

Mechanisms Underlying the Immune Response to Inhaled House Dust Mite

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Abstract

Asthma is a chronic inflammatory disease of the airways and is characterised by airway hyperresponsiveness (AHR), inflammation and remodelling. House dust mite (HDM; *Dermatophagoides pteronyssinus*) is a complex aeroallergen, commonly associated with development of allergic asthma. Animal models have been utilised extensively to model the traits of asthma and HDM-induced allergic airways disease was established in mice following serial HDM challenge via the respiratory mucosa. Mice exposed to HDM developed pulmonary eosinophilia, characterised by Th2 cytokine production, concomitant with AHR and airway remodelling.

Following the establishment and characterisation of this model of allergic airways disease, it was next investigated which features of the HDM allergen were responsible for disease pathogenesis. Using genetically modified mice and pharmacological approaches, it was found that the TLR-TRIF signalling pathway played a crucial role in HDM-induced allergic airways disease. Intrinsic protease activity of the HDM extract was also observed to be vital for disease pathogenesis, whereby mice exposed to boiled HDM developed a less severe asthma phenotype.

Utilising a pan neutralising antibody directed towards transforming growth factor- β (TGF- β), TGF- β was shown to play a critical role in regulating HDM-induced airway inflammation *in vivo*. Following therapeutic blockade of TGF- β , the numbers of CD4⁺CD25⁺FoxP3⁺ regulatory T cells and CD4⁺IL-10⁺ cells were decreased resulting in exacerbation of AHR and BAL inflammation compared to HDM-treated isotype control mice. However, HDM-induced airway remodelling progressed independently of TGF- β .

In conclusion, it has been determined that HDM-induced Th2-driven inflammation, AHR and airway remodelling in mice is induced by multiple features of the allergen and that TGF- β regulates HDM-driven airway inflammation. Thus these findings provide an insight into the mechanisms by which the aeroallergen HDM promotes allergic disease and will aid in the development of potential new therapeutic strategies for the treatment of asthma.

Statement of Contribution

All of the laboratory work in this thesis is my own, with the following exceptions. I received assistance from Lisa Gregory in analysis of the *in vivo* experiment in Chapter 3 and from Gaynor Campbell in setting up the *in vitro* protease assay in Chapter 5. Leukocyte Biology Histology Service and Lorraine Lawrence embedded, sectioned and stained all the lung sections with haematoxylin and eosin, periodic acid schiff and sirius red.

Benjamin Causton 2011.

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Abbreviations

Symbols	
α	Anti
β	Beta
δ	Delta
γ	Gamma
κ	Kappa
μ	Micro
A	
Ab	Antibody
AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
AHR	Airway hyperresponsiveness
AJ	Adherens junction
APC	Antigen presenting cell
APC (Flow cytometry)	Allophycocyanin
ASM	Airway smooth muscle
ASMC	Airway smooth muscle cell
ATP	Adenosine triphosphate
B	
BAL	Bronchoalveolar lavage
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
C	
CCL	CC chemokine
CCR	CC chemokine receptor
CD	Cluster of differentiation
CD200R	CD200 receptor
cDNA	Complementary deoxyribonucleic acid
Cdyn	Dynamic compliance
CLR	C-type lectin receptor
CXCL	CXC chemokine
D	
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
E	
E-64	Epoxy succinyl-L-leucylamido(4-guanidino)butane, L- <i>trans</i> -3-Carboxyoxiran-2-carbonyl-L-leucylagmatine, N-(<i>trans</i> -Epoxy succinyl)-L-leucine 4-guanidinobutylamide
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
EMTU	Epithelial mesenchymal trophic unit

F	
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box protein-3
G	
G-CSF	Granulocyte-colony stimulating factor
GATA-3	GATA-binding protein 3
GM-CSF	Granulocyte/monocyte-colony stimulating factor
GPCR	G-protein coupled receptor
H	
H&E	Haematoxylin and eosin
HDM	House dust mite
I	
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
IP-10	Interferon-gamma-induced protein-10
IRF	Interferon response factor
i.t.	Intratracheal
K	
KC	Keratinocyte-derived chemokine
KO	Knock out
L	
LAP	Latency activated peptide
LBP	Lipopolysaccharide-binding protein
LN	Lymph node
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LT	Leukotriene
M	
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCh	Methacholine
mMCP-1	Mast cell protease-1
MCP-1	Monocyte chemoattractant protein-1
MCPT7	Mast cell tryptase 7
mDC	Myeloid dendritic cell
MDC	Monocyte-derived chemokine
MDCK	Madin-Darby canine kidney
MIP-3 α	Macrophage inflammatory protein-3 alpha
MMP	Matrix metalloproteinase
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
N	
NBP-VANA	N-Benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B

	cells
NK	Natural killer
NLR	Nucleotide oligomerisation domain-like receptor
NOD	Nucleotide oligomerisation domain
O	
OVA	Ovalbumin
P	
PAMP	Pathogen-associated molecular pattern
PAR	Protease activated receptor
PAS	Periodic acid schiff
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
R	
RANTES	Regulated upon activation normal T cell expressed and secreted
RBM	Reticular basement membrane
RI	Dynamic lung resistance
RIG	Retinoic-acid-inducible gene-I
RLR	Retinoic-acid-inducible gene-I-like receptor
RNA	Ribonucleic acid
ROR γ t	RAR-related orphan receptor gamma
RPMI	Roswell park memorial institute medium
S	
SAPS	1-stearoyl-2-arachidonoyl- <i>sn</i> -glycero-3-[phospho-L-serine]
SEM	Standard error of the mean
SP	Surfactant protein
Syk	Spleen tyrosine kinase
T	
TARC	Thymus activated and regulated chemokine
Tbet	T-box family transcription factor
TGF- β	Transforming growth factor-beta
Th	T helper
TIMP	Tissue inhibitors of metalloproteinase
TIR	Toll/Interleukin-1 receptor
TJ	Tight junction
TL	Tethered ligand
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor-alpha
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
W	
<i>wt</i>	Wild-type
Z	
ZO	Zonula occludens

Chapter 1 - Introduction

1.1. Asthma

Approximately 300 million people worldwide suffer from asthma, with an estimated 8 million sufferers in the UK alone (Holgate, 2008; Holgate and Polosa, 2008). The prevalence of allergic sensitisation and severity of asthma has rapidly increased to epidemic proportions over the last 50 years and disease control has become associated with an escalating economic burden to the NHS (Eder et al., 2006; Bahadori et al., 2009).

Asthma is a complex heterogeneous condition, believed to arise from interplay between environmental and genetic factors. These interactions culminate in an inappropriate inflammatory response to normally harmless airborne allergens (Nelson, 2001; von Mutius, 2009) (summarised in Figure 1.1). Asthma is a chronic immune-mediated inflammatory disorder, which is characterised by attacks of wheezing and breathlessness due to bronchoconstriction, airway inflammation, mucus secretion, airway hyperresponsiveness to non-specific stimuli and airway wall remodelling.

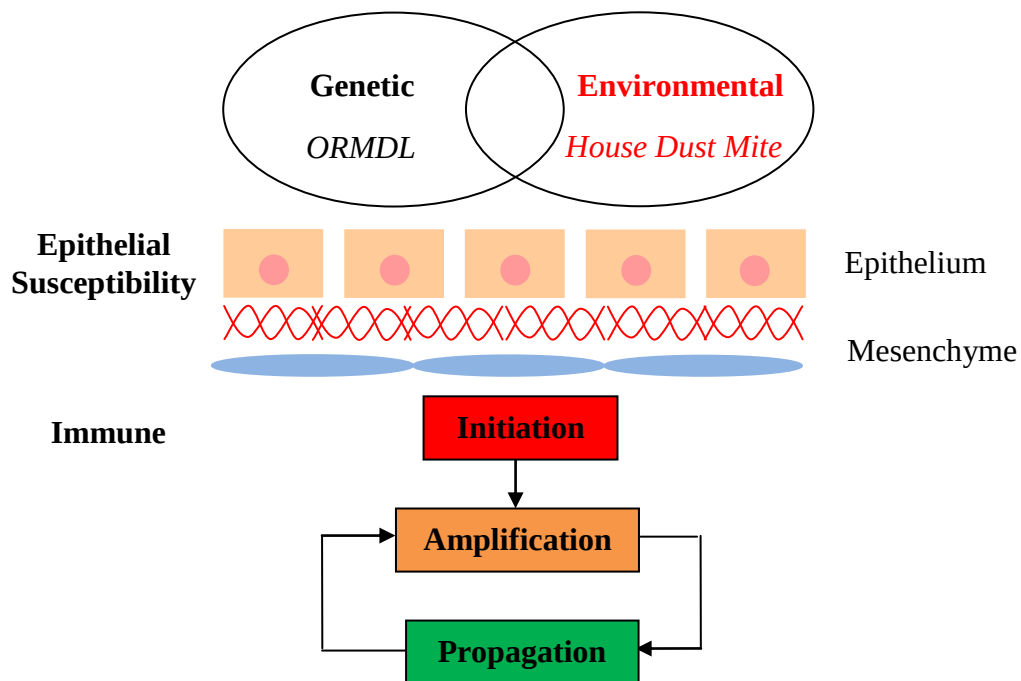


Figure 1.1. The heterogeneity of asthma.

Asthma is a complex disease caused by multiple factors. Local airway susceptibility factors together with allergen specific immune responses interact both in the induction and development of the disease phenotype.

Asthma encompasses several distinct clinical phenotypes that can be divided into different disease entities with specific characteristics. The different disease phenotypes can be further defined by specific underlying pathophysiological mechanisms and, using cluster analysis, five individual clinical phenotypes of asthma have been identified (Moore et al., 2010). Following these developments it is believed that these new classifications can be used in clinical study and drug development design to direct existing and novel therapies to patients in a more specific and tailored manner (Fajt and Wenzel, 2009; Hastie et al., 2010).

Clinically, the major focus of treatment and research over the past 25 years has been on allergic asthma, the most common form of the disease (Nelson, 2001; Karjalainen et al., 2003). Atopy is defined as the genetic predisposition to mount an allergen-specific immunoglobulin (Ig) type E (IgE) response and atopics have positive skin prick tests to common allergens. This IgE-mediated response leads to the development of inflammatory responses in the lung, altered pulmonary physiology and structural changes termed airway remodelling. In atopic asthmatics, airway inflammation is initiated by antigen-specific triggers. House dust mite (HDM) is the most common allergen associated with atopy and allergic asthma (Fernández-Caldas, 2002; Cates et al., 2007; Arshad, 2010), with 50 – 85% of atopic asthmatics HDM allergic (Nelson et al., 1996). In addition to this, the airway epithelium is now believed to be central to asthma pathogenesis (Holgate, 2008). In conjunction with clinical studies, mouse models have been used to investigate the immunopathogenesis of asthma and studies of such models have been invaluable in dissecting numerous immunological processes underlying the pathophysiology of allergen sensitisation, disease propagation and regulation. A murine model of HDM-induced allergic airways disease is the focus of this thesis.

1.1.1. Allergic Airway Hyperresponsiveness

Asthma is defined clinically by breathlessness, wheeze and a variable airflow obstruction (Bousquet et al., 2000). This is believed to account for the dramatic increase in responsiveness to stimulus of the conducting airways known as airway hyperresponsiveness (AHR) (Hargreave et al., 1986). AHR is a key characteristic of the asthma phenotype and is defined as ‘the ease with which the airways narrow in

response to a bronchoconstrictive challenge and manifests as a combination of increased sensitivity and reactivity for a given stimulus' (Juniper et al., 1981). Although AHR is a fundamental component of asthma, the specific causes leading to altered airway responsiveness in asthmatics are still unclear (Holgate, 2008). AHR is believed to be mediated by impaired function and contraction of the underlying airway smooth muscle (ASM) as most acute asthmatic symptoms can be improved by use of smooth muscle relaxants such as β -agonists (Gil and Lauzon, 2007). Although airway inflammation can modify the behaviour of ASM, the extent of airway inflammation does not correlate with the degree and severity of AHR (Jiang et al., 1992; Mitchell et al., 1994; Crimi et al., 1998; Fernandes et al., 2003). This has led to the hypothesis that inflammation induces alterations in the airway wall that make the structure more sensitive and responsive to stimuli. These airway changes include increases in muscle content, increased mucus secretion and desquamation of airway epithelium (Wardlaw et al., 1988; Blosser et al., 1994; Du et al., 1996; Liggett., 1997).

1.1.2. Allergic Airway Inflammation

The inflammatory immune response in the asthmatic airways is diverse and heterogeneous and aspects of both the innate and adaptive immune systems have been implicated in the development of asthma (Holgate, 2008; Holt and Sly, 2011; Sly and Holt, 2011). The allergic inflammatory cascade features mediator release and multi-cellular leukocyte infiltration to both the lung tissue and airway lumen. These inflammatory infiltrates contribute to and mediate many of the immunopathological features of asthma (Kraft et al., 1999; Kay, 2005; Holgate, 2008). The allergic response is dominated by an adaptive T helper 2 (Th2) response, IgE antibody production and eosinophil infiltration of the airways (summarised in Figure 1.2).

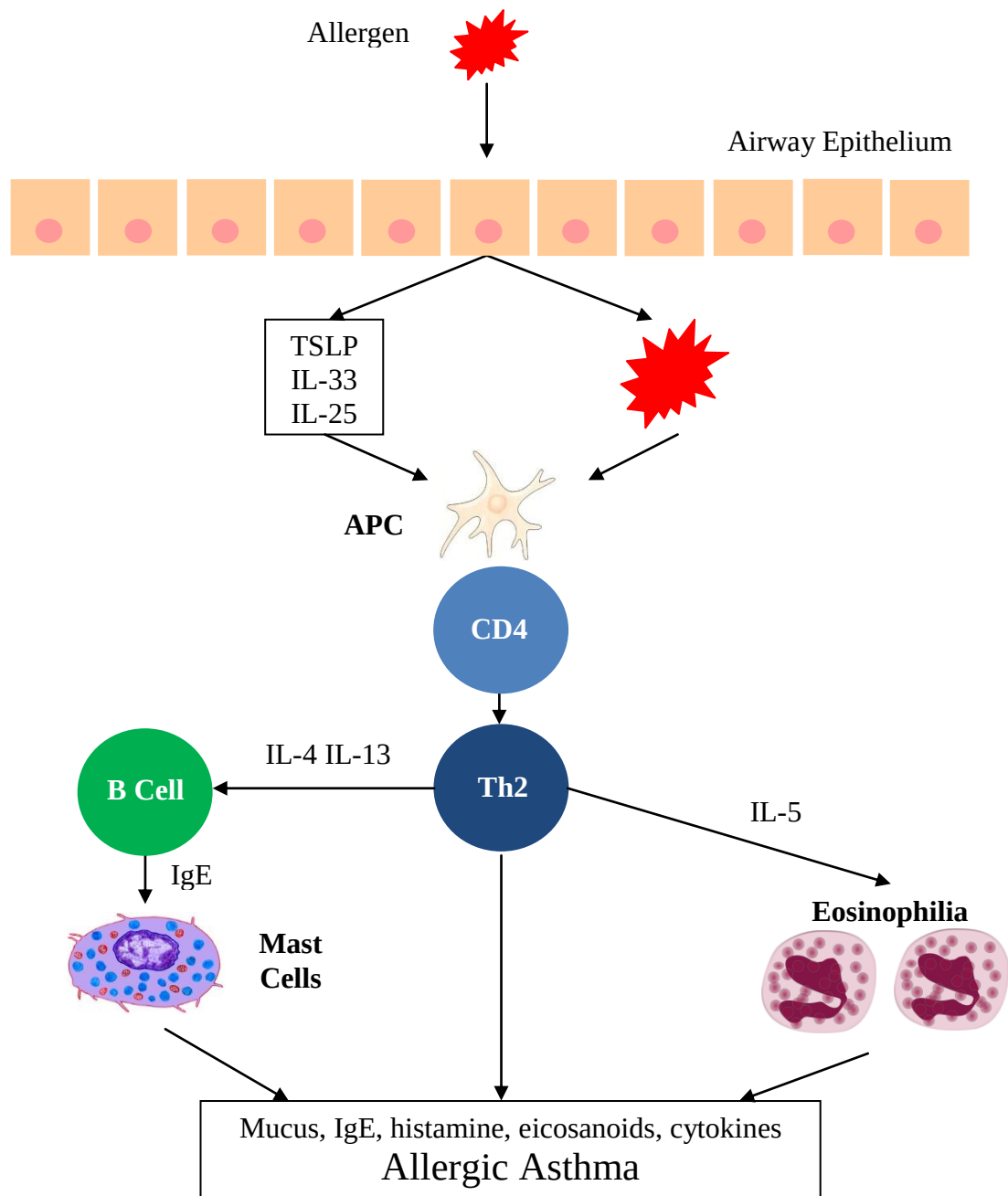


Figure 1.2. Immune cells and the inflammatory cascade in allergic asthma

Allergen exposure drives an adaptive Th2 response. This response can be initiated either by the allergen acting directly on antigen presenting cells (APCs) residing in the airway mucosa or, alternatively, these APCs can be induced via the release of epithelial cell-derived cytokines TSLP, IL-33 and IL-25 (IL-17E). Subsequent allergen exposures cause inflammatory cell recruitment, activation and mediator release. IgE-sensitised mast cells degranulate, releasing mediators such as histamine, leukotrienes and cytokines, which promote vascular permeability, smooth-muscle contraction and mucus production.

1.1.2.1. Initiation of Allergic Airway Inflammation

The pulmonary epithelium is the first point of contact for inhaled environmental allergens in the lung and is now hypothesised as a central player in the development of Th2-driven inflammation (Holgate, 2008). The relationship and interactions between the pulmonary innate immune system and surrounding tissue are essential for allergen sensitisation (Suarez et al., 2008). Residing in the airway mucosa are dendritic cells (DCs) that are essential APCs for priming, initiation and maintenance of Th2-driven inflammation following allergen exposure (Hammad and Lambrecht, 2008). Both airway epithelial cells and DCs express a number of receptors that enable these cells to detect a wide range of inhaled allergens. These receptors include pattern-recognition receptors (PRRs) which recognise a broad range of invariant structures such as microbial components, known as pathogen-associated molecular patterns (PAMPs) (Gordon, 2002; Akira et al., 2006). Many allergens are contaminated with PAMPs enabling them to induce DC maturation. DC maturation itself involves the up-regulation of co-stimulatory molecules and enables DCs to migrate to the draining lymph nodes (LNs) to prime an antigen-specific T cell response (de Heer et al., 2005; Condon et al., 2011). However, not all allergens are intrinsically immunogenic or activate PRRs. Danger signals or damage-associated molecular patterns (DAMPs) in the absence of infection can also influence DC activation (Rubartelli and Lotze, 2007; Willart and Lambrecht, 2009).

Epithelial cells can directly modulate DC function through direct cell–cell interaction and via the release of mediators (Hammad and Lambrecht, 2008). A group of pro-allergic innate cytokines are central to the recruitment and activation of DCs and the initiation of Th2 responses. Allergens can directly stimulate the production of thymic stromal lymphopoietin (TSLP), IL-25 (IL-17E), IL-33 and CCL20/MIP-3 α from airway epithelial cells. These mediators are able to direct a polarised Th2 response and further maintain the salient features of asthma. However, it is important to note that although airway epithelial cells are important cellular sources of TSLP, IL-25 (IL-17E), IL-33 and CCL20/MIP-3 α , other cells present in the allergic inflammatory cell infiltrate, such as eosinophils, also produce these cytokines.

TSLP

TSLP is a 140-amino-acid cytokine of the IL-7 family and is a powerful stimulus of Th2 inflammation (Kato et al., 2007; Ziegler and Artis, 2010). TSLP potently modulates DCs by binding to the TSLP receptor complex on DCs, which is composed of IL-7 receptor and the TSLP receptor (TSLPR) (Liu et al., 2007). TSLP can activate DCs and induce maturation, thereby promoting Th2 inflammation, by increasing DC expression of OX40L (Soumelis et al., 2002; Ito et al., 2005; Seshasayee et al., 2007; Liu, 2007). The polarisation of Th2 cells induced by TSLP-matured DCs is further enhanced by IL-25 (IL-17E), which is produced by epithelial cells, basophils and eosinophils (Wang et al., 2007).

TSLP expression is detected in the airways of asthmatics and TSLP mRNA expression correlates with disease severity (Ying et al., 2005). In mice, TSLP mRNA expression is higher in the lungs of ovalbumin (OVA)-treated mice and lung-specific expression of TSLP induces airway inflammation and AHR (Zhou et al., 2005). Mice over-expressing TSLP in the airway epithelium develop features of allergic airways disease (Zhou et al., 2005), whereas TSLPR-deficient mice have attenuated OVA-induced AHR (Al-Shami et al., 2005), likely as a consequence of a reduction in the migration of airway DCs, resulting in diminished CD4⁺ T cell priming (Shi et al., 2008).

IL-25 (IL-17E)

IL-25 (IL-17E) is a member of the IL-17 cytokine family (Fort et al., 2001). IL-25 (IL-17E) is produced from a variety of cells including epithelial cells (Angkasekwinai et al., 2007) and mast cells (Ikeda et al., 2003) in mice in response to allergen exposure, as well as from activated eosinophils and basophils (Hurst et al., 2002; Angkasekwinai et al., 2007). Greater IL-25 (IL-17E) levels are detected in the bronchial submucosa of asthmatics compared to non-asthmatics (Letuve et al., 2006; Wang et al., 2007).

