RI) during differentiation of mouse mast cells and human basophils. *The Journal of clinical investigation* **85**: 1227–1233.

Thomson, N. C., Chaudhuri, R. & Livingston, E. (2004) Asthma and cigarette smoking. *European Respiratory Journal* **24**: 822 –833.

Thomson, N. C., Chaudhuri, R., Heaney, L. G., Bucknall, C., Niven, R. M., Brightling, C. E., Menzies-Gow, A. N., Mansur, A. H. & McSharry, C. (2013) Clinical outcomes and inflammatory biomarkers in current smokers and exsmokers with severe asthma. *The Journal of allergy and clinical immunology*.

Tiku, K., Tiku, M. L. & Skosey, J. L. (1986) Interleukin 1 production by human polymorphonuclear neutrophils. *Journal of immunology (Baltimore, Md.: 1950)* **136**: 3677–3685.

To, Y., Ito, K., Kizawa, Y., Failla, M., Ito, M., Kusama, T., Elliott, W. M., Hogg, J. C., Adcock, I. M. & Barnes, P. J. (2010) Targeting phosphoinositide-3-kinase-delta with theophylline reverses corticosteroid insensitivity in chronic obstructive

pulmonary disease. *American journal of respiratory and critical care medicine* **182**: 897–904.

Tomkinson, A., Cieslewicz, G., Duez, C., Larson, K. A., Lee, J. J. & Gelfand, E. W. (2001) Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *American journal of respiratory and critical care medicine* **163**: 721–730.

Tomlinson, J. E. M., McMahon, A. D., Chaudhuri, R., Thompson, J. M., Wood, S. F. & Thomson, N. C. (2005) Efficacy of low and high dose inhaled corticosteroid in smokers versus non-smokers with mild asthma. *Thorax* **60**: 282–287.

Torén, K. & Hermansson, B. A. (1999) Incidence rate of adult-onset asthma in relation to age, sex, atopy and smoking: a Swedish population-based study of 15813 adults. *The international journal of tuberculosis and lung disease: the official journal of the International Union against Tuberculosis and Lung Disease* **3**: 192–197.

Torphy, T. J. (1994) Beta-adrenoceptors, cAMP and airway smooth muscle relaxation: challenges to the dogma. *Trends in pharmacological sciences* **15**: 370–374.

Torphy, T. J., Freese, W. B., Rinard, G. A., Brunton, L. L. & Mayer, S. E. (1982) Cyclic nucleotide-dependent protein kinases in airway smooth muscle. *The Journal of biological chemistry* **257**: 11609–11616.

Torrent, M., Sunyer, J., Muñoz, L., Cullinan, P., Iturriaga, M. V., Figueroa, C., Vall, O., Taylor, A. N. & Anto, J. M. (2006) Early-life domestic aeroallergen exposure and IgE sensitization at age 4 years. *The Journal of allergy and clinical immunology* **118**: 742–748.

Torrent, M., Sunyer, J., Garcia, R., Harris, J., Iturriaga, M. V., Puig, C., Vall, O., Anto, J. M., Newman Taylor, A. J. & Cullinan, P. (2007) Early-life allergen exposure and atopy, asthma, and wheeze up to 6 years of age. *American journal of respiratory and critical care medicine* **176**: 446–453.

Tournoy, K. G., Kips, J. C., Schou, C. & Pauwels, R. A. (2000) Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology* **30**: 79–85.

Tovey, E. R., Almqvist, C., Li, Q., Crisafulli, D. & Marks, G. B. (2008) Nonlinear relationship of mite allergen exposure to mite sensitization and asthma in a birth cohort. *The Journal of allergy and clinical immunology* **122**: 114–118, 118.e1–5.

Townley, R. G., Southard, J. G., Radford, P., Hopp, R. J., Bewtra, A. K. & Ford, L. (1990) Association of MS Pi phenotype with airway hyperresponsiveness. *Chest* **98**: 594–599.