In mouse models of asthma, several reports have shown that IL-25 (IL-17E) amplifies Th2 cytokine production and eosinophilia and that IL-25 (IL-17E) acts on both the

innate and adaptive immune systems to amplify Th2 responses. Elevated IL-25 (IL-17E) mRNA expression has been shown in the lungs of allergen challenged mice (Angkasekwinai et al., 2007) and blockade of IL-25 (IL-17E) signalling, prior to allergen exposure, results in attenuated AHR, Th2 cytokine production and allergen-specific IgE (Ballantyne et al., 2007). Direct instillation of IL-25 (IL-17E) to the airways of mice has been shown to promote AHR and over expression of IL-25 (IL-17E) by epithelial cells leads to mucus production and airway inflammation (Sharkhuu et al., 2006). IL-25 (IL-17E) enhances OVA-induced AHR (Tamachi et al., 2006) and IL-17RB⁺ NKT cell numbers (Terashima et al., 2008; Stock et al., 2009). Furthermore, the administration of recombinant IL-25 (IL-17E) induces the production of IL-4, IL-5 and IL-13 from an innate non-B cell, non-T cell c-Kit⁺FcεRI⁻ cell population (Fallon et al., 2006).

IL-33

IL-33 is a member of the IL-1 cytokine family and is expressed by bronchial epithelial cells, fibroblasts, smooth muscle cells, macrophages, DCs, basophils and mast cells (Schmitz et al., 2005; Liew et al., 2010). IL-33 has also been shown to be released from cells undergoing necrosis to then act as a pro-inflammatory endogenous danger signal (Luthi et al., 2009). Thus, in addition to its direct pro-inflammatory role, IL-33 could also act as a DAMP or an 'alarmin' (Moussion et al., 2008). IL-33 is the ligand for the receptor ST2 and has been strongly associated with the promotion of Th2 responses (Schmitz et al., 2005). Greatest ST2 expression is seen on mast cells and Th2 cells (Moritz et al., 1998; Coyle et al., 1999), which has led to the association of the ST2/IL-33 axis with asthma. Interaction of IL-33 with ST2 on a range of different leukocytes promotes a number of key inflammatory pathways that have the potential to initiate and propagate allergic inflammation (Liew et al., 2010). IL-33 acts in synergy with stem cell factor and the IgE receptor to activate human mast cells and basophils (Silver et al., 2010). Incubation of human basophils with IL-33 increases mRNA expression of IL-4 and IL-13 (Suzukawa et al., 2008). IL-33 also enhances the survival of eosinophils and eosinophil degranulation in humans (Cherry et al., 2008; Chow et al., 2010; Stolarski et al., 2010). IL-33 expression has been shown in airway smooth muscle cells (ASMCs) and airway epithelial cells and higher

expression of IL-33 was seen in asthmatics than non-asthmatics (Prefontaine et al., 2009; Prefontaine et al., 2010).

Studies from mouse models have shown a vital role for IL-33 in the development of Th2-driven inflammation. Administration of IL-33 concurrent with antigen sensitisation induces the salient features of allergic airways disease (Kondo et al., 2008; Kurowska-Stolarska et al., 2008; Kurowska-Stolarska et al., 2009) and genetic overexpression of IL-33 also leads to eosinophilic inflammation (Zhiguang et al., 2010). Blockade of ST2 abrogated Th2 cytokine production, eosinophilic inflammation and AHR (Coyle et al., 1999) and IL-33 neutralising antibodies attenuated OVA-induced eosinophil recruitment, Th2 cytokine production, serum IgE and mucus production (Liu et al., 2009).

However, the role of the IL-33 signalling in promoting allergic airway disease is complex, as ST2 knockout (KO) mice develop OVA-induced airway inflammation and eosinophilia (Hoshino et al., 1999; Townsend et al., 2000) and IL-33 can also cause airway inflammation in the absence of T and B cells (Kurowska-Stolarska et al., 2008; Kurowska-Stolarska et al., 2009). In the gut of mice IL-33 has been shown to cause IL-4 and IL-13 production from either nuocytes (Neill et al., 2010), multipotent progenitor type-2 cells (Saenz et al., 2010) or natural helper cells (Moro et al., 2010). Since the gut is also a mucosal surface, IL-33 induced production of Th2 cytokines from these innate cells may also occur in the lung, thereby bypassing any requirement for T and B cells.

CCL20/MIP-3 α

Rapid recruitment of DCs into the bronchial mucosa has been observed in animal studies (Vermaelen and Pauwels, 2003) and in human subjects in response to allergen challenge (Jahnsen et al., 2001). CCL20/MIP-3 α is central to early DC recruitment acting via CCR6 (Greaves et al., 1997; Power et al., 1997; Dieu-Nosjean et al., 2000). CCL20/MIP-3 α is expressed by airway epithelial cells (Starner et al., 2003) and CCL20/MIP-3 α expression is increased in airways of asthmatics (Pichavant et al., 2005). Production of CCL20/MIP-3 α can be induced from airway epithelial cells by multiple stimuli, such as ambient particle matter (Reibman et al., 2003) and the

CCL20/MIP-3 α -CCR6 axis can also lead to mucus production (Kim et al., 2011). CCL20/MIP-3 α -CCR6 has been shown to increase Th2-type airway inflammation in response to antigen and CCR6-deficient mice are resistant to cockroach-induced airway inflammation (Lukacs et al., 2001).

1.1.2.2. Cells and Mediators of Allergic Airway Inflammation

Airway inflammation in asthma is a multi-cellular process involving mainly mast cells, eosinophils, basophils, neutrophils and CD4⁺ T cells. Eosinophilic infiltration is a striking feature of allergic airway inflammation, with non-asthmatics usually possessing sputum eosinophil counts of less than 1.9% with this elevated in approximately 60% of asthmatics (Agbetile and Green., 2011).

Mast cells are effector cells in asthma pathogenesis and are located in both the airway epithelium and deeper layers of the mucosa (Yu et al., 2006). In the asthmatic airways, the majority of IgE is bound by Fc ϵ R1 on mast cells making them highly responsive to inhaled antigen (Robinson, 2004). Mast cells can provide an early source of pro-inflammatory mediators such as IL-4, which can influence the proximal events during allergen-specific effector T cell sensitisation (Bradding et al., 2006), and IL-5 which promotes eosinophil recruitment and activation (Lorentz et al., 1999).

Eosinophilia is a feature of allergic airway inflammation (Kay, 2005). Eosinophils are found in both the lung tissue and the bronchoalveolar lavage (BAL) fluid of asthmatic patients (Lemiere, 2004; Kay, 2005). Recruitment of eosinophils to the airways is mediated by eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26 acting via CCR3 (Ying et al., 1997; Humbles et al., 2002). Although eosinophilia is the most characteristic type of inflammation in asthma, this is neither an exclusive feature nor the only type of inflammation observed and eosinophilic asthma is now considered a distinct phenotype of asthma associated with thickening of the basement membrane and by corticosteroid responsiveness (Fahy, 2009).

Basophils share many of their recruitment mechanisms with eosinophils (Gangur et al., 2003) and are implicated in the development of Th2 inflammation (Siracusa et al., 2011). Basophils express Fc ϵ RI and contribute to the local symptoms of inflammation

and AHR through degranulation and release of eicosanoids and histamines (Falcone et al., 2000). Basophils have also been shown to promote Th2 inflammation via the release of cytokines such as IL-4 (Yoshimoto et al., 1999) and by possessing antigen presenting function (Sokol et al., 2009; Yoshimoto et al., 2009; Perrigoue et al., 2009).

Elevated numbers of neutrophils, without eosinophilia has been reported in the airways of asthmatics (Drews et al., 2009). Elevated neutrophil numbers are associated with severe asthma (Jatakanon et al., 1999; Wenzel, 2006), asthma exacerbations (Wark and Gibson, 2006) and non-atopic asthma (Baines et al., 2011). However, neutrophilic asthma and eosinophilic asthma are not mutually exclusive and considerable overlap has been reported between each (Fahy, 2009).

A fundamental feature of allergen sensitisation is the uptake and processing of inhaled allergens by DCs and the capacity of DCs to generate IL-12 determines the balance between Th1 and Th2 responses, with IL-12 polarising T-cell differentiation in favour of a Th1 response (Kuipers and Lambrecht, 2004). Once polarised, Th2 cells migrate back to the airways under the influence of the chemokines thymus and activation regulated chemokine (TARC)/CCL17 and monocyte-derived chemokine (MDC)/CCL22, which interact with the receptor CCR4 (Garcia et al., 2005; Kallinich et al., 2005). Allergen-specific Th2 cells are central to asthma pathogenesis (Kay, 1997; Lloyd and Hessel, 2010; Robinson, 2010) and there is vast experimental and clinical evidence supporting the critical importance of Th2 cells during allergic sensitisation and propagation (Robinson et al., 1992; Del Prete et al., 1993). In asthmatics, an increase in effector Th2 cells and their mediators have been reported in the bronchial mucosa and BAL fluid and elevated Th2 numbers have also been correlated with disease severity (Walker et al., 1991; Corrigan and Kay, 1992).

Th2 cells are potent producers of a range of cytokines, the majority of which are expressed on the long arm of chromosome 5, namely IL-4, IL-5 and IL-13 (Kay, 2006; Robinson, 2010). Together these cytokines promote the salient features of asthma such as AHR, airway inflammation and tissue remodelling (Robinson et al., 1992; Robinson et al., 1993; Larche et al., 2003). IL-4 and IL-13 are involved in the class-switching of B cells to IgE synthesis (Finkelman et al., 1990) and promote mucus secretion and fibrosis (Kumar et al., 2004; Fattouh and Jordana, 2008). Th2

cytokines also activate secondary effector cells in asthma including the recruitment of mast cells (IL-4 and IL-13) and basophils (IL-4) (Bradding et al., 2006), while IL-5 supports growth, differentiation, and activation of eosinophils (Paul and Seder, 1994; Ying et al., 1997). IL-13 is also strongly linked to the development of AHR (Grunig et al., 1998; Wills-Karp, 2004; Kumar et al., 2004).

Traditionally, the asthmatic response was considered to be dominated by Th2 lymphocytes. However, it is now clear that additional pro-inflammatory and regulatory T cell subsets are present in the allergic lung (Afshar et al., 2008), and it is now believed that asthma may not be controlled by Th2 cells (Lloyd and Hessel, 2010). Th1 and Th2 effector cell subsets once represented the paradigm of functional naïve CD4⁺ T cell differentiation (Mosmann and Coffman, 1989; Mosmann et al., 2009). However, the classical Th1-Th2 paradigm of CD4⁺ T cell differentiation has been reorganised following the discovery of Th17 cells, which rendered the original Th1/Th2 dichotomy too simplistic (Dong, 2008; Murphy and Stockinger, 2010). Th17 cells, identified by the unique transcription factor ROR γ t and ability to secrete IL-17, have been implicated in neutrophil-dominated asthma (Molet et al., 2001; Hellings et al., 2003; Bullens et al., 2006). Furthermore Th effector differentiation was originally considered a terminal event (Murphy et al., 1996; Grogan et al., 2001) and this view has since been revised following the recent identification of Th9 effector cells. Th9 cells were induced from differentiated Th2 cells, suggesting that functional plasticity may exist within the Th subsets (Dardalhon et al., 2008; Veldhoen et al., 2008). The Th source of IL-9 was originally thought to be Th2 cells, however the precise Th source of IL-9 requires clarification following the identification of Th9 cells. Two recent reviews by Mucida and Cheroutre, and Zygmunt and Veldhoen give the reader an up to date overview of CD4⁺ differentiation and plasticity that can occur between subsets (Mucida and Cheroutre., 2010; Zygmunt and Veldhoen, 2011).

1.1.2.3. Immune Tolerance and Regulation: Regulatory T Cells, TGF- β and IL-10

The lung is exposed to the external environment and numerous innocuous antigens on a daily basis. Therefore, the lung maintains a state of immune tolerance to these harmless stimuli to preserve pulmonary homeostasis and to prevent chronic

inflammation. Homeostasis within the lung is an active process that is maintained by site-specific mechanisms by lung resident cells (Snelgrove et al., 2011). Immune homeostasis in the lung is achieved by the balance of inflammatory and modulatory cytokines, as well as the presence of a key subset of regulatory T cells (Tregs) (O'Garra et al., 2004; Li et al., 2006b; O'Garra et al., 2008). It is vital that these homeostatic mechanisms distinguish harmless airborne antigens, entering via the airways, from pathogens.

Tregs are essential for the maintenance of immunological tolerance and immune homeostasis in the airways. The induction of tolerance in the airways in animal models has been correlated with the induction of Tregs, with repeated exposure of mice to low-dose allergen promoting the development of a regulatory CD4⁺ T cell population that expressed transforming growth factor- β (TGF- β) and Forkhead box protein-3 (FoxP3) (Ostroukhova et al., 2004). A higher dose of inhaled allergen stimulated the development of a regulatory T cell population that secreted IL-10 (Akbari et al., 2002). Further to this, repeated exposure of mice to inhaled antigen stimulated pulmonary DCs to produce IL-10, which were then able to induce the development of IL-10-producing Tregs (Akbari et al., 2002). Adoptive transfer of IL-10-producing DCs prevented the development of allergic inflammation after subsequent allergen challenge of recipient mice (Akbari et al., 2002).

Tregs also regulate Th1, Th2, and Th17 effector functions and thus play a key role in controlling inflammatory responses (O'Garra et al., 2004; O'Garra and Vieira, 2004). Tregs form part of the constitutive T cell population in both mouse and humans, accounting for 5-10% of peripheral CD4⁺ T cells (Belkaid et al., 2002). Tregs can either be naturally occurring (nTreg), derived from the thymus, or induced (iTreg) outside of the thymus from naïve CD4⁺ cells during chronic inflammation and during homeostasis. Thymically derived regulatory CD4⁺ T cells that express high levels of the IL-2 receptor- α chain, CD25, are maintained by TGF- β (Wan and Flavell, 2007). The transcription factor, FoxP3 is important in the generation of CD4⁺CD25⁺FoxP3⁺Tregs and is also used as an intracellular identification marker (Fontenot et al., 2003; Khattri et al., 2003). iTregs are divided into 3 subsets: (1) FoxP3⁺ iTregs, (2) IL-10-secreting CD4⁺CD25⁺FoxP3⁻ Tr1 cells and (3) TGF- β expressing CD4⁺FoxP3⁻ Tr3 cells (Ray et al., 2010).

Tregs elicit regulatory function by a variety of mechanisms. Interactions between Treg stimulatory (IL-2, CTLA-4) and inhibitory (GITR, CD28) receptors with their ligands on DCs (CD80/CD86) have a suppressive function on antigen presentation (Read et al., 2006). Furthermore, direct cell-cell inhibition can be achieved via membrane-bound TGF- β (Wilczynski et al., 2008). Tregs can also induce anergy in effector cells through secretion of immunosuppressive cytokines such as IL-10 and TGF- β in a process known as "bystander suppression" (Wilczynski et al., 2008). TGF- β is also required for FoxP3 expression and for the differentiation of naive CD4 cells to CD4⁺CD25⁺FoxP3⁺ nTregs (Chen et al., 2003). Alveolar epithelial cells promote the development of FoxP3⁺ cells via a TGF- β -dependant mechanism in mice (Gereke et al., 2009) and mice carrying a dominant-negative mutation in the TGF- β receptor on CD4⁺ T cells show profound inflammation in the lungs, as well as the liver and the pancreatic islets, with impaired development of Tregs (Li et al., 2006a). Together these observations showed a vital role for TGF- β and Tregs in maintaining T cell and lung homeostasis.

An important role for CD4⁺CD25⁺FoxP3⁺Tregs in immune regulation of asthma has been reported (Ling et al., 2004). CD4⁺CD25⁺FoxP3⁺Tregs from non-allergic donors but not allergic donors suppressed proliferation and Th2 cytokine secretion by CD4⁺CD25⁻T cells (Ling et al., 2004). A direct link between CD4⁺CD25⁺FoxP3⁺Tregs and suppression of allergen-induced AHR (Strickland et al., 2006; Joetham et al., 2007) and inflammation has now been demonstrated *in vivo* (Leech et al., 2007). Together with these observations, depletion of CD4⁺CD25⁺Tregs in mice elevates susceptibility to AHR (Lewkowich et al., 2005). Transfer of antigen specific Tregs suppressed features of established allergic airway inflammation (Kearley et al., 2005) and prevented the development of airway remodelling (Kearley et al., 2008). This effect was found to be dependent on IL-10 since increased levels of IL-10 were observed after cell transfer and regulation was reversed by blocking the IL-10 receptor. However, the CD4⁺CD25⁺ Tregs were found not to be the source of the IL-10 since the transfer of these cells from IL-10 deficient mice yielded the same result (Kearley et al., 2005).

TGF- β is a key regulatory cytokine and a central role for TGF- β in maintaining immune homeostasis and peripheral tolerance was illustrated when TGF- β KO mice were observed to die soon after birth from multi-organ inflammation (Shull et al., 1992). In addition to promoting FoxP3 expression (Chen et al., 2003), TGF- β also regulates lymphocyte homeostasis, inhibits Th2 and Th1 cell responses, inhibits IgE production and promotes IgA production (Li et al., 2006a). Several *in vivo* studies have illustrated a vital role for endogenous TGF- β in suppressing the development of allergic airways disease. Allergen sensitisation and challenge in mice with reduced TGF- β expression resulted in exacerbated airway disease compared to wild-type (*wt*) animals (Scherf et al., 2005). Intratracheal (i.t.) delivery of TGF- β suppresses allergen-induced inflammation (Joetham et al., 2007) and CD4⁺ T cells engineered to secrete latent TGF- β suppress allergen-specific airway inflammation and AHR (Hansen et al., 2000). Blockade of TGF- β signalling specifically in T cells also results in enhanced AHR, airway inflammation, and increased Th2 cytokine production (Nakao et al., 2000). Collectively, these data show that TGF- β regulates immune responses in the lung and that alterations in the degree of expression of either the cytokine, its receptor, or molecules within its signalling pathway have profound consequences for the maintenance of pulmonary homeostasis.

Although TGF- β has been implicated in CD4⁺CD25⁺FoxP3⁺ control of allergic airways disease (Ostroukhova et al., 2004;Joetham et al., 2007), the majority of studies have described a central role for IL-10, with or without TGF- β in the control of allergic airway inflammation. IL-10 is a key homeostatic and immunosuppressive cytokine (Moore et al., 1993). IL-10 is secreted by many cells including several T cell subsets (Tregs, Th2, Th1) (O'Garra et al., 2004), macrophages and DCs (Moore et al., 1993) and has been shown to inhibit T cell responses through down-regulation of costimulatory molecules on APCs (de Waal Malefyt et al., 1991). IL-10 is thought to contribute to homeostasis in the airways (Borish et al., 1996) and has been shown to modulate many effector functions that are associated with allergic asthma including Th2 activation, IgE production (Nouri-Aria et al., 2004) and eosinophil and mast cell function (Takanashi et al., 1994;Arock et al., 1996;Royer et al., 2001). In human asthmatics, it was found that the numbers of allergen-specific cells producing IL-10 were significantly lower than healthy controls, suggesting that a defect in IL-10 production may contribute to the asthmatic phenotype (Akdis et al., 2004).

Diminishing IL-10 can break tolerance to allergens suggesting a role in regulation of responses to allergens (Borish et al., 1996).

1.1.3. Allergic Airway Remodelling

In addition to AHR and airway inflammation, asthma is also associated with pathological alterations of the bronchial airway structures. Airway remodelling in asthmatic subjects represents a complex multi-cellular process that leads to structural changes involving the composition, content, and organisation of many of the cellular and molecular constituents of the bronchial wall (Boulet et al., 2000;Holgate et al., 2001;Girodet et al., 2011). These structural changes are believed to contribute significantly to the pathophysiology of asthma (James and Carroll, 2000;Durrani et al., 2011) and these modifications are partly reversible in mild asthma, but are mostly irreversible in chronic severe asthma (Vignola et al., 2003).

Airway remodelling encompasses a wide range of structural changes in the airways including epithelial detachment, compromised epithelial function, sub-epithelial fibrosis and increased fibroblast proliferation (Jeffery et al., 1989;Knight et al., 1994;Trautmann et al., 2002). Changes to the underlying mesenchyme occur including dysregulated extracellular matrix (ECM) protein deposition (Boulet et al., 1997), sub-epithelial fibrosis (Roche et al., 1989;Kaminska et al., 2009) and increased peri-bronchiolar ASM mass (Lambert et al., 1993). Goblet cell hyperplasia and angiogenesis are also features of airway remodelling (Ordoñez et al., 2001;Vermeer et al., 2003;Vignola et al., 2003). Current asthma therapies such as inhaled corticosteroids are the most effective anti-inflammatory therapy for asthma, however, there is minimal impact on airway remodelling (Chakir et al., 2003;Ward and Walters, 2005) and this has led to problems for disease management (Kariyawasam et al., 2007;Kelly et al., 2010). For a current overview on the development of airway remodelling in asthma and the effects of current asthma therapies treatment on these processes, these have been recently reviewed (Al-Muhsen et al., 2011;Durrani et al., 2011).

The mechanisms that regulate airway remodelling and the precise sequence of events that take place in the course of the remodelling process are not fully understood.

Opinion is divided as to whether inflammation drives airway remodelling or whether an intrinsic alteration in the bronchial wall induces chronic inflammation (Holgate, 2008). Airway remodelling was traditionally thought to be initiated as a repair process in response to airway injuries resulting from sustained airway inflammation. Exposure to allergens was hypothesised to be responsible for impaired regulation of ECM proteins, via dysregulation of regeneration and repair mechanisms following airway inflammation. Airway structural cells, such as ASM cells, epithelial cells and fibroblasts were believed to participate in enhancing inflammation and remodelling through the release of cytokines, chemokines and proteins of the ECM (Hakonarson et al., 1999;Johnson, 2001;Panettieri, 2002;Bhandari et al., 2006).