Trifilieff, A., El-Hashim, A. & Bertrand, C. (2000) Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *American journal of physiology. Lung cellular and molecular physiology* **279**: L1120–1128.

Trimble, N. J., Botelho, F. M., Bauer, C. M. T., Fattouh, R. & Stämpfli, M. R. (2009) Adjuvant and anti-inflammatory properties of cigarette smoke in murine allergic

airway inflammation. *American journal of respiratory cell and molecular biology* **40**: 38–46.

Trompette, A., Divanovic, S., Visintin, A., Blanchard, C., Hegde, R. S., Madan, R., Thorne, P. S., Wills-Karp, M., Gioannini, T. L., Weiss, J. P. & Karp, C. L. (2009) Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* **457**: 585–588.

Tsaprouni, L. G., Ito, K., Adcock, I. M. & Punchard, N. (2007) Suppression of lipopolysaccharide- and tumour necrosis factor-alpha-induced interleukin (IL)-8 expression by glucocorticoids involves changes in IL-8 promoter acetylation. *Clinical and experimental immunology* **150**: 151–157.

Tsujimura, Y., Obata, K., Mukai, K., Shindou, H., Yoshida, M., Nishikado, H., Kawano, Y., Minegishi, Y., Shimizu, T. & Karasuyama, H. (2008) Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity* **28**: 581–589.

Turi, G. J., Ellis, R., Wattie, J. N., Labiris, N. R. & Inman, M. D. (2011) The effects of inhaled house dust mite on airway barrier function and sensitivity to inhaled methacholine in mice. *American journal of physiology. Lung cellular and molecular physiology* **300**: L185–190.

Uchida, D. A., Ackerman, S. J., Coyle, A. J., Larsen, G. L., Weller, P. F., Freed, J. & Irvin, C. G. (1993) The effect of human eosinophil granule major basic protein on airway responsiveness in the rat in vivo. A comparison with polycations. *The American review of respiratory disease* **147**: 982–988.

Ulanova, M., Tarkowski, A., Hahn-Zoric, M. & Hanson, L. A. (2001) The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infection and immunity* **69**: 1151–1159.

Ulrich, K., Hincks, J. S., Walsh, R., Wetterstrand, E. M. C., Fidock, M. D., Sreckovic, S., Lamb, D. J., Douglas, G. J., Yeadon, M., Perros-Huguet, C. & Evans, S. M. (2008) Anti-inflammatory modulation of chronic airway inflammation in the murine house dust mite model. *Pulmonary pharmacology & therapeutics* **21**: 637–647.

Ulrik, C. S. & Frederiksen, J. (1995) Mortality and markers of risk of asthma death among 1,075 outpatients with asthma. *Chest* **108**: 10–15.

Undem, B. J., Myers, A. C. & Weinreich, D. (1991) Antigen-Induced Modulation of Autonomic and Sensory Neurons in vitro. *International Archives of Allergy and Immunology* **94**: 319–324.

Underwood, S. L., Haddad, E.-B., Birrell, M. A., McCluskie, K., Pecoraro, M., Dabrowski, D., Webber, S. E., Foster, M. L. & Belvisi, M. G. (2002) Functional characterization and biomarker identification in the Brown Norway model of allergic airway inflammation. *British journal of pharmacology* **137**: 263–275.

Usmani, O. S., Ito, K., Maneechotesuwan, K., Ito, M., Johnson, M., Barnes, P. J. & Adcock, I. M. (2005) Glucocorticoid receptor nuclear translocation in airway cells after inhaled combination therapy. *American journal of respiratory and critical care medicine* **172**: 704–712.

Usui, T., Preiss, J. C., Kanno, Y., Yao, Z. J., Bream, J. H., O'Shea, J. J. & Strober, W. (2006) T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *The Journal of experimental medicine* **203**: 755–766.

Van der Vaart, H., Postma, D. S., Timens, W. & ten Hacken, N. H. T. (2004) Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* **59**: 713–721.

Varga, E. M., Wachholz, P., Nouri-Aria, K. T., Verhoef, A., Corrigan, C. J., Till, S. J. & Durham, S. R. (2000) T cells from human allergen-induced late asthmatic responses express IL-12 receptor beta 2 subunit mRNA and respond to IL-12 in vitro. *Journal of immunology (Baltimore, Md.: 1950)* **165**: 2877–2885.

Vermaelen, K. Y., Carro-Muino, I., Lambrecht, B. N. & Pauwels, R. A. (2001) Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *The Journal of experimental medicine* **193**: 51–60.

Vestbo, J., Søorensen, T., Lange, P., Brix, A., Torre, P. & Viskum, K. (1999) Long-term effect of inhaled budesonide in mild and moderate chronic obstructive pulmonary disease: a randomised controlled trial. *The Lancet* **353**: 1819–1823.

Vieira, P. L., de Jong, E. C., Wierenga, E. A., Kapsenberg, M. L. & Kaliński, P. (2000) Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *Journal of immunology (Baltimore, Md.: 1950)* **164**: 4507–4512.

Vieira, V. G. & Prolla, J. C. (1979) Clinical evaluation of eosinophils in the sputum. *Journal of clinical pathology* **32**: 1054–1057.

Vignola, A. M., Riccobono, L., Mirabella, A., Profita, M., Chanez, P., Bellia, V., Mautino, G., D'accardi, P., Bousquet, J. & Bonsignore, G. (1998) Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *American journal of respiratory and critical care medicine* **158**: 1945–1950.

Vignola, A. M., Humbert, M., Bousquet, J., Boulet, L.-P., Hedgecock, S., Blogg, M., Fox, H. & Surrey, K. (2004) Efficacy and tolerability of anti-immunoglobulin E therapy with omalizumab in patients with concomitant allergic asthma and persistent allergic rhinitis: SOLAR. *Allergy* **59**: 709–717.

Voehringer, D., Shinkai, K. & Locksley, R. M. (2004) Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* **20**: 267–277.

Vozoris, N. T. & Stanbrook, M. B. (2011) Smoking prevalence, behaviours, and cessation among individuals with COPD or asthma. *Respiratory medicine* **105**: 477–484.

Wakashin, H., Hirose, K., Maezawa, Y., Kagami, S., Suto, A., Watanabe, N., Saito, Y., Hatano, M., Tokuhisa, T., Iwakura, Y., Puccetti, P., Iwamoto, I. & Nakajima, H. (2008) IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *American journal of respiratory and critical care medicine* **178**: 1023–1032.

Walker, C., Kaegi, M. K., Braun, P. & Blaser, K. (1991) Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *Journal of Allergy and Clinical Immunology* **88**: 935–942.

Wallace, J., D'silva, L., Brannan, J., Hargreave, F. E., Kanaroglou, P. & Nair, P. (2011) Association between proximity to major roads and sputum cell counts. *Canadian respiratory journal: journal of the Canadian Thoracic Society* **18**: 13–18.

Walter, D. M., McIntire, J. J., Berry, G., McKenzie, A. N., Donaldson, D. D., DeKruyff, R. H. & Umetsu, D. T. (2001) Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *Journal of immunology (Baltimore, Md.: 1950)* **167**: 4668–4675.

Wan, H., Winton, H. L., Soeller, C., Tovey, E. R., Gruenert, D. C., Thompson, P. J., Stewart, G. A., Taylor, G. W., Garrod, D. R., Cannell, M. B. & Robinson, C. (1999*a*) Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *The Journal of clinical investigation* **104**: 123–133.

Wan, H., Winton, H. L., Soeller, C., Tovey, E. R., Gruenert, D. C., Thompson, P. J., Stewart, G. A., Taylor, G. W., Garrod, D. R., Cannell, M. B. & Robinson, C. (1999*b*) Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *The Journal of clinical investigation* **104**: 123–133.