However, emerging theories have challenged the concept that airway inflammation drives the development of airway remodelling. Reactivation of the epithelial mesenchymal trophic unit (EMTU) is a key feature of induction of airway remodelling and has led to the hypothesis that inflammation and remodelling occur in parallel rather than as sequential events. Communication between the epithelium and the underlying fibroblast layer is thought to be similar to the processes that drive branching morphogenesis in the foetus, where the epithelium and the mesenchyme function as a trophic unit. Indeed it has been proposed that the EMTU becomes reactivated during chronic asthma to drive pathological airway remodelling (Holgate et al., 2000;Davies et al., 2003). It is, therefore, conceivable that airway remodelling might precede airway inflammation associated with asthma and this has been investigated in studies of childhood asthma. Reticular basement membrane (RBM) thickening, an early feature of airway remodelling, has been shown in bronchial biopsies from children with moderate asthma (Cokugras et al., 2001) and this was of similar degree to that seen in adult asthma (Warner et al., 1998;Payne et al., 2003). There is also evidence for early bronchial smooth muscle hypertrophy in biopsies from schoolchildren (Hogg et al., 1970;Bai., 1990). Eosinophilic inflammation and RBM thickening, that are characteristics of asthma in older children and adults, are not present in infants with reversible airflow obstruction and there is no correlation with atopy (Sagiani et al., 2005). Indeed, unlike inflammatory markers, ASM remodelling is correlated with asthma severity (Benayoun et al., 2003). Taken together, these findings suggest that structural changes develop very early in the course of the disease and that remodelling may precede the development of chronic

inflammation, and, therefore, could be a crucial component for the development of asthma (Sagiani et al., 2005; Sagiani et al., 2007).

TGF- β and Airway Remodelling

TGF- β has long been associated with airway remodelling in asthma. Numerous clinical studies have shown increased TGF- β expression as detected in lung biopsies by immunohistochemistry and RNA expression (Ohno et al., 1996; Magnan et al., 1997; Minshall et al., 1997; Vignola et al., 1997) and by absolute protein levels in the BAL of asthmatics (Redington et al., 1997). The active signalling of TGF- β , as determined by the nuclear immunostaining of phosphorylated Smad 2 (pSmad 2), and the increased nuclear expression of Smad 4 has also been reported in the bronchial mucosa of asthmatics (Torrego et al., 2007). The airway epithelium is the major site of TGF- β 1 expression in non-asthmatics (Magnan et al., 1994) however, the main source of TGF- β in an allergic airway remains controversial. Some researchers have shown the airway epithelium to be the major source of TGF- β in both asthmatics (Sagara et al., 2002; Phipps et al., 2004) and in animal models of allergic airways disease (Rosendahl et al., 2001; Kelly et al., 2005; Alcorn et al., 2007), whereas others have proposed that eosinophils are the main source of TGF- β in asthmatic airways (Minshall et al., 1997; Vignola et al., 1997; Flood-Page et al., 2003).

TGF- β has been linked directly to the underlying processes in airway remodelling, specifically epithelial changes, sub-epithelial fibrosis, ASM remodelling and goblet cell hyperplasia. In asthmatics, TGF- β –induced apoptosis results in the detachment of epithelial cells (Undevia et al., 2004) and TGF- β has also been shown to induce epithelial-mesenchymal transition (EMT) in primary airway epithelial cells from asthmatics (Hackett et al., 2009). TGF- β signalling promotes ECM synthesis from fibroblasts and ASM (Morishima et al., 2001; Gu et al., 2007). Fibroblasts and myofibroblasts are the primary source of ECM deposition in sub-epithelial fibrosis (Matsumoto et al., 2005) and TGF- β promotes the differentiation of fibroblasts to myofibroblasts, as well as promoting myofibroblast proliferation (Michalik et al., 2009). In addition, TGF- β induces the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), both of which regulate ECM deposition (Mattos et al., 2002). TGF- β can also contribute to fibrosis indirectly

by triggering the production of IL-6, which is linked with increased collagen synthesis and AHR (Gomes et al., 2005). TGF- β can directly promote ASM hypertrophy, which can influence airway responsiveness (Chen and Khalil., 2006; Goldsmith et al., 2006). TGF- β has also been shown to increase the proliferation of ASM cells and TGF- β also enhances the migration of ASM cells, which contributes to tissue remodelling (Chen and Khalil., 2006). TGF- β has also been shown to promote goblet cell hyperplasia, by inducing the transcription and translation of mucin in bronchial epithelial cells (Chu et al., 2004).

In addition to asthmatic patient findings, TGF- β expression is increased in murine models of allergic airway disease, induced by fungal extracts (Blease et al., 2002) and OVA respectively (Kumar et al., 2004; McMillan and Lloyd, 2004; Kelly et al., 2005). Blockade of TGF- β using a TGF- β neutralising Ab reduced OVA-induced airway remodelling (McMillan et al., 2005; Alcorn et al., 2007). TGF- β signalling through Smad 2/3 also mediated fibroblast α -smooth muscle actin expression, which is a marker of myofibroblast differentiation, and Smad 3 KO mice demonstrated reduced OVA-induced airway remodelling (Kobayashi et al., 2006; Le et al., 2007). Following the induction of allergic airway disease, decreases in airway fibrosis were associated with a reduction in myofibroblasts without associated changes in inflammation (Le et al., 2007). Furthermore, overexpression of TGF- β in the lung induced severe fibrosis with ECM deposition (Sime et al., 1997; Kolb et al., 2002).

1.2. The Airway Epithelium: Central to Asthma Pathogenesis

1.2.1. Structure of the Airway Epithelium

The airway epithelium is the first point of contact of inhaled pathogens with the lung and forms a complex physicochemical barrier complemented by the mucociliary escalator to provide an important first line of defence against inhaled pathogens (Vareille et al., 2011). The airway epithelium is pseudostratified in the large airways, becoming columnar and cuboidal in the small airways, and the major cell types are ciliated columnar, undifferentiated, secretory and basal cells (Crystal et al., 2008). Ciliated columnar epithelial cells are the predominant cell type within the airways, accounting for over 50% of all epithelial cells, and the primary role of these cells is the directional transport of mucus from the lung to the throat (Crystal et al., 2008).

Mucus overlying the airway epithelium provides protection by creating a semi-permeable barrier that enables the exchange of nutrients, water, and gases while being impermeable to most pathogens (Voynow and Rubin, 2009). The epithelial basement membrane acts as an anchor for the epithelium and facilitates adhesion and migration of epithelial cells, as well as being essential for regulating the phenotype of epithelial cells and establishing and maintaining polarity. The basement membrane also acts as a barrier between the surface epithelium and the underlying mesenchymal compartments (Terranova et al., 1980; Boudreau et al., 1996).

Epithelial cells are connected by cell-cell junctions located at the apex of columnar cells (Mullin et al., 2005). These junctions are composed of tight junctions (TJs), adherens junctions (AJs), gap junctions and desmosomes that maintain structural integrity (Roche et al., 1993; Knight, 2002). Inter-epithelial junctions form an impermeable and effective mechanical barrier and permit maintenance of an ionic gradient for directional secretion of many substances (Davies and Garrod., 1997), as outlined in Figure 1.3. TJs are the most important for maintaining epithelial integrity and consist of a series of interacting proteins and receptors which ensure impermeability of the barrier and also enable communication between adjacent cells and regulate intercellular transport (Roche et al., 1993). TJs are composed of the proteins claudins, occludin, tricellulins, junctional adhesion molecules, cytoplasmic linker proteins zonula occludens (ZO)-1, ZO-2, and ZO-3, and associated signalling molecules and cell cycle regulators that control proliferation and differentiation (Steed et al., 2010). Under normal circumstances the epithelium forms a highly regulated and almost impermeable barrier through the formation of TJs (Godfrey, 1997). Located below TJs, AJs provide important adhesive contacts between neighbouring epithelial cells. E-cadherin, a component of AJs, is an important regulator of airway epithelial innate immune function (Nawijn et al., 2011). Gap junctions are cell-to-cell channels that allow diffusion of small metabolites, second messengers and ions between neighbouring cells (Mese et al., 2007). Desmosomes are intercellular junctions that provide strong adhesion between cells and form adhesive bonds in a network which gives mechanical strength to tissues (Green and Simpson, 2007). Thus, through the physical presence and the network formed at the surface of the airway mucosa, epithelial cells represent an efficient and crucial first-line of defence against aeroallergens.

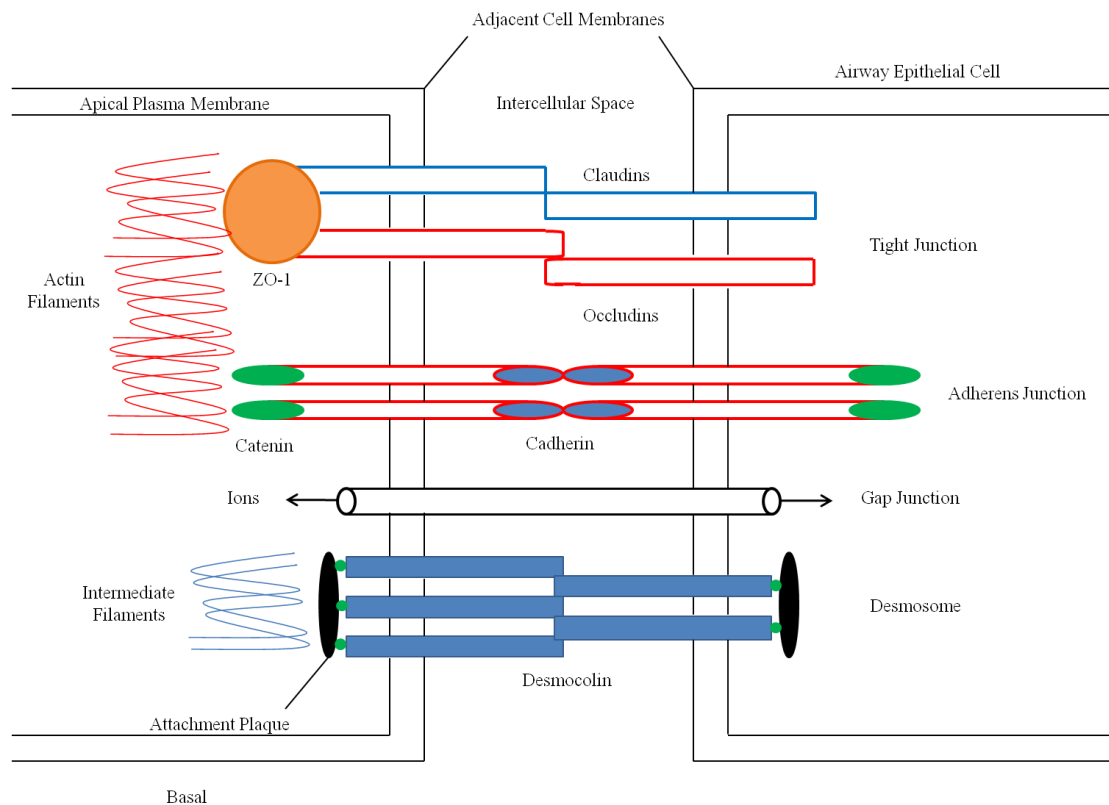


Figure 1.3. An overview of inter-epithelial junctions.

Key components of epithelial tight junctions, adherens junctions, gap junctions and desmosomes are shown. Together these structures maintain an almost impermeable barrier and prevent transepithelial delivery of allergens and other inhaled substances into the basal regions.

1.2.2. Airway Epithelium and Asthma

Previously, the epithelium was considered to be only a physical barrier between the external environment and the inner tissues of the lung. However, the epithelium is now regarded as playing a pivotal role in asthma pathogenesis (Holgate, 2008). Building further on this concept, a new paradigm for persistent asthma has emerged by which a damaged epithelium repairs incompletely and leads to a chronic wound scenario with the secretion of a range of growth factors capable of driving structural changes linked to airway remodelling (Holgate et al., 2009; Crosby and Waters, 2010).

Epithelial Damage and Airway Remodelling

Epithelial desquamation is a pathological feature of asthma (Shebani et al., 2005; Shahana et al., 2006). In asthma, the epithelium is more fragile, compared to non-asthmatics, with easy loss of the columnar cells due to disruption of both TJs and desmosomal attachments (Montefort et al., 1992; Shebani et al., 2005; Shahana et al., 2006; Barbato et al., 2006). Bronchial biopsy studies from asthmatics demonstrate physical damage to the columnar cell layer, epithelial metaplasia and damage, thickening of the sub-epithelial basal lamina and increased numbers of myofibroblasts (Fedorov et al., 2005; Saglani et al., 2005; Barbato et al., 2006). Evidence from asthmatics suggests that a dysfunctional epithelium displaying an abnormal structure and immunological profile can result in a compromised airway epithelial barrier (Diamond et al., 2000; Fokkens and Scheeren, 2000; Knight and Holgate, 2003), with the barrier function of the airway epithelium being shown to be impaired in asthmatics (Knight, 2002; Swindle et al., 2009) and increased epithelial permeability has also been shown *in vivo* (Ilowite et al., 1989). Epithelial TJs in asthmatics have been shown to be severely disrupted and this is correlated with increased epithelial permeability (Wan et al., 2000). It has been reported that expression of ZO-1 and E-cadherin is lower in asthmatics compared to non-asthmatics, suggesting a broad defect in adhesion mechanisms (de Boer et al., 2008; Xiao et al., 2011). Impaired wound healing and failure to form adequate TJs suggest that the epithelium is primarily defective in asthma (Holgate, 2007; de Boer et al., 2008).

The expression of markers of cell stress are also a further indication of epithelial injury in asthmatics (Bertorelli et al., 1998;Comhair et al., 2001;Ercan et al., 2006;Mak et al., 2007;Cohen et al., 2007) and in murine models of allergic airways disease (Truong-Tran et al., 2002). These markers of cell stress include enhanced surface expression of epidermal growth factor receptors (EGFRs) (Polosa et al., 2002;Hamilton et al., 2003;Hamilton et al., 2005) and toll-like receptors (TLRs) (Ritter et al., 2005). Markers of cell stress have also been observed in moderate and severe childhood asthma (Fedorov et al., 2005), suggesting that epithelial injury and aberrant repair is involved at the disease initiation. Evidence of epithelial damage with upregulation of EGFRs and features of impaired proliferation, such as reduced expression of proliferative markers (Puddicombe et al., 2003) suggest that the epithelium is chronically injured and unable to repair properly (Bucchieri et al., 2002;Kicic et al., 2006).

Asthma Susceptibility Genes and the Airway Epithelium

Genetic studies have revealed molecules and pathways that may underlie the origins of asthma (Meurer et al., 2006;Holloway et al., 2010). A number of recently identified asthma susceptibility genes are preferentially expressed in either the epithelium or underlying mesenchyme and smooth muscle. This is in addition to asthma susceptibility genes located in the immune or inflammatory pathways (Cookson, 2004;Vendelin et al., 2005). Asthma susceptibility genes expressed in the epithelium and/or the underlying ASM include *POSTN*, *SERPINB2*, *CLCA1*, *COL29A1*, *DPP10*, *GPRA*, *HLA-G*, *IRAKM* and *PHF11* (Woodruff et al., 2007;Vercelli, 2008). Indeed, many of the molecules that are involved in disordered epithelial–mesenchymal signalling in asthma are also utilised in fetal branching morphogenesis of the lung, suggesting that asthma at least in some of its manifestations has morphogenetic origins (Holgate et al., 2006;Eder et al., 2006). These findings strengthen the idea that asthma occurs as a result of aberrant gene expression within the epithelium and that structural cells are key drivers of the allergic response.

Sentinel Role of the Airway Epithelium

Epithelial cells lining the respiratory tract constitute the primary cellular barrier and orchestrate immune responses to inhaled allergens. Airway epithelial cells possess microbial detection mechanisms, and these cells express a specifically localised set of PRRs (Hammad and Lambrecht, 2008). The PRRs present on the airway epithelium include TLRs, the nucleotide-binding oligomerisation domain protein (NOD)-like receptors (NLRs), and the RNA retinoic-acid-inducible gene-I (RIG)-like receptors (RLRs), as well as protease-activated receptors (PARS) 1 – 4 that allow recognition of protease active allergens. TLRs are a family of PRRs that are type I membrane glycoproteins, consisting of extracellular leucine rich repeats (LRRs) and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, required for PAMP recognition and downstream signalling, respectively (Kawai and Akira, 2010). For a current update on TLRs, NLRs and RLRs and the association with allergic diseases, these have recently been reviewed (Heine, 2011). PARs are a family of cell surface, proteolytically activated G-protein coupled receptors (GPCRs) that have a unique mechanism of activation that distinguishes them from other GPCRs (Macfarlane et al., 2001). When PARs are activated by proteolytic cleavage of a specific site within the receptor N-terminus, a tethered ligand (TL) is produced that binds to and activates the receptor (Coughlin, 2005;Steinhoff et al., 2005;Adams et al., 2011).

The ligation of TLRs and PARs expressed by epithelial cells leads to a cascade of events that culminates in the production of chemokines that attract neutrophils, monocytes and DCs to the airways, and to the production of cytokines that can induce DC maturation including TSLP, IL-25 (IL-17E) and IL-33 (Stumbles et al., 2001;Kiss et al., 2007;Ebeling et al., 2007). The expression of TLRs and PARs enables airway epithelial cells to respond to antigens and allergens, thereby initiating the first step in the host–pathogen interaction. The epithelial barrier influences the development of antigen-specific immunity, suggesting that the type of immune response elicited by the host highly depends on the nature of the response defined by the local tissue microenvironment. Innate immune recognition by barrier cells determines the functional properties of tissue-residing DCs, thereby instructing the outcome of antigen-specific immunity (Hammad and Lambrecht, 2008).

Epithelial Communication with DCs

Airway DCs form a dense network in the lung where they are ideally placed to sample inhaled antigens (Holt et al., 1990; Huh et al., 2003). Mucosal DCs are situated in the basolateral space and are only separated from the inhaled air by the epithelial TJ barrier (GeurtsvanKessel and Lambrecht, 2008). In the airways, DCs can extend dendrites between epithelial cells directly into the airway lumen, thus performing a periscope function enabling continuous immune surveillance of the airway lumen (Jahnsen et al., 2006). In mice intraepithelial airway CD103⁺ DCs express the TJ proteins claudin-1, claudin-7 and ZO-2, which form TJs with airway epithelial cells, thereby allowing DCs to sample the content of the airway lumen without disturbing the function and integrity of the epithelial barrier (Sung et al., 2006). Airway epithelial cells regulate both innate and adaptive immunity through production of functional molecules and physical interactions with cells of the immune system. Activation of epithelial cells results in immediate host defence responses that include production of pro-inflammatory cytokines which recruit and activate other mucosal innate immune cells and initiate mechanisms of adaptive immunity (Hiemstra, 2001). The epithelium can produce a diverse array of lipid mediators, growth factors, and bronchoconstricting peptides, as well as chemokines and cytokines (Stumbles et al., 2001; Reibman et al., 2003; Pichavant et al., 2005). As part of the innate immune function in response to allergens and TLR ligands, epithelial cells produce cytokines that attract and activate DCs including TSLP, IL-25 (IL-17E), IL-33 and CCL20/MIP-3 α , as outlined in 1.1.2.1.

1.3. House Dust Mite

House dust mites (HDM) are arthropods belonging to the subphylum Chelicerata, class Arachnida, order Acari, and suborder Astigmata (Arlan and Platts-Mills., 2001). *Dermatophagoides* was first identified as the source of allergen associated with house dust in 1967 (Voorhorst et al., 1967) and thirteen species have since been found in house dust. The most common of these species are *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* that are found in temperate climates (Arlan and Platts-Mills., 2001). *Dermatophagoides pteronyssinus* is generally found in Europe, and *Dermatophagoides farina* is generally found in North America (Thomas., 2010; Thomas et al., 2010).

Mite bodies and mite faeces are the sources of many allergens (Tovey et al., 1981; Arlian et al., 1987). The allergens associated with mite faecal matter are enzymes that originate from the digestive tracts of the mites. However, some allergens may be components of mite saliva that is left in the environment on food substrates where mites feed. Secretions from the supracoxal glands that are involved in the active uptake of water likely contain proteins, as well as sodium and potassium chloride (Wharton and Furumizo, 1977). After death, soluble protein in body fluids may be released as the mite body disintegrates.

Allergens derived from HDM are perennial and are one of the most frequently associated with allergic asthma, in temperate climates (Fernández-Caldas., 2002; Thomas et al., 2010). House dust can contain a huge number of proteins and structures, many of which have been shown to induce IgE production and many asthmatics have raised levels of HDM-specific IgE (Maunsell et al., 1968; McAllen et al., 1970). The allergens associated with HDM have been identified by analysing aqueous extracts of whole mites, nymphs, faecal pellets and eggs (Thomas et al., 2010).

1.3.1. HDM Allergenicity

Mite allergens are divided into specific groups on the basis of their biochemical composition, sequence homology, and molecular weight, as summarised in Table 1.1. Absolute IgE-binding measurements have been used to establish the hierarchy of the isolated *Dermatophagoides pteronyssinus* allergens (Hales et al., 2006). Laboratory investigations have used preparations of HDM extract in order to elicit allergic responses *in vitro* and *in vivo*. The majority of the allergenicity has been attributed to group 1 (*Der p 1* or *Der f 1*) and group 2 (*Der p 2* or *Der f 2*) allergens (Robinson et al., 1997). The cellular and molecular mechanisms driving the allergic phenotype and sensitisation in mouse models of HDM allergic asthma have recently begun to be elucidated.