Wan, H., Winton, H. L., Soeller, C., Gruenert, D. C., Thompson, P. J., Cannell, M. B., Stewart, G. A., Garrod, D. R. & Robinson, C. (2000) Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **30**: 685–698.

Wang, J. M., Rambaldi, A., Biondi, A., Chen, Z. G., Sanderson, C. J. & Mantovani, A. (1989) Recombinant human interleukin 5 is a selective eosinophil chemoattractant. *European journal of immunology* **19**: 701–705.

Wang, Y.-H., Voo, K. S., Liu, B., Chen, C.-Y., Uygungil, B., Spoede, W., Bernstein, J. A., Huston, D. P. & Liu, Y.-J. (2010) A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *The Journal of experimental medicine* **207**: 2479–2491.

Ward, J. K., Barnes, P. J., Tadjkarimi, S., Yacoub, M. H. & Belvisi, M. G. (1995) Evidence for the involvement of cGMP in neural bronchodilator responses in humal trachea. *The Journal of physiology* **483 (Pt 2)**: 525–536.

Wardlaw, A. J., Dunnette, S., Gleich, G. J., Collins, J. V. & Kay, A. B. (1988) Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *The American review of respiratory disease* **137**: 62–69.

Waserman, S., Olivenstein, R., Renzi, P., Xu, L. J. & Martin, J. G. (1992) The relationship between late asthmatic responses and antigen-specific immunoglobulin. *The Journal of allergy and clinical immunology* **90**: 661–669.

Watanabe, J., Miyazaki, Y., Zimmerman, G. A., Albertine, K. H. & McIntyre, T. M. (2003) Endotoxin contamination of ovalbumin suppresses murine immunologic responses and development of airway hyper-reactivity. *The Journal of biological chemistry* **278**: 42361–42368.

Watson, A. M., Benton, A. S., Rose, M. C. & Freishtat, R. J. (2010) Cigarette smoke alters tissue inhibitor of metalloproteinase 1 and matrix metalloproteinase 9 levels in the basolateral secretions of human asthmatic bronchial epithelium in vitro. *Journal of investigative medicine: the official publication of the American Federation for Clinical Research* **58**: 725–729.

Watts, S. W. & Cohen, M. L. (1993) Time-dependent improvement in guinea pig tracheal contractility: modification by cyclooxygenase inhibitors. *Journal of Pharmacology and Experimental Therapeutics* **266**: 950–957.

Weatherall, M., Wijesinghe, M., Perrin, K., Harwood, M. & Beasley, R. (2010) Meta-analysis of the risk of mortality with salmeterol and the effect of concomitant inhaled corticosteroid therapy. *Thorax* **65**: 39–43.

Weaver, C. T., Hatton, R. D., Mangan, P. R. & Harrington, L. E. (2007) IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annual review of immunology* **25**: 821–852.

Wegmann, M. (2008) Animal models of chronic experimental asthma - strategies for the identification of new therapeutic targets. *Journal of occupational medicine and toxicology (London, England)* **3 Suppl 1**: S4.

Weigand, L. A., Myers, A. C., Meeker, S. & Undem, B. J. (2009) Mast cell-cholinergic nerve interaction in mouse airways. **587**: 3355–3362.

Weiss, J. W., Drazen, J. M., Coles, N., McFadden, E. R., Jr, Weller, P. F., Corey, E. J., Lewis, R. A. & Austen, K. F. (1982) Bronchoconstrictor effects of leukotriene C in humans. *Science (New York, N.Y.)* **216**: 196–198.

Weiss, K. B., Gergen, P. J. & Hodgson, T. A. (1992) An economic evaluation of asthma in the United States. *The New England Journal of Medicine* **326**: 862–866.

Weller, P. F., Lee, C. W., Foster, D. W., Corey, E. J., Austen, K. F. & Lewis, R. A. (1983) Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C4. *Proceedings of the National Academy of Sciences of the United States of America* **80**: 7626–7630.