Allergen	Biological Action/Homology	Molecular Weight (kDa)	Frequency of Reactivity	Reference
<i>Der p 1</i>	Cysteine protease	24	>90%	(Chruszcz et al., 2009)
<i>Der p 2</i>	MD-2 homologue	15	>90%	(Gafvelin et al., 2001)
<i>Der p 3</i>	Serine protease	31	57 – 90%	(Cheong et al., 2003)
<i>Der p 4</i>	Alpha amylase	60	25 – 46%	(Hales et al., 2007)
<i>Der p 5</i>	Alpha helical protein	14	45 – 60%	(Mueller et al., 2010b)
<i>Der p 6</i>	Serine protease	25	40 – 60%	(Chapman et al., 2007)
<i>Der p 7</i>	Lipid binding protein	26, 30 and 31	50%	(Mueller et al., 2010a)
<i>Der p 8</i>	Glutathione S-transferase	27	40%	(O'Neill et al., 1994)
<i>Der p 9</i>	Serine protease	29	80%	(King C et al., 1996)
<i>Der p 10</i>	Tropomyosin	36	60%	(Yi et al., 2002)
<i>Der p 11</i>	Paramyosin	103	41-67%	http://www.allergen.org/viewallergen.php?aid=291
<i>Der p 12</i>	Chitinase			(Thomas et al., 2010)
<i>Der p 13</i>	Lipocalin			(Batard et al., 2006)
<i>Der p 14</i>	Apolipoprotein	177	70%	(Thomas et al., 2005)
<i>Der p 15</i>	Chitinase	59-61	70%	(O'Neil et al., 2006)
<i>Der p 18</i>	Chitinase	49.2	63%	(O'Neil et al., 2006)
<i>Der p 20</i>	Arginine kinase			(Hales et al., 2007)
<i>Der p 21</i>	Alpha-helical protein (Similar to <i>Der p 5</i>)	15	26%	(Weghofer et al., 2008)
<i>Der p 23</i>	Peritrophin-A	14	Unknown	http://www.allergen.org/viewallergen.php?aid=296

Table 1.1. Characterisation of HDM allergens.

Information was derived from the respective scientific publication or the following allergen websites; www.allergen.org – the official site for the systematic allergen nomenclature approved by the World health organisation and International Union of Immunological Societies. www.allergome.org – contains information on allergenic molecules causing allergic diseases. <http://pfam.sanger.ac.uk>. - protein classification database www.meduniwien.ac.at/allergens/allfam – database for classifying allergens into protein families.

HDM Proteases

Group 1 allergens (*Der p 1* and *Der f 1*) are cysteine proteases and those allergens of groups 3, 6 and 9 are serine proteases (*Der p 3, 6, 9* and *Der f 3, 6, 9*) (Chapman et al., 2007). The protease activity of these allergens accounts for 79% of the proteolytic activity of house dust (Stewart et al., 1994). The protease activity of groups 1, 3, 6 and 9 allows HDM to evoke a wide range of biological effects which may be critical to development of allergen sensitisation. *Der p 1* is one of the common allergens associated with HDM and it has been the most widely and frequently studied. However, in addition to *Der p 1*, the other protease active allergens within HDM and their links to allergy have been investigated.

Der p 1 can directly program Th2 responses by interacting with cells of the immune system. The proteolytic activity of *Der p 1* has been shown to promote the activation, function and behaviour of DCs and the enzymatic activity of this allergen is pivotal in directing DCs to induce Th2 responses, while concomitantly dampening Th1 responses. *Der p 1* increased the expression of CD86 on DCs from HDM allergic patients, which was associated with elevated T cell proliferation and production of the pro-inflammatory cytokines TNF- α and IL-1 β (Hammad et al., 2001). *Der p 1* has also been shown to cleave and decrease the expression of DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and DC-SIGN-receptor (DC-SIGN-R) on DCs, which resulted in reduced Th1 cytokine production (Furmonaviciene et al., 2007; Huang et al., 2011). DCs matured with *Der p 1* also produced significantly less IL-12, due to the cleavage of CD40 by the proteolytic activity of *Der p 1*, which rendered DCs less responsive to stimulation through the CD40L-CD40 pathway (Ghaemmaghmi et al., 2002). Concomitantly, DCs matured in the presence of *Der p 1* produced less IFN- γ and more IL-4 by CD4⁺ T cells, compared to DCs that were matured in the presence of proteolytically inactive *Der p 1* (Hammad et al., 2001; Ghaemmaghmi et al., 2002). *Der p 1*-pulsed DCs from allergic patients also showed increased production of the Th2-associated chemokines TARC/CCL17 and MDC/CCL22, without altering the release of the Th1 associated chemokine IFN- γ -induced protein-10 (IP-10)/CXCL10 (Hammad et al., 2003). The pro-inflammatory effects of *Der p 1* are not restricted to DCs, as basophils upon exposure to *Der p 1* can produce IL-4, IL-5 and IL-13 (Phillips et al., 2003). *Der p 1*

has been shown to further promote Th2 responses by cleaving CD25 on T cells, which results in diminished IFN- γ production (Shakib et al., 1998;Schulz et al., 1998;Harris et al., 2004). A direct link between HDM and IgE production has also been demonstrated, whereby *Der p* 1 cleaves the low affinity IgE receptor (Fc ϵ RII) CD23 on B cells. Since CD23 regulates IgE uptake, by interfering with the negative feedback loop regulating IgE, *Der p* 1 stimulates IgE production as a consequence (Hewitt et al., 1995;Schulz et al., 1995). In addition to having a direct effect on B cells and IgE production, human T cells exposed to *Der p* 1 augment IgE synthesis by B cells (Ghaemmaghami et al., 2001).

Natural protective mechanisms present in the airway are also targeted by *Der p* 1, whereby *Der p* 1 degrades lung surfactant proteins (SP)-A and SP-D that are critical components of the host defence. SP-A and SP-D are calcium-dependent carbohydrate-binding proteins predominantly synthesised and secreted in the lung by alveolar type II cells and Clara cells (Hickman-Davis and Matalon, 1999;Hansen and Holmskov., 2002). In addition to playing a crucial role in innate immune defence by modulating leukocyte function, SP-A and SP-D have also been implicated in the allergic response. SP-A and SP-D interact with *Der p* 1 by competing with their binding to cell-sequestered IgE resulting in inhibition of mast cell degranulation. Thus, the degradation and consequent inactivation of SP-A and SP-D by *Der p* 1 may also contribute to elevated IgE levels and to the potent allergenicity of HDM (Deb et al., 2007). α 1-anti-trypsin protects the lower respiratory tract against damage by proteases and *Der p* 1 catalytically inactivates α 1-anti-trypsin, thereby reducing the defensive capabilities of the airway (Kalsheker et al., 1996). α 1-anti-trypsin deficiency is associated with the onset of childhood asthma and cleaved α 1-anti-trypsin has been shown to be recruit neutrophils *in vivo* (Banda et al., 1988;Lindmark et al., 1990). Thus inactivation of the major natural inhibitor of neutrophil enzymes by *Der p* 1 may exacerbate tissue damage and contribute to inflammation.

The most widely investigated impact of *Der p* 1, 3, 6 and 9, has been on airway epithelial cells, with protease active allergens from HDM increasing epithelial permeability (Herbert et al., 1990;Herbert et al., 1995) and promoting pro-inflammatory cytokine release (Kauffman et al., 2006). *Der p* 1, 3, 6 and 9 have been shown to degrade epithelial TJs and increase epithelial permeability. The protease

active allergens disrupted intercellular TJs, via proteolysis of occludin and ZO-1, desmosomes and adherens junctions, thereby altering the structure and organisation of the airway epithelium (Herbert et al., 1995; Wan et al., 1999; Wan et al., 2001). Importantly, these findings suggest that the disruption of epithelial TJs may be the initial step in the development of asthma to a variety of allergens. By increasing epithelial permeability, conditions would be optimum for the transepithelial delivery of allergens allowing for greater access to APCs residing in the airway mucosa. However, the impaired barrier function observed in asthmatics (Bhure et al., 2009) is not replicated following a single dose of HDM extract when administered to mice *in vivo* (Turi et al., 2011) and, therefore, may be a function of chronic as opposed to acute exposure. Airway epithelial cells upon exposure to *Der p* 1, 3, 6 and 9 release a vast array of pro-inflammatory cytokines and chemokines, including MCP-1/CCL2, IL-6, IL-8, GM-CSF, eotaxin-1/CCL11 (Tomee et al., 1998; King et al., 1998; Sun et al., 2001; Asokanathan et al., 2002a; Asokanathan et al., 2002b; Gough et al., 2003; Pichavant et al., 2005; Adam et al., 2006; Kauffman et al., 2006) and TSLP (Kouzaki et al., 2009), that attract neutrophils, eosinophils, monocytes and DCs to the airways, and induce DC maturation.

The release of pro-inflammatory mediators from the airway epithelium has been shown to require PAR2 (Asokanathan et al., 2002a; Kauffman et al., 2006; Adam et al., 2006). In addition to airway epithelial cells (Asokanathan et al., 2002a), PAR2 is also expressed by fibroblasts (Akers et al., 2000), macrophages (Colognato et al., 2003), mast cells (D'Andrea et al., 2000) and DCs (Ramelli et al., 2010). The expression of PAR2 by DCs and airway epithelial cells is of particular significance as this allows the recognition of protease active allergens. Asthmatics have increased expression of PAR2 on respiratory epithelial cells compared to non-asthmatics (Knight et al., 2001) and proteases can activate PAR2 in the airways to generate leukocyte infiltration and to amplify the response to allergens (Cocks and Moffatt, 2001; Lan et al., 2002; Reed and Kita, 2004). Activation of PARs on eosinophils and mast cells cause these cells to degranulate and release pro-inflammatory mediators (Schmidlin et al., 2002; Stenton et al., 2002). Fibroblasts mature and proliferate and produce collagen in response to activation of PARs on the cell surface (Akers et al., 2000). Proteases can induce bronchial smooth muscle contraction and proliferation (Hauck et al., 1999; Miglino et al., 2011) and are capable of activating basophils in the

absence of antigen-specific IgE (Siracusa et al., 2010). An important function of PARs in the respiratory system involves the regulation of airway tone by causing either the contraction or relaxation of smooth muscle cells (Lan et al., 2002). PAR2 involvement has also been demonstrated in remodelling of the lung, through the secretion of pro-inflammatory and pro-fibrotic mediators (Mercer et al., 2007), the production of ECM components and through stimulating cell mitogenesis (Moffatt et al., 2004). HDM proteases stimulate EMT in airway epithelial cells, leading to airway remodelling (Heijink et al., 2010a;Heijink et al., 2010b). The redistribution of E-cadherin in human bronchial epithelial cells by mite protease allergens has been shown to be mediated through EGFR-dependent activation of PAR2 (Heijink et al., 2010a).

In several other studies, there have been mechanisms proposed by which other HDM proteases can further polarise the immune response to an allergic, Th2-driven phenotype. These include the findings that, *Der p* 3 cleaves the C3 and C5 components of complement pathway to produce anaphylatoxins (Maruo et al., 1997). Products of C3 and C5 cleavage are C3a and C5a respectively, which can cause smooth muscle contraction, histamine release from mast cells, and inflammatory cell recruitment (Wills-Karp, 2005).

HDM and TLR Signalling

TLRs are strategically located on structural cells, such as epithelial cells, and immune cells, including DCs, macrophages and T cells, to detect PAMPs (Akira et al., 2006). LPS is the primary ligand for TLR4 and, although not a HDM-specific component, LPS is routinely detected in HDM extracts (Douwes et al., 2000). The role of LPS and asthma has been widely investigated and a complex role has been highlighted whereby LPS exposure has been shown to both protect against and promote the development of asthma. An up-to-date review on LPS and association with asthma has recently been carried out (Doreswamy and Peden, 2011). Mechanistically, the role of LPS in either promoting or being protective against asthma is believed to be as a consequence of differing exposure levels. This has been addressed *in vivo*, with Eisenbarth and colleagues showing that mice primed with low-dose LPS before OVA exposure developed Th2 responses via TLR4 signalling and activation of DCs.

However, high-dose LPS was shown to induce a Th1, IL-12-driven response and in the absence of LPS there was no inflammatory response (Eisenbarth et al., 2002).

With respect to HDM, two studies have suggested that LPS present in the inhaled HDM extract is pivotal in the generation of HDM-induced allergic airway inflammation. Mice deficient in either TLR4 or a key TLR adaptor protein myeloid differentiation primary response gene 88 (MyD88) have attenuated HDM-induced AHR, eosinophilia and Th2 responses (Phipps et al., 2009). An essential role for TLR4 signalling on airway epithelial cells, but not DCs, has also been shown to be required for the development of HDM-specific Th2 responses (Hammad et al., 2009). TLR activation on epithelial cells also results in transactivation of the EGFR (Koff et al., 2008), which can cause inflammatory cell migration and production of pro-allergic cytokines. EGFR signalling in the airway epithelium plays an important role in mediating HDM-induced AHR and ASM remodelling (Le Cras et al., 2011).

In addition to LPS, HDM allergens have been shown to signal via TLRs or have been strongly linked with TLR signalling. The group 2 allergens, *Der p 2* and *Der f 2*, have the highest rates of skin test positivity in atopic patients (Heymann et al., 1989). Sequence homology has shown that group 2 allergens share structural similarities with the MD-2, which is the LPS-binding component of the TLR4 signalling complex (Inohara and Nunez, 2002; Derewenda et al., 2002; Ohto et al., 2007; Kim et al., 2007). *Der p 2* has been shown to facilitate TLR4 signalling, in both in a low dose LPS setting and in MD-2 deficient mice, thereby suggesting that *Der p 2* has auto-adjuvant properties contributing to allergenicity (Trompette et al., 2009). The ability of *Der p 2* to reconstitute LPS-driven TLR4 signalling in the absence of MD-2 is of critical importance in the lung, since airway epithelial cells express TLR4 but not MD-2 (Jia et al., 2004). Administration of *Der p 2* has been shown to induce the characteristics of allergic airways directly *in vivo*, including eosinophilia, inflammatory cell recruitment, goblet cell hyperplasia and IgE production (Trompette et al., 2009; Ye et al., 2011). *Der p 2* has also been shown to promote pro-inflammatory cytokine release via NF- κ B and MAPK pathways in primary cultured ASMCs (Chiou and Lin, 2009) and epithelial cells (Osterlund et al., 2009).

Der p 5, 7 and 14 elicit strong IgE and T cell responses in patients with HDM allergy (Chew et al., 1999; Epton et al., 2001; Thomas and Hales., 2007). Airway epithelial cells exposed to *Der p* 5 secrete the pro-inflammatory cytokines IL-6 and IL-8 (Kauffman et al., 2006). *Der p* 5 has also been shown to create large hydrophobic cavities (Kuo et al., 2003), which could have functional significance as these cavities could represent ligand binding sites that allow the transport of lipid based PAMPs, similar to *Der p* 2. Since many allergens are known to bind hydrophobic cavities, *Der p* 5 is believed to stimulate the innate immune system and have adjuvant-like effects on IgE-mediated inflammatory responses (Mueller et al., 2010b). Although no studies to date have evaluated the direct stimulation of innate immune cells by group 7 allergens, *Der p* 7 is of a similar structure to LBP, which interacts with TLRs after binding LPS and *Der p* 7 binds the bacterially derived lipid product, lipopeptide polymyxin B (Mueller et al., 2010a). *Der p* 14 is a member of the apolipoprotein-like group 14 allergens which are also lipid binding proteins and is a major constituent of the lipid bodies that can act as Th2 adjuvants (Brewer et al., 1998). Allergens from HDM of groups 13 and 21 can, according to sequence homology, also be considered lipid-binding proteins (Thomas et al., 2010).

HDM and Carbohydrate Structures

Carbohydrate moieties contained in common allergens function as strong Th2 inducers through activation of a variety of C-type lectin receptors (CLRs) on DCs. Glucans are a diverse class of naturally occurring glucose polymers and are commonly found in the cell walls of fungi, pollens and bacteria (Hay et al., 1992) and β -glucans are also present within HDM extracts (Douwes et al., 2000). HDM has been shown to induce the release of the chemokine CCL20/MIP-3 α from human airway epithelial cells, which resulted in the recruitment of immature DCs via CCR6. This was shown to occur through β -glucan and spleen tyrosine kinase (Syk)-dependent signalling pathways and independently of TLR activation and protease activity (Nathan et al., 2009). Importantly, neither ragweed nor cockroach allergens were able to elicit CCL20/MIP-3 α production, suggesting that this allergenic property may be unique to HDM (Nathan et al., 2009). Although the exact lectin receptor mediating these effects was not identified, the results suggested that β -glucan moieties contained in HDM were central to immature DC recruitment to the airways.

Dectin-2 is a receptor for glycans in allergen extracts from HDM and has been shown to mediate cysteinyl leukotriene generation from DCs, thus providing a link between Dectin-2 and the initiation of Th2 inflammation elicited by HDM (Barrett et al., 2009;Barrett et al., 2011).

Glycosylation of natural *Der p 1* and *Der p 2* promoted the uptake of these allergens by DCs following binding to the mannose receptor (MR), which plays a key role in Th2 polarisation (Royer et al., 2010). Ligation of the MR also polarises human DCs towards a Th2 promoting phenotype and, consistent with a role for lectins in promoting Th2 immune responses, blockade of the MR significantly reduced *Der p 1* uptake by DCs (Deslee et al., 2002). Binding of the naturally glycosylated *Der p 2* to DC-SIGN on DCs elicited the release of TNF- α , whereas unglycosylated *Der p 2* cannot activate this receptor (Hsu et al., 2010).

Chitin and Chitinases

The exoskeleton of HDM contains chitin, a widespread environmental biopolymer of *N*-acetyl- β -D-glucosamine and provides structural rigidity to mites (Elias et al., 2005;Lee et al., 2011). Chitin is considered a size-dependent PAMP that stimulates TLR2, Dectin-1 and the MR (Koller et al., 2011) and chitin differentially stimulates the production of pro- and anti-inflammatory cytokines (Lee et al., 2011). Chitin acts as a recognition element for tissue infiltration by innate cells and induces the accumulation of IL-4-expressing eosinophils and basophils in mice (Reese et al., 2007). Thus the chitin presence within the dust mite itself may provide another pathway by which HDM can elicit activation of the immune system.

Der p 15 and *Der p 18* are chitinase allergens and show a high frequency of binding to IgE in allergic patients and are therefore potentially important in the development of allergy to HDM (McCall et al., 2001;O'Neil et al., 2006). *Der p 15* and *18* thus are a different class of epitope within HDM and chitinases catalyse the hydrolysis of *N*-acetyl-D-glucosamine 1,4- β -linkages in chitin polymers. Chitinases are essential for the arthropod life cycle and important for arthropod gastrointestinal epithelia (O'Neil et al., 2006).

An additional avenue by which HDM could promote airway inflammation is through the release of DAMPS, such as uric acid and ATP that are normally located intracellularly but are released into the extracellular regions upon physical or metabolic stress (Matzinger, 2002). HDM exposure has also been shown to release uric acid into the BAL of asthmatics and uric acid promotes DC activation directly through a Syk/PI3K δ signalling pathway (Kool et al., 2011).

ATP is released in the airways of allergen-challenged patients and contributes to disease pathogenesis via signalling at purinergic receptors, expressed at the epithelial surface (Idzko et al., 2007). *Der p* 1-mediated stimulation of eosinophils and DCs derived from HDM allergic subjects resulted in increased P2Y₂ receptor expression that is not observed in non-atopic patients, and is accompanied by eosinophil and DC chemotaxis to ATP (Muller et al., 2010).

1.3.2. HDM and the Immune Response

HDM is the most common allergen associated with atopic asthma (Nelson et al., 1996; Lodge et al., 2011). However, there is still an incomplete understanding of the allergenic components of HDM and the precise mechanisms by which HDM facilitates the development of allergic airways disease *in vivo* at the site of the airway epithelium. A more complete understanding of both HDM allergenicity and the contribution of airway structural cells and aspects of the innate immune system directing such robust Th2 inflammatory responses will likely lead to the development of new therapeutic strategies, particularly targeting airway remodelling. A summary of the known interactions of HDM with the airway epithelium is shown in Figure 1.4.

Thus the establishment and characterisation of a murine model of HDM-induced allergic airways disease will be the initial basis of this thesis. Using this model, the contribution of TLR signalling and the role of intrinsic protease in the development of allergic airways disease will be investigated. This murine model of HDM-induced allergic airways disease will also be utilised to determine the role of TGF- β in disease pathogenesis.

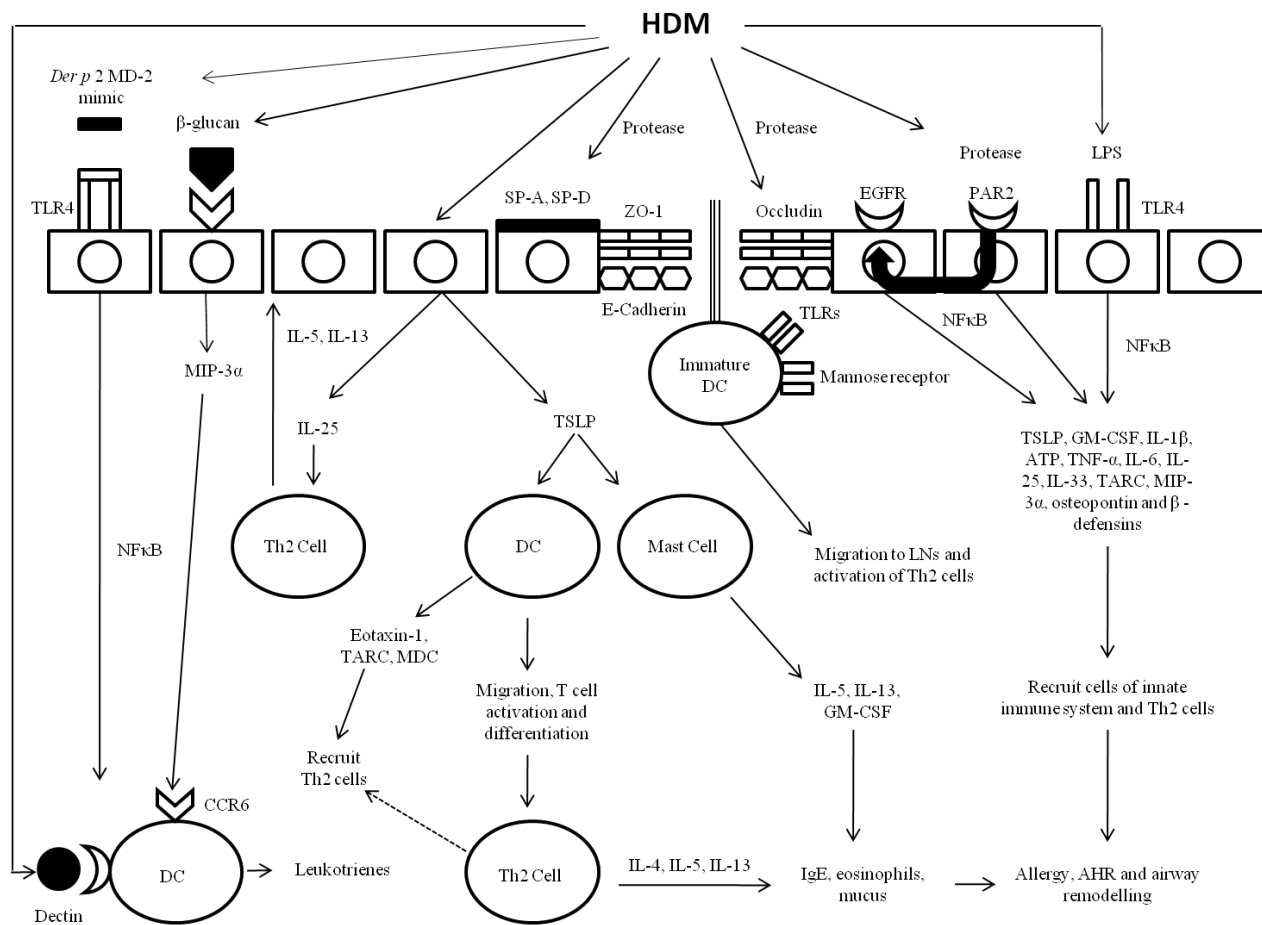


Figure 1.4. A summary of the interactions of HDM with the airway epithelium.

HDM is a complex aeroallergen that contains multiple antigenic epitopes that are capable of triggering pro-inflammatory responses at the site of the airway epithelium. These facets include proteases, signalling via PAR2-dependent and –independent mechanisms, and ligands that signal via TLRs. Together HDM triggers the release of TSLP, IL-25 (IL-17E), IL-33 and CCL20/MIP-3α that are central to DC recruitment and Th2 polarisation.

1.4. Hypothesis and Aims

1.4.1. Hypothesis

HDM exposure to immunologically naïve mice will lead to the development of AHR, airway inflammation and airway remodelling, characterised by the pulmonary expression of Th2 cytokines. Since HDM is a multi-faceted allergen, no single feature of HDM will be responsible for the development of disease pathogenesis and several aspects of the allergen are responsible for breaching the airway epithelium and promoting allergic airways disease.

1.4.2. Aims

The aims of this thesis are to:

Aim 1:

Characterise a murine model of allergic airways disease, whereby continuous exposure of immunologically naïve mice to HDM will lead to the development of the salient features of human asthma *in vivo*.

Aim 2:

Utilise this model to determine the contribution of TLR signalling and protease activity to the development of HDM-induced allergic airways disease pathogenesis *in vivo*.

Aim 3:

Determine the role of TGF- β in the development of HDM-induced allergic airways disease.

Chapter 2 – Materials and Methods

2.1. Animals

Female BALB/c, C3H-HeN, C3H-HeJ and C57BL/6 mice were purchased from Harlan Olac Ltd (Bicester, UK) and were used at age 6-8 weeks in all experiments. MyD88 knockout (KO) animals were originally generated and kindly provided by Cancer Research UK. MyD88 KO mice were on a C57BL/6 background, backcrossed at least 20 generations and genotyped. Animals were housed at Imperial College animal facility with food and water *ad libitum*. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals.

2.2. Murine models of allergen induced airway inflammation

House dust mite extract (HDM; *Dermatophagoides pteronyssinus*; Greer Laboratories, Lenoir, NC) was used as the allergen to induce allergic airways disease in mice as described below. The *Der p 1* content was 31.4 µg per mg of whole protein and the LPS content was 20 U per mg of whole protein.

2.2.1. House dust mite challenge

2.2.1.1. Allergen exposure

Mice were repeatedly challenged by intranasal (i.n.) instillation of either 15 µl or 25 µl of a 1 mg/ml solution of whole HDM allergen extract in PBS under inhalational of isoflurane anaesthesia. This volume of allergen equates to either 15 µg or 25 µg of protein, respectively.

2.2.1.2. HDM exposure

Female mice were exposed to purified HDM extract (Greer Laboratories, Lenoir, NC) i.n. for 5 consecutive days per week for up to 5 weeks, based on the model by Johnson and colleagues (Johnson et al., 2004). Control animals received either 15 µl or 25 µl PBS i.n. Disease parameters were assessed either 2h, 4h, 8h or 24h post final challenge.

2.3. Manipulation of mouse models

2.3.1. HDM challenge – Chapter 3

Female BALB/c mice were administered either 25 µl PBS or 25 µg (in 25 µl) HDM i.n. 5 times a week for 3 weeks and culled either 2h, 4h, 8h or 24h post final challenge.

2.3.2. HDM challenge in TLR4 signalling deficient mice – Chapter 4

Female wt C3H-HeN or TLR4 signalling deficient C3H-HeJ mice were given either 25 µl PBS or 25 µg (in 25 µl) HDM i.n. 5 times a week for 3 weeks and culled 24h post final challenge.

2.3.3. Prophylactic treatment with a TLR4 Antagonist – Chapter 4

Female BALB/c mice administered either 25 µl PBS or 25 µg (in 25 µl) HDM i.n. 3 times a week for 3 weeks. Prior to each PBS or HDM challenge, mice received either vehicle (water) or a TLR4 antagonist (Invivogen, San Diego, CA) 1 µg via the intratracheal (i.t.) route. The vehicle or TLR4 antagonist were administered in a volume of 20 µl. Mice were culled 24h post final challenge.

2.3.4. HDM challenge in MyD88 knockout mice – Chapter 4

Female wt C57BL/6 or MyD88 KO mice were administered either 25 µl PBS or 25 µg (in 25 µl) HDM i.n. 3 times a week for either 1 week, 3 weeks or 5 weeks. Mice were culled 24h post final challenge.

2.3.5. Prophylactic treatment with a SAPS Inhibitor – Chapter 4

Female BALB/c mice given either 25 µl PBS or 25 µg (in 25 µl) HDM i.n. 3 times a week for 3 weeks. Prior to each PBS or HDM challenge, mice received either vehicle (PBS) or SAPS inhibitor (gift from Anthony Berry, Vaccine Technology Ltd, UK) 1 mg/Kg i.n. The vehicle or SAPS inhibitor were administered in a volume of 50 µl. Mice were culled 24h post final challenge.

2.3.6. Boiled HDM challenge – Chapter 5

Female BALB/c mice were given either 25 µl PBS, 25 µg (in 25 µl) HDM or 25 µg (in 25 µl) boiled HDM (bHDM) i.n. 5 times a week for 5 weeks and culled 24h post final challenge. HDM was boiled for 10 minutes at 100°C prior to challenge (Lombardero et al., 1990; King et al., 1998).

2.3.7. Epithelial Permeability – Chapter 5

Female BALB/c mice were administered either 25 μ l PBS, 25 μ g (in 25 μ l) HDM or 25 μ g (in 25 μ l) bHDM i.n. 5 times a week for one week. 24h after the final PBS, HDM or bHDM challenge, each mouse was given 20 μ l of 50 mg/ml FITC-Dextran (4 kDa; Sigma, UK) i.n. and culled 1h later. The amount of FITC-Dextran present in the lung homogenate was determined by Fluostar Galaxy software (MTX Lab Systems, Virginia, USA). Lung homogenate samples were run against a standard curve of FITC-dextran that was generated after being read at an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

2.3.8. Labelled HDM – Chapter 5

Female BALB/c mice were administered either 25 μ l PBS, 25 μ g (in 25 μ l) HDM or 25 μ g (in 25 μ l) bHDM i.n. 3 times a week for 1 week. All mice were then given 25 μ g (in 25 μ l) of Alexa Fluor-488-labelled HDM and were culled 24h post final challenge. HDM was labelled with Alexa Fluor 488 Protein Labeling Kit (Invitrogen, UK). A group of PBS-treated mice did not receive the Alexa-488 labelled HDM.

2.3.9. Therapeutic Blockade of TGF- β – Chapter 6

Female BALB/c mice were administered either 15 μ l PBS or 15 μ g (in 15 μ l) HDM 3 times a week for 5 weeks. A therapeutic regimen of anti-TGF- β antibody (Ab), which neutralised all mouse isoforms of TGF- β (Clone 1D11, Genzyme Corporation, MA, USA), was instigated at the beginning of week 3 and mice were given 100 μ l of either 0.5 mg/Kg or 5 mg/Kg intraperitoneal (i.p.) 30 minutes before each allergen challenge. Mice received an isotype (IgG₁) control Ab instead of anti-TGF- β Ab and served as the controls. Mice were culled either at 4h, for cell analysis, or at 24h, for lung function analysis, post final challenge.

2.3.10. Prophylactic Blockade of TGF- β – Chapter 6

Female BALB/c mice were administered either 15 μ l PBS or 15 μ g (in 15 μ l) HDM 3 times a week for 3 weeks. A prophylactic regimen of anti-TGF- β antibody (Ab), which neutralised all mouse isoforms of TGF- β (Clone 1D11, Genzyme Corporation, MA, USA), was instigated at the beginning of week 1 and mice were given 100 μ l of either 0.5 mg/Kg or 5 mg/Kg i.p. 30 minutes before each allergen challenge. Mice

received an isotype (IgG₁) control Ab instead of anti-TGF- β Ab and served as the controls. Mice were culled 24h post final challenge.

2.4. Non-invasive measurements of lung function

2.4.1. Whole body plethysmography

AHR was measured by whole body plethysmography, and presented as enhanced pause (Penh), which is a mathematically derived value as described by Hamelmann and colleagues (Hamelmann et al., 1997). Following allergen challenge, Penh was measured in response to increasing doses of aerosolised methacholine (MCh; Sigma, UK).

2.5. Invasive measurements of lung function

2.5.1. Resistance Compliance

Direct measurements of dynamic lung resistance (RI) and pulmonary compliance (Cdyn) were measured in anaesthetised and tracheostomised mice. Anaesthesia was induced with an i.p. injection of 10 mg/ml Pentobarbitone (50 mg/Kg) (Pfizer, UK) and intramuscular (i.m.) injection of 100 mg/ml Ketamine (200 mg/Kg) (Fortdodge Animal Health Ltd, Southampton, UK). Mice were ventilated using a small animal ventilator (Harvard Apparatus, Kent, UK) and measurements were taken in response to inhalational doses of the cholinergic agonist methacholine (MCh - Sigma, UK) at increasing concentrations (3 mg/ml – 100 mg/ml) (Martin et al., 1988) in an EMMS system (Electro-Medical Measurement Systems, Bordon, Hants, UK). The trachea was exposed and cannulated then mice were ventilated at 160 breaths/minute with a stroke volume of 150 μ l, comparable with normal breathing. Baseline measurements were obtained for 2 minutes followed by a PBS dose. Each subsequent MCh dose lasted 10 seconds, and the response to each dose was measured for 5 minutes with measurements taken every 25 breaths. Lung RI and Cdyn values were averaged for each 5 minute period following MCh challenge and expressed as cmH₂O/mls/s and ml/cmH₂O respectively. Resistance Compliance was used to measure lung function in Figures 3.1, 4.1 and 6.11.

2.5.2. Flexivent

Measurements of dynamic resistance and compliance were performed using a Flexivent system (Scireq, Montreal, Canada). After induction of anaesthesia with an

i.p. injection of 10 mg/ml Pentobarbitone (50 mg/Kg) (Pfizer, UK) and i.m. injection of 100 mg/ml Ketamine (200 mg/Kg) (Fortdodge Animal Health Ltd, Southampton, UK), mice were tracheostomised and connected to the flexivent ventilator via a blunt-ended 19-gauge needle. Mice were ventilated using the following settings; tidal volume of 7 ml/kg body weight, 150 breaths/minute; positive end-expiratory pressure approximately 2 cm H₂O. Mice were initially ventilated for 5 minutes. Standardisation of the lung volume history was done by performing two deep inflations. Subsequently, measurements of airway function were made. Measurements of resistance and compliance were determined from a user defined protocol using the snapshot-150 perturbation, which is a single frequency sinusoidal wave at a frequency equivalent to ventilation rate (i.e., 2.5 Hz). Resultant data was fitted using multiple linear regression to the single compartment model in the form: pressure = resistance x flow + elastance x volume + fitting constant. Fitting the data to this model provides readings of resistance and compliance. Changes in resistance and compliance were measured in response to increasing concentrations of nebulised MCh from either 3 mg/ml – 100 mg/ml or 10 mg/ml - 300 mg/ml, delivered into the inspiratory line of the flexivent ventilator. Airway resistance was expressed as cmH₂O.s/ml and compliance was expressed as mL/cmH₂O. Flexivent was used to measure lung function in Figures 4.8, 4.14, 4.21, 5.5 and 6.2.

2.6. Blood Removal

2.6.1. Exanguination and serum isolation

Mice were bled under terminal anaesthesia by cardiac puncture, recovering approximately 0.5 - 1 ml per mouse. After overnight clotting, samples were centrifuged at 13000 rpm (10000 g) for 15 minutes at 4°C, serum removed and stored at -80°C for use in ELISA.

2.6.2. Serum isolation from the tail

Mice were also bled from the tail, recovering 50 µl per mouse. After overnight clotting, samples were centrifuged at 13000 rpm (10000 g) for 15 minutes at 4°C, serum removed and stored at -80°C for use in ELISA.

2.7. Cell recovery

Following isolation of leukocytes from each tissue below, cells were resuspended in complete media (RPMI containing 10% FCS, 2 mM L-Glutamine, 100 U/ml Penicillin/Streptomycin, was used throughout all leukocyte preparations) and cell counts were performed in a haemocytometer using white blood cell counting fluid (Turks Solution; trace gentian violet in 1% v/v acetic acid in H₂O) (2.1. Appendix of buffers).

2.7.1. Bronchoalveolar lavage of airway lumen

Bronchoalveolar lavage (BAL) was performed by flushing out the lungs three times with 0.4 ml of PBS via a tracheal canulae as described previously (Kearley et al., 2005). The fluid collected was centrifuged for 5 minutes (1200 rpm (120 g), 5 minutes, 4°C) and recovered cells re-suspended in 0.5 ml complete media. The supernatant was aliquoted and stored at -80°C for use in downstream applications.

2.7.2. Lung parenchyma

Either the right hand lob or inferior left lobe of lung tissue was removed, weighed and mechanically chopped prior to being incubated at 37°C for 1 hour in digest reagent; 150 µg/ml Collagenase (Roche Diagnostics, Lewes, UK), 25 µg/ml DNase (Type 1, Roche Diagnostics, Lewes, UK) in 4 ml of media. Cells were recovered by mechanical disruption and filtration through a 70 µm nylon sieve (Falcon, Marathon Lab Supplies, London, UK), washed twice by centrifugation (1900 rpm (150 g), 10 minutes, 4°C) and re-suspended in 1ml complete media.

2.7.3. Lung draining lymph nodes

Lung draining lymph nodes (LNs) were removed, weighed, cells extracted by mechanical disruption and filtered through a 70 µm nylon sieve before washing twice by centrifugation (1900 rpm (150 g), 10 mins, 4°C). The resulting pellet was re-suspended in 1 ml complete media.

2.8. Differential leukocyte counts

BAL and lung leukocytes (5×10^4 cells) were applied to glass slides by cytocentrifugation for 4 minutes at 400 rpm (60 g) (Cytospin 4, ThermoShandon, UK). Slides were allowed to air dry and were fixed in 100% methanol for 5 minutes.

2.8.1. Wright-Giemsa staining of cytocentrifuge preparations

Following cytocentrifuge application to glass slides, BAL and lung tissue leukocytes were stained with Wright-Giemsa stain (Sigma, UK) for 5 minutes followed by a rinse with water. Percentages of macrophages, lymphocytes/monocytes, eosinophils and neutrophils were determined under light microscopy (40x magnification) by counting cells in 8 randomly selected fields and dividing this number by the total number of cells counted. To obtain absolute numbers, this percentage was multiplied by the total number of cells recovered in the BAL fluid and lung digest suspension. All counts were blinded to group designation.

2.9. Lung tissue pathology and immunohistochemistry

After removal from the animal, one lobe of lung (either the right hand lobe or inferior left hand lobe) was inflated with PBS. Lungs were fixed in 10% normal buffered formalin for 24h. Specimens were paraffin embedded and transverse sectioned (4 µm) onto glass slides. Embedding, sectioning and staining with Haematoxylin & Eosin, Periodic Acid Schiff and sirius red were performed in house by the Leukocyte Biology Histology Service. All histological and immunohistochemical assessments were done blinded to group designation.

2.9.1 Cellular infiltration – Haematoxylin & Eosin

The degree of cellular infiltration was determined on Haematoxylin & Eosin (H&E) stained lung sections using an arbitrary scoring system as previously described (Lloyd et al., 2001). Cellular infiltration in the lung was scored as; 0=no infiltrate, 1= one small pocket of infiltrate, 2= small infiltrate around more than one airway or vessel, 3= large infiltrate (>3 cells deep) around >one airway or vessel, 4= most airways and vessels have large infiltrates and 5= most airways and vessels have infiltrates and cells are present in the alveolar bed.

2.9.2. Mucus production – Periodic Acid-Schiff

Mucus containing cells were counted on Periodic Acid-Schiff (PAS)-stained sections using an arbitrary scoring system as previously described (Grunig et al., 1998). Goblet cells in the airway epithelium were quantified according to the following scoring system; 0=<0.5%, 1=5-25%, 2=25-50%, 3=50-75% and 4=>75% of positive

purple cells. The total scores for each section were divided by the number of airways examined and expressed as mucus index in arbitrary units.

2.9.3. Collagen deposition – Sirius Red

Sirius red is an anionic dye with sulfonic acid side chain groups that react with the basic amino acid side chain groups of collagen, allowing detection and quantification of types I through V collagen (Lee et al., 2001). Collagen deposition was assessed on sirius red stained sections. Peri-bronchiolar collagen deposition was quantified on sirius red stained sections viewed under polarised light using Leica QWin Image analysis software package (Leica Microsystems, Milton Keynes, UK). Digital photographs of four intact bronchioles per lung section were taken at 40X magnification. Image analysis was performed using Leica DM2500 microscope with 21 40x HC xPL 4.0 x 0.85 corr lens, at 25°C and Leica DFC300F digital Camera and Leica suite software. These data is expressed as the mean intensity of peri-bronchial collagen deposition (Saglanı et al., 2009).

2.9.4. Staining of Mast Cell Tryptase 7 by Immunohistochemistry

Tissue sections were deparaffinised with xylene and rehydrated through 100%, 90% and 75% changes of ethanol to H₂O. For antigen retrieval, slides were placed in 10 mM sodium citrate and heated at a high temperature, three times for 3 minutes. Slides were washed (PBS x 2, 5 minutes) and endogenous peroxidise was removed using hydrogen peroxide in methanol for 30 minutes. Following this, slides were washed (PBS x 2, 5 minutes) prior to addition of an avidin/biotin binding kit (Vector Laboratories, Peterborough, UK). Slides were then washed (PBS x 2, 5 minutes) and blocked using 10% donkey serum in PBS (Sigma, UK). Serum block was removed and slides were incubated with either 50 µl/slide of 0.2 µg/ml of anti-mouse tryptase beta-1/MCPT7 primary antibody (R&D Systems, UK) or 0.2 µg/ml rabbit Ig isotype control (Sigma, UK) in PBS containing 10% donkey serum (Sigma, UK) overnight. Slides were then washed twice for 5 minutes with PBS and 0.05% Tween-20. After washing, slides were incubated with the secondary biotinylated goat anti-rabbit antibody (Jackson Labs, distributed by Stratech Scientific Limited, Suffolk, UK) for 45 minutes at room temperature (RT). After washing, this was followed by addition of streptavidin made according to the manufacturer's instructions (Vectastain ABC method, Vector Laboratories, Peterborough, UK) for 45 minutes, RT before

visualisation with a DAB peroxidase substrate prepared according to the manufacturer's instructions (Vectorlabs, Peterborough, UK). Tissues were counterstained with Gill's haematoxylin (Polysciences Inc, Germany) and dehydrated through 75%, 90% ethanol to H₂O then 100% ethanol before mounting.

2.9.5. Staining of ZO-1 by Immunofluorescence

Tissue sections were deparaffinised with xylene and rehydrated through 100%, 90% and 75% changes of ethanol to H₂O. For antigen retrieval, slides were incubated with 2 mg/ml protease K (Sigma, UK) at 37°C for 10 minutes. Endogenous biotin and avidin binding sites were blocked using a commercially available kit and used according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK). Slides were then washed (PBS x 2, 5 minutes) and blocked using 10% donkey serum (Sigma, UK). Serum block was removed and slides were incubated with 5 µg/ml rabbit anti-mouse ZO-1 polyclonal primary antibody (Invitrogen, UK) or 5 µg/ml rabbit Ig isotype control (Sigma, UK) in PBS containing 1% mouse serum (Sigma, UK) was added overnight at 4°C. Slides were washed twice (5 minutes each) with PBS containing 0.1% Tween. After washing, slides were incubated with the secondary biotinylated goat anti-rabbit antibody (Jackson Labs, distributed by Stratech Scientific Limited, Suffolk, UK) for 45 minutes at room temperature (RT). This was followed by addition of AlexaFluor-488 Streptavidin Complex (Sigma, UK) for 30 minutes. The slides were washed twice in PBS and 0.1% Tween, immersed in water and mounted with Prolong Gold anti-fade that contains DAPI and acts as a nuclear counterstain (Invitrogen, UK). Slides were then viewed by confocal microscopy (Leica SP2 upright, Leica Microsystems, UK).