Wenzel, S. (2005) Severe asthma in adults. *American journal of respiratory and critical care medicine* **172**: 149–160.

Wenzel, S., Szefler, S., Leung, D. M., Sloan, S., Rex, M. & Martin, R. (1997) Bronchoscopic Evaluation of Severe Asthma . Persistent Inflammation Associated with High Dose Glucocorticoids. *Am. J. Respir. Crit. Care Med.* **156**: 737–743.

Wenzel, S. E. & Busse, W. W. (2007) Severe asthma: lessons from the Severe Asthma Research Program. *The Journal of allergy and clinical immunology* **119**: 14–21; quiz 22–23.

Wenzel, S. E., Westcott, J. Y., Smith, H. R. & Larsen, G. L. (1989) Spectrum of prostanoid release after bronchoalveolar allergen challenge in atopic asthmatics and in control groups. An alteration in the ratio of bronchoconstrictive to bronchoprotective mediators. *The American review of respiratory disease* **139**: 450–457.

Wenzel, S. E., Balzar, S., Cundall, M. & Chu, H. W. (2003) Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with

asthma severity, neutrophilic inflammation, and wound repair. *The Journal of allergy and clinical immunology* **111**: 1345–1352.

Wenzel, S. E., Schwartz, L. B., Langmack, E. L., Halliday, J. L., Trudeau, J. B., Gibbs, R. L. & Chu, H. W. (1999) Evidence That Severe Asthma Can Be Divided Pathologically into Two Inflammatory Subtypes with Distinct Physiologic and Clinical Characteristics. *Am. J. Respir. Crit. Care Med.* **160**: 1001–1008.

Wichmann, H. E. (1996) Possible explanation for the different trends of asthma and allergy in East and West Germany. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **26**: 621–623.

Wieringa, M. H., Weyler, J. J., Van Bastelaer, F. J., Nelen, V. J., Van Sprundel, M. P. & Vermeire, P. A. (1997) Higher asthma occurrence in an urban than a suburban area: role of house dust mite skin allergy. *The European respiratory journal* **10**: 1460–1466.

Wilder, J. A., Collie, D. D., Wilson, B. S., Bice, D. E., Lyons, C. R. & Lipscomb, M. F. (1999) Dissociation of airway hyperresponsiveness from immunoglobulin E and airway eosinophilia in a murine model of allergic asthma. *American journal of respiratory cell and molecular biology* **20**: 1326–1334.

Willart, M. A. M., Deswarte, K., Pouliot, P., Braun, H., Beyaert, R., Lambrecht, B. N. & Hammad, H. (2012) Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *The Journal of experimental medicine* **209**: 1505–1517.

Williams, J. W., Tjota, M. Y. & Sperling, A. I. (2012) The contribution of allergen-specific IgG to the development of th2-mediated airway inflammation. *Journal of allergy* **2012**: 236075.

Wills-Karp, M., Santeliz, J. & Karp, C. L. (2001) The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nature reviews. Immunology* **1**: 69–75.

Wilson, N. J., Boniface, K., Chan, J. R., McKenzie, B. S., Blumenschein, W. M., Mattson, J. D., Basham, B., Smith, K., Chen, T., Morel, F., Lecron, J.-C., Kastelein, R. A., Cua, D. J., McClanahan, T. K., Bowman, E. P. & de Waal Malefyt, R. (2007) Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nature immunology* **8**: 950–957.

Wilson, R. H., Whitehead, G. S., Nakano, H., Free, M. E., Kolls, J. K. & Cook, D. N. (2009) Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *American journal of respiratory and critical care medicine* **180**: 720–730.

Wilson, S. J., Shute, J. K., Holgate, S. T., Howarth, P. H. & Bradding, P. (2000) Localization of interleukin (IL) -4 but not IL-5 to human mast cell secretory granules by immunoelectron microscopy. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **30**: 493–500.