2.9.6. Staining for cell nuclei by Immunofluorescence

Tissue sections, from mice treated in 2.3.8., were deparaffinised with xylene and rehydrated through 100%, 90% and 75% changes of ethanol to H₂O. The sections were then mounted with Prolong Gold anti-fade that contains the nuclei stain DAPI (Invitrogen, UK).

2.10. Total lung collagen analysis

2.10.1. Lung tissue homogenisation

Snap frozen lung tissue samples were homogenised at 50 mg tissue/ml in HBSS (Invitrogen, Paisley, UK) containing a protease inhibitor cocktail (Roche Diagnostics, Lewes, UK). Samples were then centrifuged (1600 rpm (135 g), 20 minutes) and supernatant collected and stored at -80°C.

2.10.2. Total lung collagen analysis

Total lung collagen was measured using the Sircol™ Collagen Assay in accordance with the manufacturer's instructions (Biocolor Ltd, Belfast, UK). Samples of homogenised lung were acidified with 20 µl 0.5M acetic acid and Sircol dye reagent added. Samples were then left to shake for 30 minutes at room temperature. Samples were centrifuged (1400 rpm; 130 g) and the pellet resuspended in Sircol Alkali reagent. Quantification of dye reagent bound to collagen was performed using a spectrophotometer and read at a wavelength of 540nm (Tecan, Reading, UK).

2.11 Staining of leukocytes and flow cytometric analysis

2.11.1. Antibodies

Directly labelled anti-mouse antibodies to the cell surface proteins CD4, CD8, cKIT/CD117 and the intracellular cytokine IL-10 and their respective isotype controls were purchased from BD Pharmingen (BD Pharmingen, Oxford, UK). Anti-mouse T1/ST2 was purchased from Morwell Diagnostics (Zurich, CH). Anti-mouse CD11c, CD11b, Gr-1, MHCII and FcεR1 were purchased from E Bioscience (E Bioscience, UK). Anti-mouse 120G8 was purchased from Dendritics (Dendritics, Lyon, France). CD4⁺CD25⁺FoxP3⁺ Treg staining kit was purchased from E Bioscience (E Bioscience, UK). Antibodies are detailed in Table 2.1 and buffers in Appendix 2.1.

2.11.2. Extracellular staining of cell surface antigens

3 x 10⁵ cells from either BAL, lung tissue or lung draining LNs (prepared as described in Section 2.7.1 - 3), were stained in staining buffer (PBS containing 1% foetal calf serum and 0.01% sodium azide). To reduce non-specific binding, cells were incubated with rabbit serum (Sigma, UK) for 15 minutes at 4°C. Cells were then stained with cell surface antibodies or relevant isotype controls for 20 minutes at 4°C, followed by twice washing with staining buffer and fixation in CellFix™ (BD Pharmingen, Oxford, UK). Flow cytometric analysis was performed using a FACSCalibur™ (Becton Dickinson, Oxford, UK) and subsequently analysed using

FlowJo software (Treestar, Ashland, Oregon). A minimum of 50,000 events were collected and dead cells were excluded on the basis of forward and side scatter.

2.11.3. Intracellular cytokine staining

For intracellular cytokine staining, cells were first stimulated by incubation with PMA (4 µg/ml) and ionomycin (4 µM) (Merck, New Jersey, USA) in the presence of Brefeldin A (10 µg/ml) (Sigma, UK) for 4 hours 37°C in a humidified incubator (5% CO₂) before extracellular staining as described in Section 2.11.2. After fixation, cells were washed in permeabilisation buffer (staining buffer containing 0.05% saponin (Sigma, UK). Afterwards, cells were incubated in permeabilisation buffer for 5 minutes then washed and stained with antibodies against cytokines or relevant isotype controls diluted in permeabilisation buffer (listed in Table 2.1). Flow cytometric analysis was performed using a FACSCalibur™ (Becton Dickinson, Oxford, UK) and subsequently analysed using FlowJo software (Treestar, Ashland, Oregon). A minimum of 50,000 events were collected and dead cells were excluded on the basis of forward and side scatter.

2.11.4. Flow Cytometric staining of CD4⁺CD25⁺FoxP3⁺Tregs

Tregs were detected using the CD4⁺CD25⁺FoxP3⁺ staining kit available from EBioscience (EBioscience, UK) according to the manufacturer's instructions. Flow cytometric analysis was performed using a FACSCalibur™ (Becton Dickinson, Oxford, UK) and subsequently analysed using FlowJo software (Treestar, Ashland, Oregon). A minimum of 50,000 events were collected and dead cells were excluded on the basis of forward and side scatter.

2.12. Analysis of mediators by Enzyme Linked Immunosorbent Assay

Cytokine and chemokine levels were measured in BAL samples and homogenised lung tissue (prepared as described in Section 2.7.1. and 2.10.1. respectively) using paired antibodies against murine IL-13, TSLP, IL-25 (IL-17E), IL-33, MIP-3α/CCL20, MCP-1/CCL2, RANTES/CCL5, eotaxin-2/CCL24, TARC/CCL17, MDC/CCL22 and KC/mCXCL1 (R&D Systems, Abingdon, UK). Eotaxin-1/CCL11 was quantified by paired antibodies from 2 batches of Abs from R&D Systems. TGF-β, IL-4 and IL-5 were quantified by paired antibodies (BD Biosciences, Oxford, UK). IL-1β, KC/mCXCL1, IL-4, IL-5, IFN-γ, IL-10 and IL-12 were quantified using a

mouse Th1/Th2 multiplex kit and IL-17 using a mouse singleplex kit (Meso Scale Discovery, Maryland, USA). Mast cell protease-1 (mMCP-1) was quantified in serum with paired antibodies from Moredun (Moredun Scientific, Scotland, UK). IL-25 (IL-17E) and IL-1 β were also quantified using Ready Set Go kits (EBioscience, UK). Total IgE, IgG₁, IgG_{2a}, IgA and IgM were measured in the serum (isolated as described in Section 2.6) using paired antibodies (BD Biosciences, Oxford, UK). For antigen specific serum IgE, IgG₁, IgG_{2a} and IgA, plates were coated with HDM (50 μ g/ml). Buffers used are outlined in Appendix 2.1.

2.12.1. ELISA Protocol

All mediators were assessed using a standardised sandwich ELISA. Flat bottomed high binding 96 well plates (Costar, Corning, NY, USA) were coated overnight with 100 μ l/well of anti-mouse antibodies diluted to appropriate concentration in coating buffer. Wells were blocked using appropriate blocking buffer (Appendix 2.1.) followed by washing and samples or standards (recombinant protein diluted in assay buffer to the appropriate range) were added in duplicate and incubated for either 2 hours RT or overnight at 4°C. After incubation, cells were washed (x4) and the secondary biotinylated anti-mouse antibodies added for either 2 hours or overnight at 4°C. Following washes (x4) samples were then incubated with streptavidin-HRP (diluted in assay buffer) for 30 minutes. Plates were washed and K-blue substrate (Neogen, Lexington, KY, USA) or DuoSet Substrate Reagent Pack (R&D Systems, Abingdon, UK) was added to each well. The colourimetric reaction was stopped by the addition of 0.19M H₂SO₄ and optical density readings at 450 nm were measured using a spectrophotometer (Tecan, Reading, UK) within 30 minutes. Standard curves were plotted using either linear or non-linear regression depending on the fit of the data and unknowns were calculated from the standard curve.

2.13. Protease Activity of HDM

To assess the protease activity of HDM, 50 μ l of HDM extract or control proteases were assayed in triplicate to a 96 well plate. The proteases were either incubated with 10 μ l PBS or 10 μ l of 1 mM DL-Dithiothreitol (DTT; Sigma, UK) for 5 minutes to activate cysteine proteases. 20 μ l of either AEBSF (Sigma, UK) (20mM), E-64 (Sigma, UK) (100 μ M) or both AEBSF (20 mM) and E-64 (100 μ M) combined for 15 minutes at room temperature. AEBSF specifically inhibits serine proteases such as

trypsin and E-64 specifically inhibits cysteine proteases such as papain. Volumes were then made up to 150 μ l in PBS and incubated for a further 15 minutes. 50 μ l of N-Benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (NBP-VANA; Sigma, UK), which is a substrate for trypsin, thrombin and reptilase, was added at a concentration of 1 mM to each well. Absorbances were read immediately at 405 nm and then at 20 minute intervals over 2 hours and stored at 37°C in the dark between readings. Breakdown of NBP-VANA correlated with increased absorbance readouts. Trypsin (Sigma, UK) and papain (Sigma, UK), activated with DTT (Sigma, UK), acted as positive controls for serine and cysteine proteases respectively.

2.14. RNA extraction from the lung

The smallest lung lobe (post-caval) was perfused and flash frozen at -80°C. 500 μ l of Trizol (Invitrogen, UK) was added and, following homogenisation, 200 μ l chloroform was added (Sigma, UK). This mixture was manually mixed for 15 seconds, followed by 2 minutes at room temperature and then centrifuged for 15 minutes at 20000rpm (12000 g) at 4°C. The top aqueous layer was then removed and transferred to a new epindorff where an equal volume of ethanol was added. This solution was then used for RNA extraction according to the manufacturer's instructions (Qiagen RNAeasy, West Sussex, UK).

2.15. PCR Array

A Toll-like receptor (TLR) signalling pathway PCR array was carried out using RNA extracted from mouse lung tissue and carried out according to the manufacturer's instructions (SABiosciences, Maryland, USA). Briefly, 1 μ g of RNA was used for RT and the cDNA was added to the PCR master mix. Thermal cycling was performed using Roche LightCycler 480 (Roche, USA) and the changes in gene expression were analysed using the manufacturer's analysis spreadsheet (SABiosciences, Maryland, USA).

2.16. Data Analysis

Data are expressed as either median or mean $\pm$ SEM as stated. Data was not assumed to be normally distributed and statistical significance between groups was tested using a non-parametric Mann-Whitney *U* Test as stated. A p value of <0.05 was accepted as significant. All other statistical and regression analysis was performed by

using GraphPad Prism software (Version 4.00, San Diego, CA). For the gene array a Student's t-test was performed as according to the manufacturers guidelines. A p value of <0.05 was accepted as significant.

2.1. List of Buffers

Complete media

RPMI
10% FCS
2 mM L-glutamine
100 U/ml Penicillin/streptomycin

FACS staining buffer

PBS
1% FCS
0.1% NaN₃

FACS permeabilisation buffer

PBS
1% FCS
0.1% NaN₃
0.5% saponin

Blocking buffer (BD ELISAs, total Ig ELISAs and RnD kits)

PBS
1% BSA

Blocking buffer (HDM-specific Ig)

PBS
3% BSA

ELISA wash buffer

5.5l H₂O
44g NaCl
55ml 1m NaPO₄
1g Thimerasol
11ml Tween20

ELISA coat wash

5.5l H₂O
55ml 1M KPO₄
1g Thimerasol
2.75ml Tween20

White Cell Counting Fluid

Gentian violet in 1% v/v acetic acid in H₂O

Table 2.2. FACS Antibodies

Antibody	Clone	Isotype	Fluorescent label	Company
Anti-T1/ST2	DJ8	Rat IgG ₁	FITC	Morwell Diagnostics
Anti- cKit/CD117	2B8	Rat IgG _{2b}	PE	BD Pharmingen
Anti-CD8	Ly-2	Rat IgG _{2a}	PE	BD Pharmingen
Anti-CD11c	N418	Armenian Hamster IgG	PerCP Cy5.5	EBioscience
Anti-FcεR1	Mar-1	Armenian Hamster IgG	APC	EBioscience
Anti-Gr-1	RB6-8C5	Rat IgG _{2b}	Pacific Blue/Alexa- 450	EBioscience
Anti-CD11b	M1/70	Rat IgG _{2b}	PE-Cy7	EBioscience
Anti-MHCII	NIMR-4	Rat IgG _{2b}	PE	EBioscience
Anti-CD4	L3T4	Rat IgG _{2a}	APC	BD Pharmingen
Anti-IL-10	JES5-16E3	Rat IgG _{2b}	PE	BD Pharmingen
Anti-120G8	120G8.04	Rat IgG _{2a}	APC	Dendritics

Table 2.3. ELISA kits and paired antibodies

Mediator	Company	Product/Ab Ids	Figure Used
Eotaxin-1/CCL11	RnD Systems	Coat: AF-420-NA Standard: DAF-420 Detect: BAF-420	3.5; 4.3
Eotaxin-1/CCL11	RnD Systems	DY420	4.10; 4.17; 4.23; 5.8;
Eotaxin-2/CCL24	RnD Systems	DY528	All
TARC/CCL17	RnD Systems	DY529	All
KC/mCXCL1	RnD Systems	DY453	4.10; 4.17; 4.23;
RANTES/CCL5	RnD Systems	DY478	All
MCP-1/CCL2	RnD Systems	DY479	All
MDC/CCL22	RnD Systems	DY439	All
TSLP	RnD Systems	DY555	All
IL-25 (IL-17E)	RnD Systems	DY1399e	3.7; 4.5; 4.11; 4.24; 5.10
IL-33	RnD Systems	DY3626	All
CCL20/MIP-3 α	RnD Systems	DY760	All
IL-13	RnD Systems	M1300CB	All
IL-1 β , IL-12, KC/mCXCL1, IL-4, IL-5, IL-10 and IFN- γ	Mesoscale Discovery	SI6000 (Multiplex)	IL-1 β : 3.7; 4.4 IL-12: 4.4 KC/mCXCL1: 3.5; 4.3; 5.8 IL-4 and IL-5: 3.6; 4.4; 5.9; 6.8 IL-10: 3.7 IFN- γ : 4.4
IL-17	Mesoscale Discovery	SI2400 (Singleplex)	All
mMCP-1	Moredun Scientific	MS-RM3	All
IL-4	BD Bioscience	Capture: 554433 Standard: 550067 Detection: 554390	4.11; 4.24; 6.12; 6.13

IL-5	BD Bioscience	Capture: 554393 Standard: 554581 Detection: 554397	4.24; 6.12; 6.13
TGF- β	BD Bioscience	Capture: 555052 Standard: 354039 Detection: 555053	All
Total IgM	BD Bioscience	Capture: 553435 Standard: 550963 Detection: 553436	All
Total IgE	BD Bioscience	Capture: 553413 Standard: 03231D Detection: 553419	All
Total IgG ₁	BD Bioscience	Capture: 553445 Standard: 555746 Detection: 553441	All
Total IgG _{2a}	BD Bioscience	Capture: 553446 Standard: 553454 Detection: 553928	All
Total IgA	BD Bioscience	Capture: 556969 Standard: 553476 Detection: 556978	All
HDM-specific IgE	BD Bioscience	Coat: See 2.12 Detection: 553419	All
HDM-specific IgG ₁	BD Bioscience	Coat: See 2.12 Detection: 553441	All
HDM-specific IgG _{2a}	BD Bioscience	Coat: See 2.12 Detection: 553928	All
HDM-specific IgA	BD Bioscience	Coat: See 2.12 Detection: 556978	All
IL-1 β	EBioscience	88-7913	4.11; 4.18; 4.24
IL-25 (IL-17E)	EBioscience	88-7002	4.18

Chapter 3 – Establishment of a murine model of HDM-induced allergic airways disease

3.1. Introduction

Animal models have been used to outline functional roles for cytokines and cells in the characteristic pathological features of asthma. The vast majority of these studies have used a model of ovalbumin (OVA) sensitisation and challenge whereby mice are immunised peripherally with OVA in the presence of the adjuvant alum before local airway challenge with OVA. The OVA model of allergic lung inflammation can replicate many of the main features of allergic asthma including increased allergen-specific IgE production, mucus production, Th2-driven eosinophilic inflammation, AHR and airway remodelling (McMillan and Lloyd, 2004). Studies utilising the OVA model of sensitisation and challenge have provided important insights into the nature of the allergic airway inflammation, including the importance of the Th2 pathway in disease pathogenesis. However, there are several drawbacks of the OVA sensitisation and challenge model. It is necessary to immunise mice peripherally with OVA, since mice become tolerant to inhaled or aerosol challenge with OVA in the absence of systemic sensitisation (Tsitoura et al., 1999; Herrick et al., 2000; Swirski et al., 2002a; Swirski et al., 2002b; Clausen et al., 2003). Since this model relies on use of the adjuvant alum there may be bias towards promoting a Th2-mediated inflammatory response (Brewer et al., 1999; Sun et al., 2003; Piggott et al., 2005; Gavin et al., 2006; Eisenbarth et al., 2008; Kool et al., 2008a; Kool et al., 2008b). Although OVA is the main protein found in egg white and associated with human gut allergy (Sampson, 2004), OVA is not associated with any form of human asthma (Huntington and Stein, 2001). The sensitisation process in the OVA model does not replicate the scenario in the human disease and the process may distort the immunological development of disease since the initial contact with the immune system occurs in the peritoneum rather than in the lung. Thus, different populations of DCs and other types of APCs will be encountered and stimulated.

It has recently been reported that continued intranasal (i.n.) delivery of an immunologically complex allergen, house dust mite (HDM; *Dermatophagoides pteronyssinus*) extract, results in a chronic inflammatory responses and structural remodelling in the lungs of immunologically naïve, unsensitised mice (Johnson et al., 2004; Southam et al., 2007). This model represents a step forward in that it does not require a peripheral sensitisation step, uses an environmentally and clinically relevant

allergen, and replicates the relevant features of human asthma. HDM models have now been used to determine the role of key mediators in the allergic response, such as GM-CSF (Cates et al., 2004; Johnson et al., 2007). However, the temporal relationships between critical inflammatory mediators and inflammatory cells with the development of AHR have not yet been determined after inhaled HDM. Previous studies have documented the production of Th2 cytokines by splenocytes restimulated with HDM *in vitro* (Johnson et al., 2004). However, these levels may not accurately reflect the amount of mediators of interest in the lung following allergen challenge *in vivo*.

3.2. Hypothesis and Aims

3.2.1. Hypothesis

HDM exposure will result in Th2 cytokine and pro-inflammatory chemokine production in the lung tissue and BAL, concomitant with airway eosinophilia and AHR.

3.2.2. Aims

The aims of Chapter 3 are to:

- Determine the temporal and spatial relationship of leukocytes and inflammatory mediators in the lung and BAL associated with the allergic response and the development of AHR in mice after exposure to inhaled HDM
- Quantify Th2 cytokines and pro-inflammatory mediators in the lung and BAL following exposure to HDM.

3.3. Experimental Design: Induction of HDM-induced allergic airways disease

Airway inflammation and AHR were induced in female BALB/c mice in response to HDM challenge as discussed in the general methods. Mice were administered either 25 µl PBS or 25 µg (in 25 µl) HDM i.n. 5 times a week for 3 weeks and culled 2h, 4h, 8h and 24h post final challenge. At each time point PBS-treated mice were culled and these were pooled for clarity.

3.4. Results: Time course analysis of HDM exposure

3.4.1. HDM exposure results in AHR

HDM-treated mice developed AHR compared to PBS controls. HDM exposure significantly increased airway resistance at 24h post final exposure (Figure 3.1A and B), whereas lung compliance was decreased as early as 2h post challenge and was maintained throughout the 24h period, compared to PBS controls (Figure 3.1C and D).

3.4.2. HDM exposure results in the early recruitment of inflammatory leukocytes to the lung and airway lumen

Elevated numbers of total cells were observed in HDM-treated mice compared to PBS controls in the lung from 2h post final challenge and this was maintained throughout the 24h time period (Figure 3.2A). Differential cell counts determined that the majority of these cells were eosinophils (Figure 3.2B). The numbers of neutrophils, lymphocytes and monocytes recovered from the lung were also significantly higher in HDM-treated mice compared to PBS controls at all time points post final challenge (Figure 3.2C and D). Cell recruitment to the airway lumen, as determined in BAL, was maintained over the 24h period following allergen inhalation. Significant increases in total cells, eosinophils, neutrophils and lymphocytes and monocytes were observed at each time point in response to HDM (Figure 3.2E - H). In order to investigate the degree of cellular inflammation, sections of lung isolated from PBS and HDM-treated mice were examined. HDM exposure induced widespread peri-bronchial and peri-vascular inflammation (Figure 3.3A and B).

FACS analysis showed there was a population of CD8⁺ T cells recruited to the lung and BAL following HDM challenge (Figure 3.4A and B). However, the majority of T cells in the lung and BAL were of the CD4⁺ phenotype (Figure 3.4C and D). Within the CD4⁺ population, there were cells that also stained positive for the surrogate Th2 surface marker T1ST2. Increased Th2 cell numbers (CD4⁺ T1ST2⁺ cells) were observed in the lung and BAL over the 24h period investigated (Figure 3.4E and F).