Wissink, S., van Heerde, E. C., vand der Burg, B. & van der Saag, P. T. (1998) A dual mechanism mediates repression of NF-kappaB activity by glucocorticoids. *Molecular endocrinology (Baltimore, Md.)* **12**: 355–363.

Wodnar-Filipowicz, A., Heusser, C. H. & Moroni, C. (1989) Production of the haemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation. *Nature* **339**: 150–152.

Woerly, G., Lacy, P., Younes, A. B., Roger, N., Loiseau, S., Moqbel, R. & Capron, M. (2002) Human eosinophils express and release IL-13 following CD28-dependent activation. *Journal of leukocyte biology* **72**: 769–779.

Wohlsen, A., Martin, C., Vollmer, E., Branscheid, D., Magnussen, H., Becker, W. M., Lepp, U. & Uhlig, S. (2003) The early allergic response in small airways of human precision-cut lung slices. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **21**: 1024–1032.

Wolfenden, L. L., Diette, G. B., Krishnan, J. A., Skinner, E. A., Steinwachs, D. M. & Wu, A. W. (2003) Lower physician estimate of underlying asthma severity leads to undertreatment. *Archives of internal medicine* **163**: 231–236.

Woodman, L., Siddiqui, S., Cruse, G., Sutcliffe, A., Saunders, R., Kaur, D., Bradding, P. & Brightling, C. (2008) Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1. *Journal of immunology (Baltimore, Md.: 1950)* **181**: 5001–5007.

Woodruff, P. G., Khashayar, R., Lazarus, S. C., Janson, S., Avila, P., Boushey, H. A., Segal, M. & Fahy, J. V. (2001) Relationship between airway inflammation, hyperresponsiveness, and obstruction in asthma. *The Journal of allergy and clinical immunology* **108**: 753–758.

Woolcock, A. J. (1996) Corticosteroid-resistant asthma. Definitions. *American journal of respiratory and critical care medicine* **154**: S45–48.

Woolcock, A. J., Salome, C. M. & Yan, K. (1984) The shape of the dose-response curve to histamine in asthmatic and normal subjects. *The American review of respiratory disease* **130**: 71–75.

Wooldridge, L., Ekeruche-Makinde, J., van den Berg, H. A., Skowera, A., Miles, J. J., Tan, M. P., Dolton, G., Clement, M., Llewellyn-Lacey, S., Price, D. A., Peakman, M. & Sewell, A. K. (2012) A single autoimmune T cell receptor recognizes more than a million different peptides. *The Journal of biological chemistry* **287**: 1168–1177.

Xia, W., Pinto, C. E. & Kradin, R. L. (1995) The antigen-presenting activities of Ia+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. *The Journal of experimental medicine* **181**: 1275–1283.

Xu, G.-N., Yang, K., Xu, Z.-P., Zhu, L., Hou, L.-N., Qi, H., Chen, H.-Z. & Cui, Y.-Y. (2012) Protective effects of anisodamine on cigarette smoke extract-induced airway smooth muscle cell proliferation and tracheal contractility. *Toxicology and applied pharmacology* **262**: 70–79.

Yamada, N., Kadowaki, S. & Umezu, K. (1992) Development of an animal model of late asthmatic response in guinea pigs and effects of anti-asthmatic drugs. *Prostaglandins* **43**: 507–521.

Yamaguchi, E., Itoh, A., Furuya, K., Miyamoto, H., Abe, S. & Kawakami, Y. (1993) Release of tumor necrosis factor-alpha from human alveolar macrophages is decreased in smokers. *Chest* **103**: 479–483.

Yamaguchi, Y., Suda, T., Suda, J., Eguchi, M., Miura, Y., Harada, N., Tominaga, A. & Takatsu, K. (1988) Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *The Journal of Experimental Medicine* **167**: 43–56.

Yamaguchi, Y., Suda, T., Ohta, S., Tominaga, K., Miura, Y. & Kasahara, T. (1991) Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. *Blood* **78**: 2542–2547.