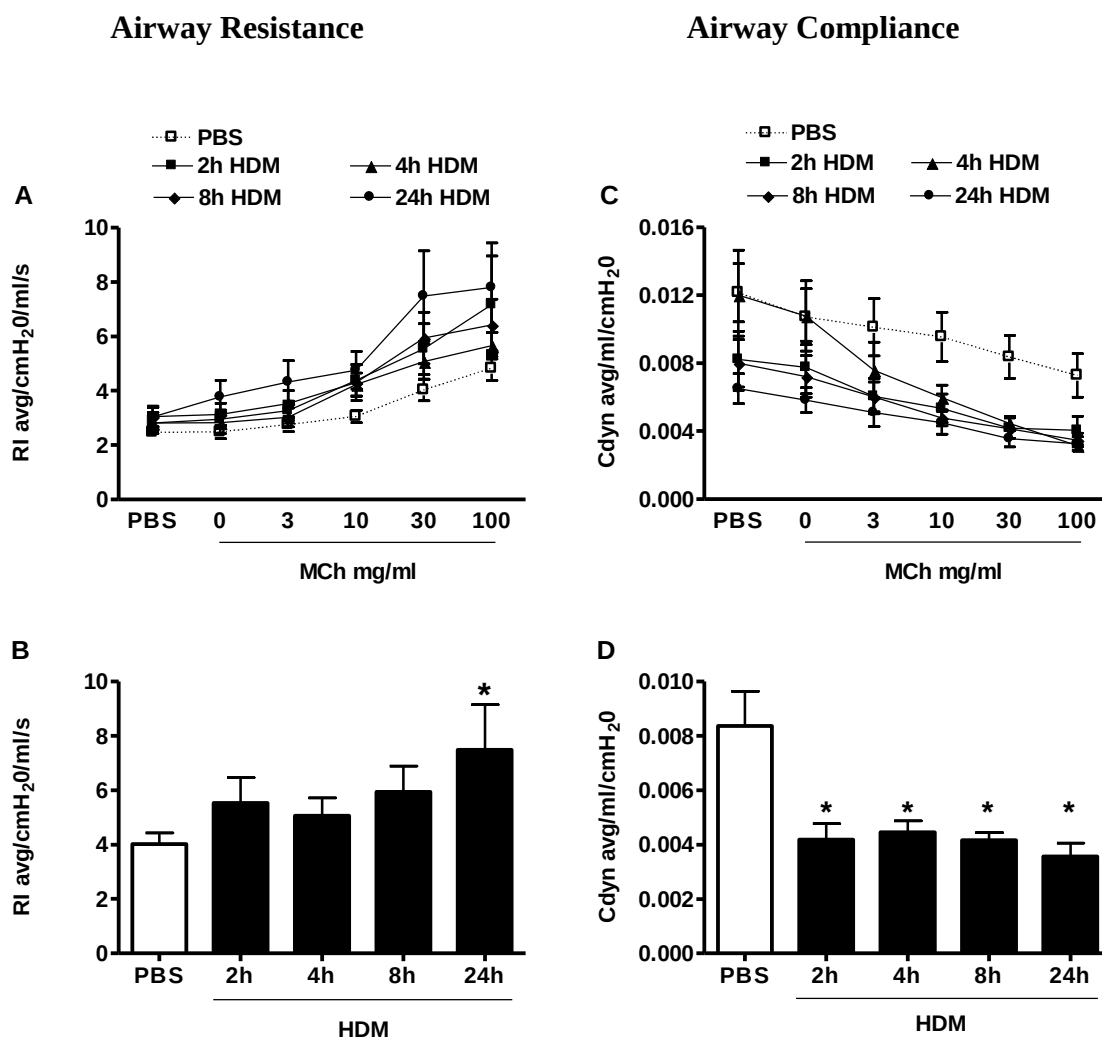


Figure 3.1. AHR in HDM-treated mice.

(A) Airway resistance (RI) of PBS and HDM-treated mice at 2, 4, 8 and 24h post final challenge. (B) Airway resistance is expressed as mean RI $\pm$ SEM for the 30mg/ml dose of methacholine. (C) Dynamic airway compliance (Cdyn) of PBS and HDM-treated mice at 2, 4, 8 and 24h post final challenge. (D) Airway compliance is expressed as mean Cdyn $\pm$ SEM for the 30mg/ml dose of methacholine. (n=12 mice treated with PBS, n=5-6 mice treated with HDM at each time point). *, $P < 0.05$ (Mann-Whitney U test) compared with PBS. Data are mean $\pm$ SEM. One experiment.

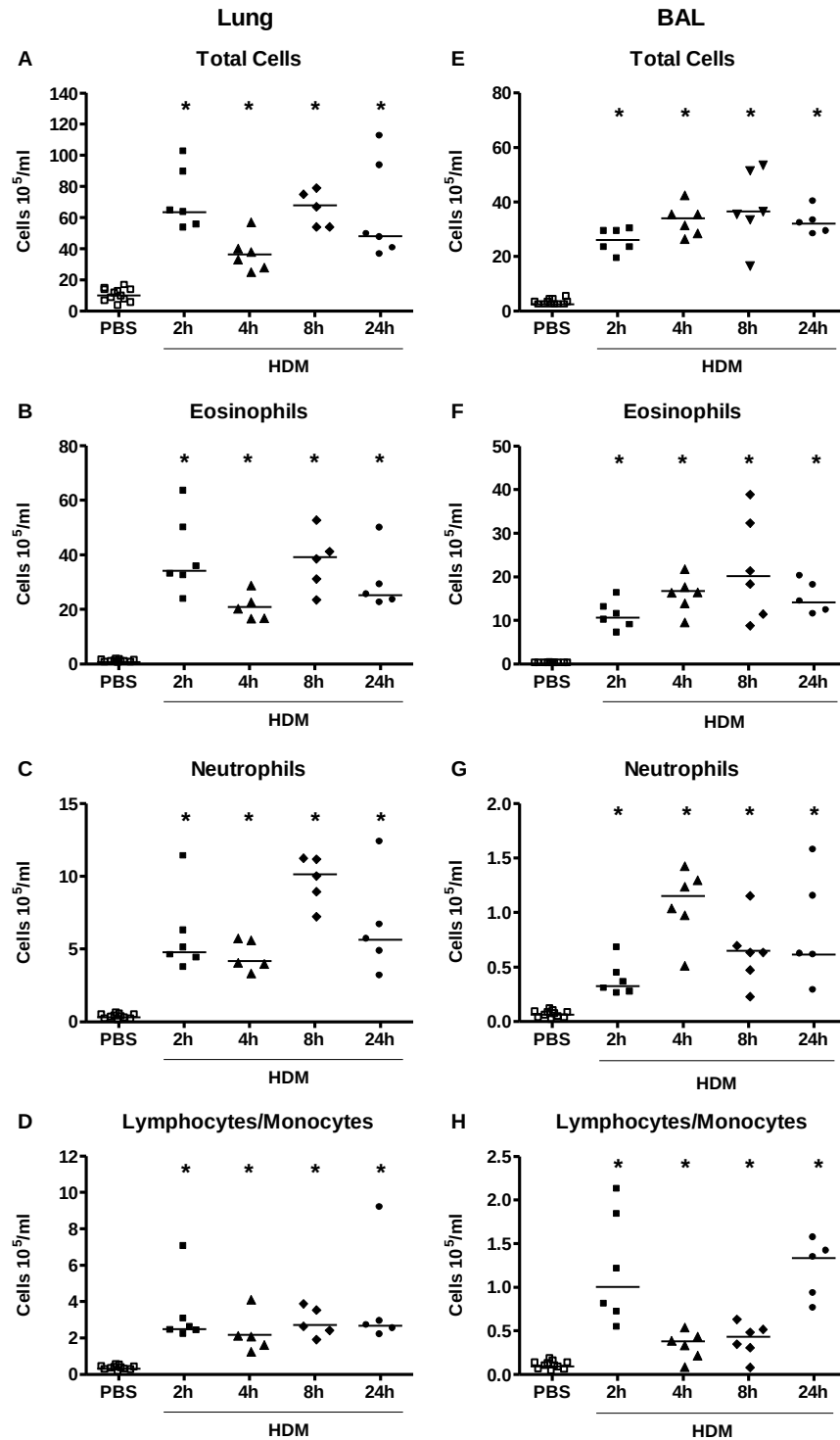
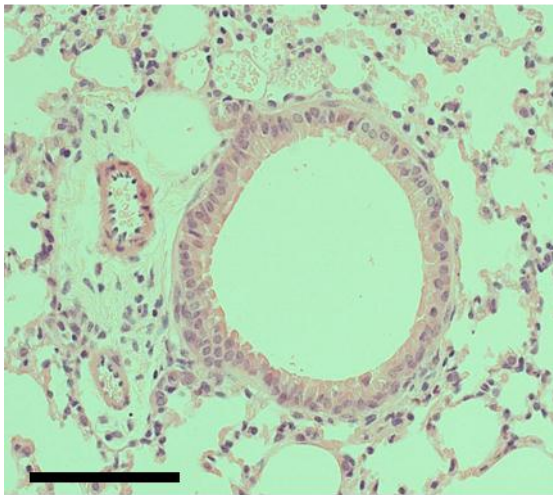


Figure 3.2. Inflammatory cell profile of HDM-treated mice.

Total cells, eosinophils, neutrophils and lymphocytes and monocytes recovered from the lung (A - D) and BAL (E - H) 2, 4, 8 and 24h after the final i.n. challenge (n=12 mice treated with PBS, n=5-6 mice treated with HDM at each time point). Total cell counts were determined using a haemocytometer with white blood cell counting fluid and eosinophils, neutrophils and lymphocytes/monocytes were quantified from Wright-Giemsa stained cytopins, as described in the Materials and Methods. *, $P < 0.05$ (Mann-Whitney U test) compared with PBS. Bars depict median of the groups. One experiment.

A. PBS



B. HDM

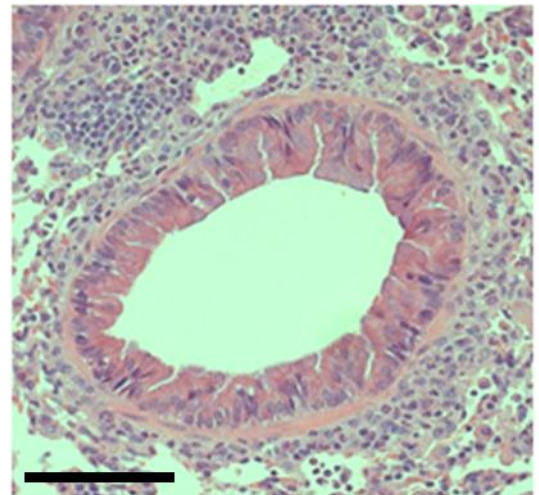


Figure 3.3. HDM-induced airway inflammation.

Representative photomicrographs of H&E stained lung sections from (A) PBS and (B) HDM-treated mice 4h post final challenge (original 20X magnification). *Scale bar* = 50 μ m.

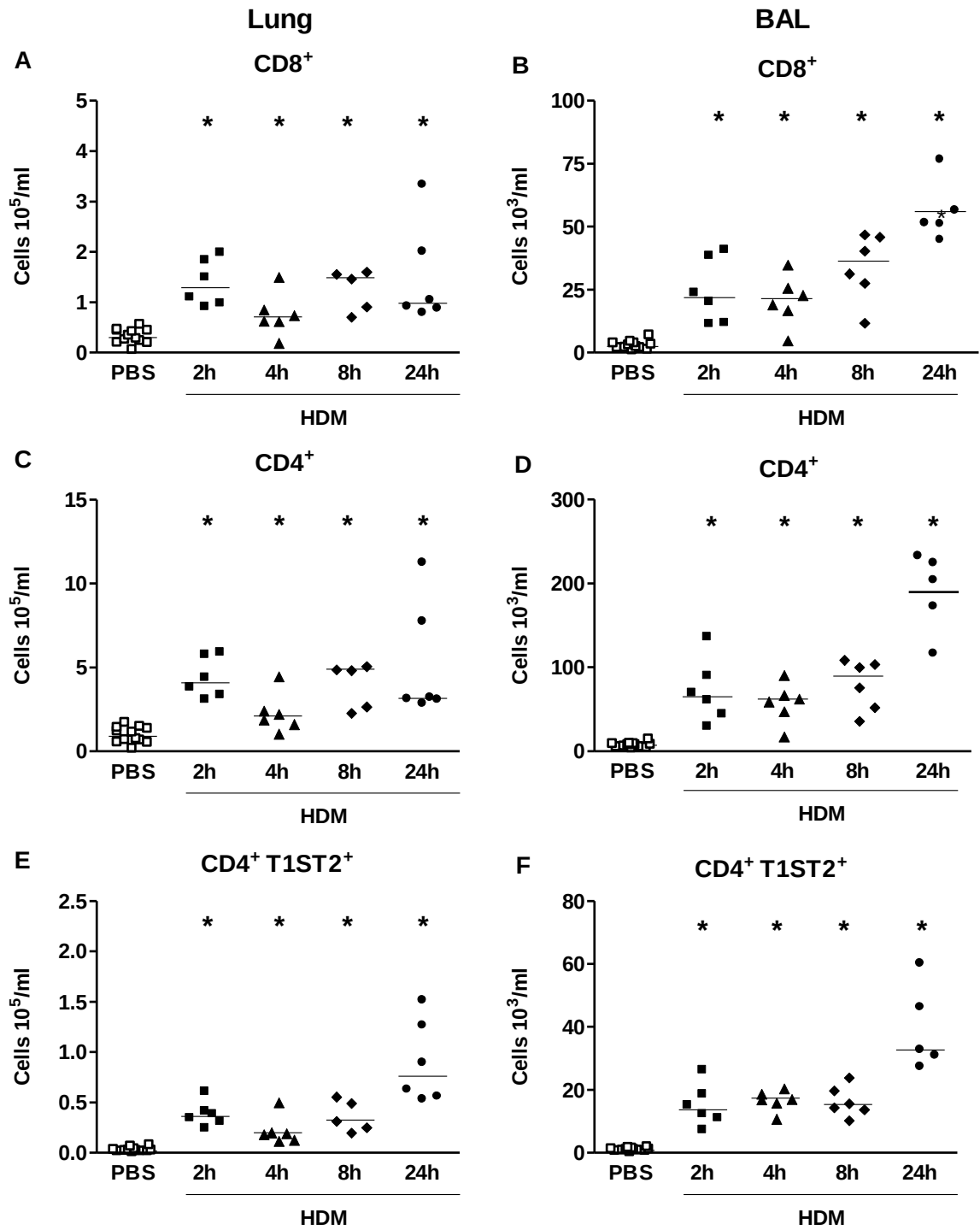


Figure 3.4. Inflammatory T Cell Profile.

CD8⁺, CD4⁺ and CD4⁺T1ST2⁺ T Cells recovered from the lung (A, C and E) and BAL (B, D and F) 2, 4, 8 and 24h after the final i.n. challenge (n=12 mice treated with PBS, n=5-6 mice treated with HDM at each time point). All cells were quantified by flow cytometry. *, *P*<0.05; (Mann-Whitney *U* test) compared with PBS. One experiment.

3.4.3. Chemokine levels correlate with cell recruitment and are maintained after allergen challenge

Concentrations of chemokines important in the development of allergen-induced airway inflammation were determined in the lung and BAL. These included the DC chemokine MCP-1/CCL2, the eosinophil chemokines eotaxin-1/CCL11 and eotaxin-2/CCL24 and the neutrophil chemoattractant KC/mCXCL1. TARC/CCL17, MDC/CCL22 and RANTES/CCL5, which are associated with Th2 cell migration and activation, were also quantified. Eotaxin-1/CCL11, TARC/CCL17 and KC/mCXCL1 were significantly elevated in the lung and BAL of HDM-treated mice, compared to PBS controls, at all time points post final challenge (Figure 3.5A – F). MCP-1/CCL2 and eotaxin-2/CCL24 were elevated in the lungs of HDM-treated mice 2h and 4h post final challenge and levels declined to baseline by 24h post final challenge (Figure 3.5G - H). MDC/CCL22 and RANTES/CCL5 was observed to be increased at all time points post final challenge in response to HDM (Figure 3.5I and J).

3.4.4. Th2 cytokines are produced in the lung following inhaled HDM challenge

The levels of Th2 and Th1 cytokines were quantified in the lung and BAL in response to HDM. HDM-treated mice developed increased levels of the Th2 associated cytokines IL-4, IL-5 and IL-13 in the lung and BAL, compared to PBS-treated mice, at all time points post final challenge, with the highest production at 4h (Figure 3.6A - F). Cytokine levels in the BAL declined to near baseline by 24h post challenge. The Th1 associated cytokine IFN- γ was detectable at very low levels only in the lungs of HDM-treated mice, also with a maximum release 4h post challenge (Figure 3.6G and H).

HDM-treated mice also showed increased levels in the lung homogenate of the pro-allergic mediators TSLP, IL-25 (IL-17E) and IL-33 when compared to PBS-treated mice (Figure 3.7A - C). IL-1 β levels were higher in the lungs of HDM-treated mice compared to PBS controls (Figure 3.7D). TGF- β and the regulatory cytokine IL-10 were also observed to be elevated in the lungs of HDM-treated mice (Figure 3.7E and F).

3.4.5. Serum immunoglobulin levels following HDM challenge

IgE has important functions in the development of allergic disorders and asthma. Following HDM challenge, total and HDM-specific IgE in the serum were significantly elevated from levels in PBS controls at all time points post final challenge (Figure 3.8A and B). Serum IgG₁ and IgG_{2a}, which are indicators of Th2 and Th1 immune responses in mice respectively, were also found to be elevated in HDM-treated mice. IgG₁ levels were observed to be greater in HDM-treated mice than PBS exposed controls at 4h, 8h and 24h post final challenge, whereas IgG_{2a} was elevated in the serum of HDM-treated mice throughout the 24h time period (Figure 3.8C and D).

3.4.6. Goblet cell hyperplasia occurred following HDM challenge

Hypersecretion of mucus from goblet cells is a feature of asthma. An increase in PAS positive cells was observed in the bronchial epithelium in HDM-treated mice compared to PBS controls (Figure 3.9A). Representative lung sections from PBS and HDM-treated mice stained with PAS are shown (Figure 3.9B - C).

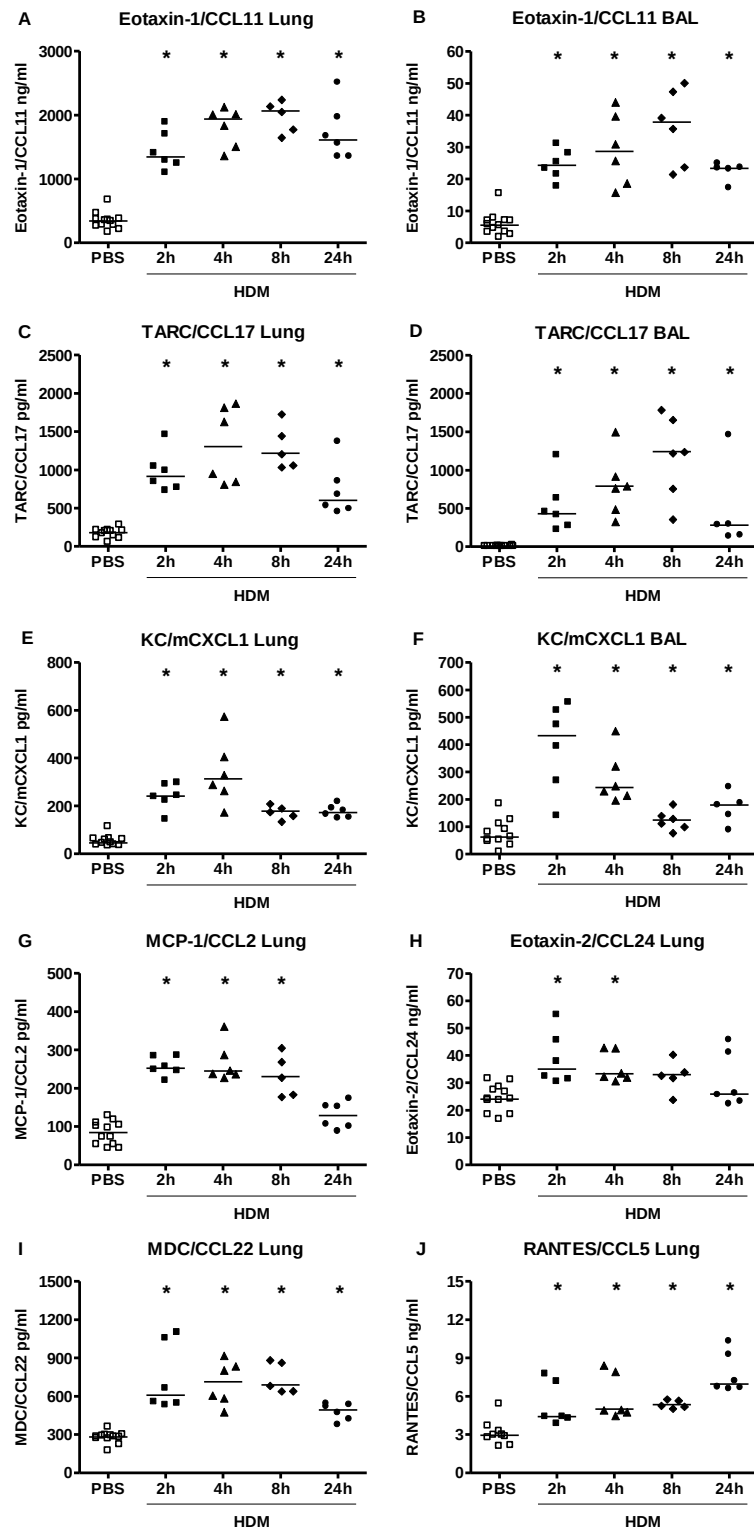


Figure 3.5. Chemokine profile of HDM-treated mice.

Eotaxin-1/CCL11, TARC/CCL17 and KC/mCXCL1 levels measured in the lung (A, C and E) and BAL (B, D and F) 2, 4, 8 and 24h after the final i.n. challenge. (G) MCP-1/CCL2, (H) eotaxin-2/CCL24, (I) MDC/CCL22 and (J) RANTES/CCL5 levels measured in the lung 2, 4, 8 and 24h after the final i.n. challenge. Eotaxin-1/CCL11, TARC/CCL17, MCP-1/CCL2, eotaxin-2/CCL24, MDC/CCL22 and RANTES/CCL5 levels were determined by ELISA and KC/mCXCL1 levels by MSD (n=12 mice treated with PBS, n=5-6 mice treated with HDM at each time point). *, $P < 0.05$; (Mann-Whitney U test) compared with PBS. Bars depict the median of the groups. One experiment.