Yanagihara, Y., Kajiwara, K., Basaki, Y., Ikizawa, K., Ebisawa, M., Ra, C., Tachimoto, H. & Saito, H. (1998) Cultured basophils but not cultured mast cells induce human IgE synthesis in B cells after immunologic stimulation. *Clinical and experimental immunology* **111**: 136–143.

Yang, G., Li, L., Volk, A., Emmell, E., Petley, T., Giles-Komar, J., Rafferty, P., Lakshminarayanan, M., Griswold, D. E., Bugelski, P. J. & Das, A. M. (2005) Therapeutic dosing with anti-interleukin-13 monoclonal antibody inhibits asthma progression in mice. *The Journal of pharmacology and experimental therapeutics* **313**: 8–15.

Yang-Yen, H. F., Chambard, J. C., Sun, Y. L., Smeal, T., Schmidt, T. J., Drouin, J. & Karin, M. (1990) Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* **62**: 1205–1215.

Yazdanbakhsh, M., van den Biggelaar, A. & Maizels, R. M. (2001) Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. *Trends in immunology* **22**: 372–377.

Yemaneberhan, H., Bekele, Z., Venn, A., Lewis, S., Parry, E. & Britton, J. (1997) Prevalence of wheeze and asthma and relation to atopy in urban and rural Ethiopia. *Lancet* **350**: 85–90.

Yiamouyiannis, C. A., Schramm, C. M., Puddington, L., Stengel, P., Baradaran-Hosseini, E., Wolyniec, W. W., Whiteley, H. E. & Thrall, R. S. (1999) Shifts in lung lymphocyte profiles correlate with the sequential development of acute allergic and chronic tolerant stages in a murine asthma model. *The American journal of pathology* **154**: 1911–1921.

Ying, S., O'Connor, B., Ratoff, J., Meng, Q., Mallett, K., Cousins, D., Robinson, D., Zhang, G., Zhao, J., Lee, T. H. & Corrigan, C. (2005) Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *Journal of immunology (Baltimore, Md.: 1950)* **174**: 8183–8190.

Yoo, J.-K., Galligan, C. L., Virtanen, C. & Fish, E. N. (2010) Identification of a novel antigen-presenting cell population modulating antiinfluenza type 2 immunity. *The Journal of experimental medicine* **207**: 1435–1451.

Yoon, C. H., Park, H.-J., Cho, Y.-W., Kim, E.-J., Lee, J. D., Kang, K. R., Han, J. & Kang, D. (2011) Cigarette Smoke Extract-induced Reduction in Migration and Contraction in Normal Human Bronchial Smooth Muscle Cells. *The Korean journal of physiology & pharmacology: official journal of the Korean Physiological Society and the Korean Society of Pharmacology* **15**: 397–403.

Yoshida, T. & Tuder, R. M. (2007) Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiological reviews* **87**: 1047–1082.

Yssel, H., Lécart, S. & Pène, J. (2001) Regulatory T cells and allergic asthma. *Microbes and infection / Institut Pasteur* **3**: 899–904.

Yu, C.-K. & Chen, C.-L. (2003) Activation of Mast Cells Is Essential for Development of House Dust Mite Dermatophagoides farinae-Induced Allergic Airway Inflammation in Mice. *The Journal of Immunology* **171**: 3808 –3815.

Yu, P., Kosco-Vilbois, M., Richards, M., Köhler, G. & Lamers, M. C. (1994) Negative feedback regulation of IgE synthesis by murine CD23. *Nature* **369**: 753–756.

Zeibecoglou, K., Macfarlane, A. J., Ying, S., Meng, Q., Pavord, I., Barnes, N. C., Robinson, D. S. & Kay, A. B. (1999) Increases in eotaxin-positive cells in induced sputum from atopic asthmatic subjects after inhalational allergen challenge. *Allergy* **54**: 730–735.

Zetterström, O., Buhl, R., Mellem, H., Perpiñá, M., Hedman, J., O'Neill, S. & Ekström, T. (2001) Improved asthma control with budesonide/formoterol in a single inhaler, compared with budesonide alone. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **18**: 262–268.

Zhang, M., Zhang, Z., Garmestani, K., Goldman, C. K., Ravetch, J. V., Brechbiel, M. W., Carrasquillo, J. A. & Waldmann, T. A. (2004) Activating Fc receptors are required for antitumor efficacy of the antibodies directed toward CD25 in a murine model of adult t-cell leukemia. *Cancer research* **64**: 5825–5829.

Zhang, X., Ma, L., Xu, Y., Liu, X. & Zhang, Z. (2011) [The effect of cigarette smoke extract on the proliferation of human airway smooth muscle cells sensitized by serum from bronchial asthmatic patients]. *Zhonghua jie he he hu xi za zhi = Zhonghua jiehe he huxi zazhi = Chinese journal of tuberculosis and respiratory diseases* **34**: 604–608.

Zhang, X.-Y., Xu, Y.-J., Liu, X.-S. & Zhang, Z.-X. (2010) Cigarette smoke extract promotes proliferation of airway smooth muscle cells in asthmatic rats via regulating cyclin D1 expression. *Chinese medical journal* **123**: 1709–1714.

Zhang, Y., Lamm, W., Albert, R., Chi, E., Henderson, W. & Lewis, D. (1997) Influence of the route of allergen administration and genetic background on the murine allergic pulmonary response. *Am. J. Respir. Crit. Care Med.* **155**: 661–669.

Zheng, W. & Flavell, R. A. (1997) The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89**: 587–596.

Zhou, B., Comeau, M. R., De Smedt, T., Liggitt, H. D., Dahl, M. E., Lewis, D. B., Gyarmati, D., Aye, T., Campbell, D. J. & Ziegler, S. F. (2005*a*) Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nature immunology* **6**: 1047–1053.

Zhou, C., Baïz, N., Zhang, T., Banerjee, S. & Annesi-Maesano, I. (2013) Modifiable exposures to air pollutants related to asthma phenotypes in the first year of life in children of the EDEN mother-child cohort study. *BMC public health* **13**: 506.

Zhou, Q., Sun, Y. & Yao, W. (2005*b*) [Characteristics of the airway inflammation and the relationship to interleukin-17 in severe asthma]. *Zhonghua jie he he hu xi za zhi =*

Zhonghua jiehe he huxi zazhi = Chinese journal of tuberculosis and respiratory diseases **28**: 630–634.

Zhu, Z., Homer, R. J., Wang, Z., Chen, Q., Geba, G. P., Wang, J., Zhang, Y. & Elias, J. A. (1999) Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *The Journal of clinical investigation* **103**: 779–788.

Zijlstra, G. J., Ten Hacken, N. H. T., Hoffmann, R. F., van Oosterhout, A. J. M. & Heijink, I. H. (2012) Interleukin-17A induces glucocorticoid insensitivity in human bronchial epithelial cells. *The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology* **39**: 439–445.

Zlotkowska, R. & Zejda, J. E. (2005) Fetal and postnatal exposure to tobacco smoke and respiratory health in children. *European journal of epidemiology* **20**: 719–727.

Zmirou, D., Gauvin, S., Pin, I., Momas, I., Sahraoui, F., Just, J., Le Moullec, Y., Bremont, F., Cassadou, S., Reungoat, P., Albertini, M., Lauvergne, N., Chiron, M. & Labbe, A. (2004) Traffic related air pollution and incidence of childhood asthma: results of the Vesta case-control study. *Journal of Epidemiology and Community Health* **58**: 18–23.

Zosky, G. R., Larcombe, A. N., White, O. J., Burchell, J. T., Janosi, T. Z., Hantos, Z., Holt, P. G., Sly, P. D. & Turner, D. J. (2008) Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology* **38**: 829–838.