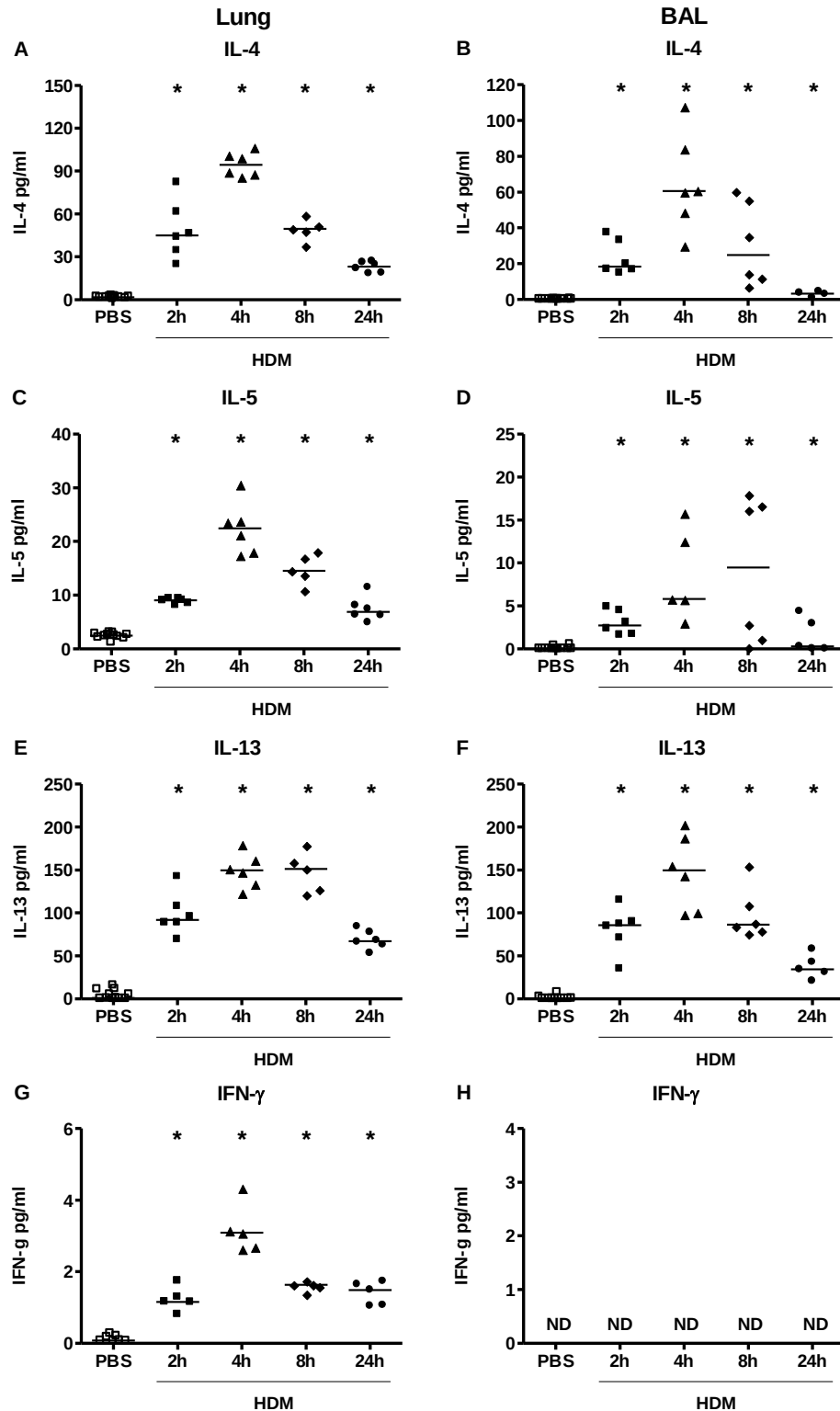


Figure 3.6. Inflammatory cytokine profile of HDM-treated mice.

IL-4, IL-5, IL-13 and IFN- γ measured in the lung (A, C, E and G) and BAL (B, D, F and H) 2, 4, 8 and 24h after the final i.n. challenge. IL-4, IL-5 and IFN- γ levels were determined by MSD and IL-13 levels by ELISA (n=12 mice treated with PBS, n=5-6 mice treated with HDM at each time point). *, $P < 0.05$; (Mann-Whitney U test) compared with PBS. Bars depict the median of the groups. N.D., not detected. One experiment.

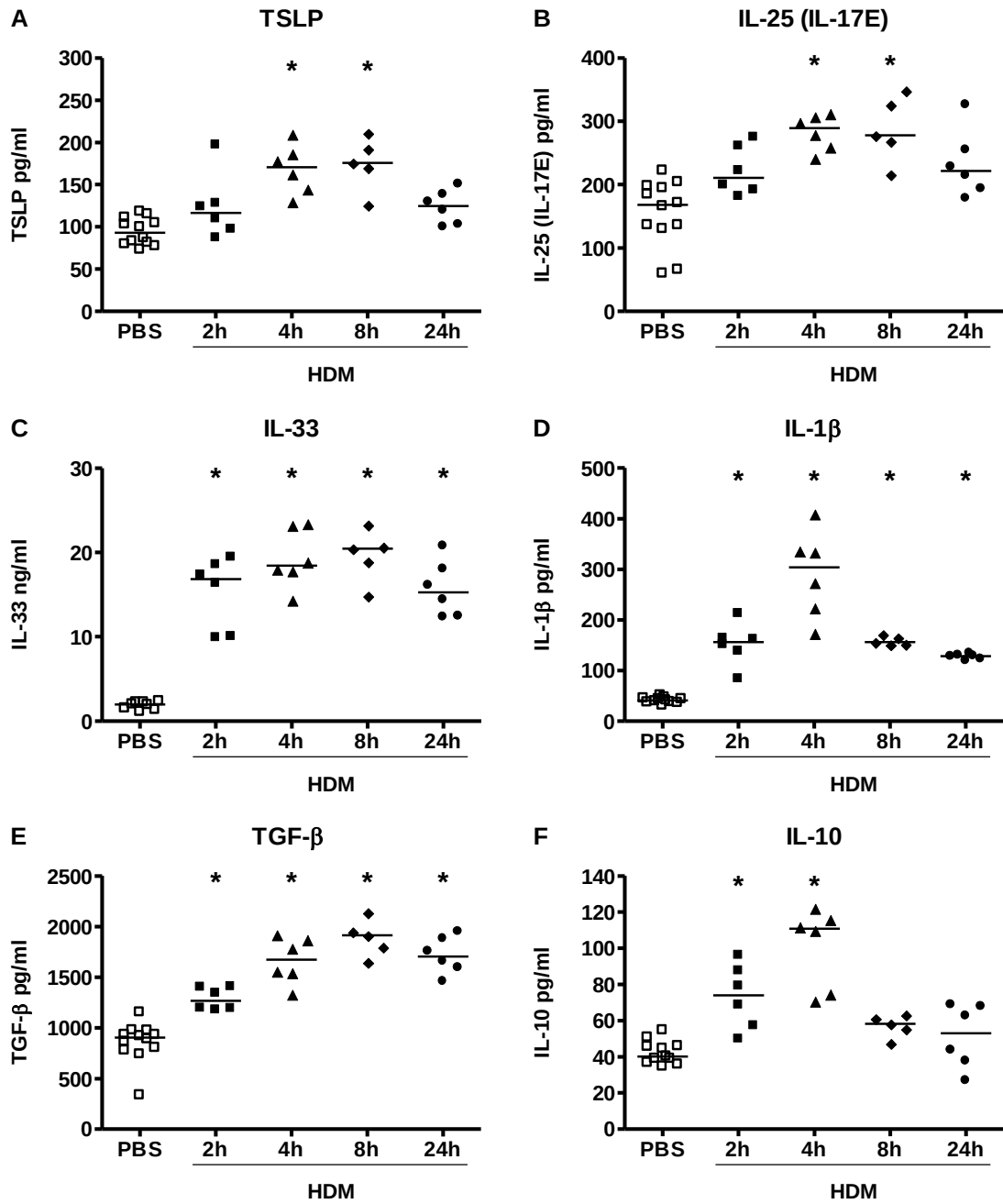


Figure 3.7. Inflammatory cytokine profile in the lung of HDM-treated mice. (A) TSLP, (B) IL-25 (IL-17E), (C) IL-33, (D) IL-1 β , (E) TGF- β and (F) IL-10 measured in the lung 2, 4, 8 and 24h after the final i.n. challenge. TSLP, IL-25 (IL-17E), IL-33 and TGF- β levels were determined by ELISA and IL-10 and IL-1 β levels by MSD (n=12 mice treated with PBS, n=5-6 mice treated with HDM at each time point). *, P<0.05; (Mann-Whitney *U* test) compared with PBS. Bars depict the median of the groups. One experiment.

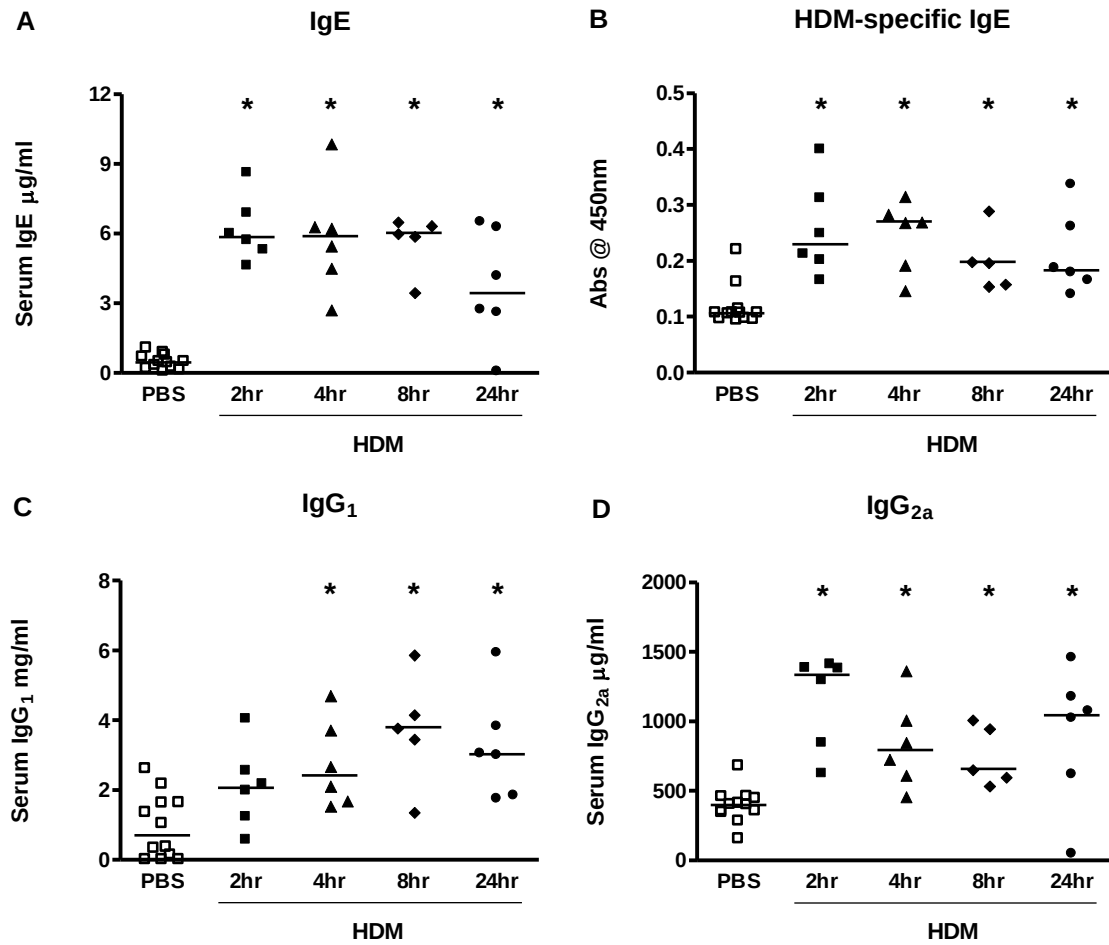
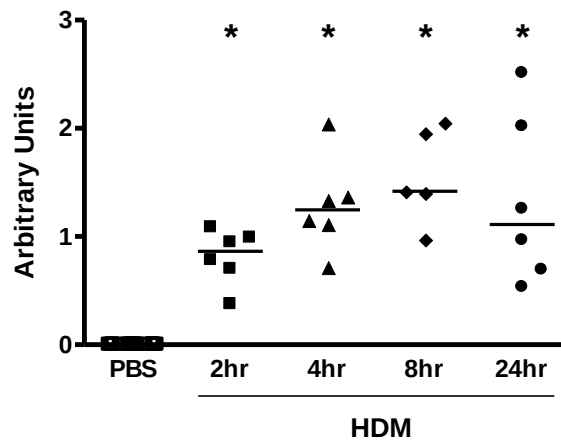


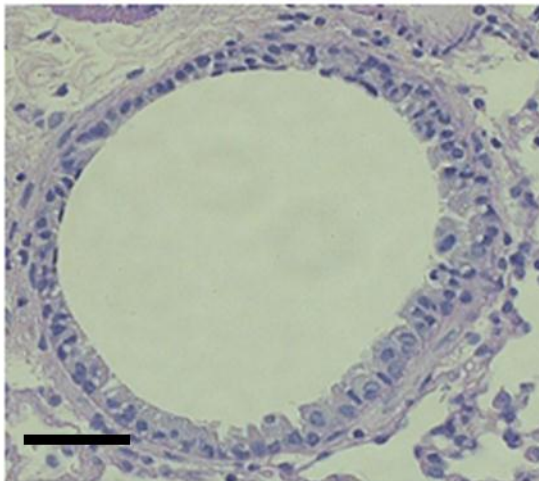
Figure 3.8. Serum immunoglobulin profile of HDM-treated mice.

(A) Total IgE, (B) HDM-specific IgE, (C) total IgG₁ and (D) total IgG_{2a} levels measured in the serum 2, 4, 8 and 24h after the final i.n. challenge. All levels were determined by ELISA. (n=12 mice treated with PBS, n=5-6 mice treated with HDM at each time point). *, $P < 0.05$; (Mann-Whitney U test) compared with PBS. Bars depict the median of the groups. One experiment.

A. Mucus Score



B. PBS



C. HDM

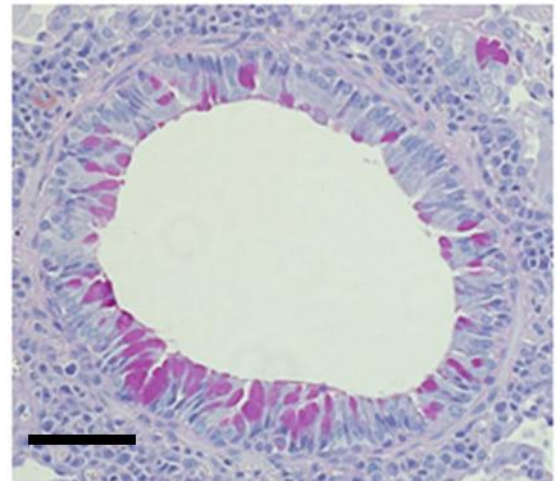


Figure 3.9. Goblet cell hyperplasia in HDM-treated mice.

(A) Semi-quantitative scoring of PAS stained lung sections. (n=12 mice treated with PBS, n=5-6 mice treated with HDM at each time point). *, $P < 0.05$; (Mann-Whitney U test) compared with PBS. Bars depict the median of the groups. Representative photomicrographs of PAS stained lung sections from (B) PBS and (C) HDM-treated mice 4h post final challenge (original 20X magnification). Scale bar = 50 μm . One experiment.

3.5. Discussion

The pathophysiological features of asthma, namely AHR, airway inflammation and airway remodelling, have been modelled in mice in order to determine the molecules responsible for disease pathogenesis. The pulmonary response to inhaled HDM in mice was shown to follow a distinct time course from the development of AHR, with Th2 cytokines in particular being produced early after inhaled challenge. The peak time to measure changes in lung function did not correspond with the highest production of Th2 cytokines which has important consequences for the study of the molecular mechanisms of tissue pathophysiology in this model of inhaled HDM challenge.

Previous to this work, HDM has been shown to sensitise immunologically naïve mice via the respiratory mucosa and that prolonged exposure to HDM can mimic certain aspects of chronic asthma, namely airway inflammation, AHR and airway remodelling (Johnson et al., 2004). Inflammatory cytokine generation was demonstrated by these authors after *in vitro* restimulation of splenocytes isolated from mice exposed to HDM (Johnson et al., 2004). Although these observations are important to define antigen-specific memory responses, local lung cytokine production was not reported and the cytokine levels determined from restimulated splenocytes may not accurately reflect the cytokine levels in the lung and BAL following allergen challenge *in vivo*. Indeed, allergen challenge in both asthmatic patients and OVA sensitised mice is characterised by AHR and increased production of IL-4, IL-5 and IL-13 in the BAL (Robinson et al., 1992; Pilette et al., 2004; McMillan and Lloyd, 2004; Koya et al., 2007). Therefore, the aim of the experiment was to determine whether key cells, inflammatory mediators and Th2 cytokines associated with the allergic response were produced locally in the lung and BAL after exposure to HDM concomitant with AHR.

Three weeks of exposure to HDM has been shown to be the earliest time point at which AHR is observed (Gregory et al., 2009). Thus to determine the Th2 cytokine profile and inflammatory mediator response to HDM locally in the lung and BAL together with AHR, mice were exposed to 3 weeks of inhaled HDM and were then sacrificed 2, 4, 8 and 24h following the final allergen challenge. It is important to

note at this time point that airway remodelling, although initiated, is not fully established (Johnson et al., 2004;Southam et al., 2007).

The results show that following continuous exposure to HDM, greatest levels of cytokines are quantified early post-final challenge in the lung and BAL and then decrease over the 24h period monitored. In contrast, levels of chemokines are maintained and persist throughout. The decline in cytokine levels at 24h, although not to baseline, contrasts with the increase in lung resistance which only reached significance at 24h post challenge. This implies that the optimum time point for cytokine analysis occurs at a time when significant changes in lung resistance are not fully established in the model. It is interesting to note that the peak of cellular influx and Th2 cytokine production in response to HDM occurs before the onset of AHR and thus demonstrates a temporal relationship between the onset of pathological and physiological changes seen in this murine model of allergic airways disease. Three weeks of HDM exposure led to the production of allergen-specific IgE production, therefore, indicating a true allergic response.

Described here is a murine model of HDM-induced allergic airways disease that was developed to reproduce the characteristics of in human allergic asthma. Several key features of human asthma (AHR and Th2-driven eosinophilic airway inflammation) are replicated following the exposure of immunologically naïve mice to HDM. Concomitant with HDM-induced AHR, Th2-associated cytokines, chemokines, inflammatory cells and pro-allergic innate cytokines (TSLP, IL-33 and IL-25 (IL-17E)) linked to the development of asthma were profiled in the lung tissue and BAL. A time-course relationship between these mediators and cells with the functional consequence of AHR was determined and the optimum time points to investigate various aspects of the inflammatory response following 3 weeks of HDM challenge were deduced.

All mediators found to be elevated in the lung and BAL of HDM-treated mice, have been linked to the pathogenesis of asthma, such as IL-13 and AHR (Wills-Karp et al., 1998;Wills-Karp, 2004). Indeed, several of these mediators have been targeted therapeutically in clinical trials, including IgE (Poole et al., 2005;Holgate et al., 2005), eosinophils (Nair et al., 2009;Halder et al., 2009) and Th2 cytokines (Leckie et

al., 2000; Flood-Page et al., 2007; Corren et al., 2010; Corren et al., 2011). The data from the current study have important implications for the analysis of a murine model that is more closely related to allergen exposure and sensitisation that occurs in humans and can build on knowledge from OVA sensitisation and challenge models. These findings are of fundamental importance because mouse models are used to explore the molecular and biochemical pathways leading to immunopathophysiology. In particular, when used for delineation of mechanisms of action of potential novel therapies.

The relationship between chronic inflammation, airway remodelling, and airway dysfunction is complex because these are interacting processes each with distinct kinetics. Thus, an experimental model mimicking the phenotype of chronic asthma is valuable as it will allow further investigations into the mechanisms by which allergic airways disease is initiated and maintained. However, it is interesting to note that HDM-driven inflammation is not solely a Th2-associated response, since IFN- γ and the regulatory cytokines IL-10 and TGF- β were also quantified in the lung tissue.

HDM is a complex aeroallergen and the inflammatory profile in response to HDM is composed of a variety of cells, such as eosinophils and neutrophils, and characterised by a diverse cytokine profile. Whole HDM extract was used in these experiments to mimic human pulmonary exposure and it is unlikely that one single feature of the allergen is responsible for the immune response. The observed disease pathology leads to several important questions regarding the properties of the HDM allergen and what roles these features play in the development of the immune response.

Using the model of HDM exposure as described in Chapter 3 to elicit the characteristics of allergic asthma in mice, the focus and aims of Chapters 4 and 5 are investigating the contribution of TLR signalling and protease activity to the immune response to HDM. In addition to this, determining the role of TGF- β in HDM-induced allergic airways disease was also undertaken in Chapter 6.

Chapter 4 – Contribution of Toll-like receptor signalling to HDM-induced allergic airways disease

4.1. Introduction

Activation of pattern recognition receptors (PRRs) on epithelial cells and DCs provide the signalling information for appropriate programming of the adaptive immune response to inhaled antigens. Toll-like receptors (TLRs) are the most widely studied family of PRRs in allergic inflammation and TLRs recognise antigens that express TLR ligands (Kawai and Akira, 2010). Mice express TLRs 1-9 and 11-13 (Akira et al., 2006). TLR1, 2, 4, 5 and 6 are primarily expressed on the cell surface and recognise pathogen-associated molecular patterns (PAMPs) derived from bacteria, fungi and protozoa, whereas TLR3, 7, 8 and 9 are exclusively expressed within endocytic compartments and primarily recognise nucleic acid PAMPs derived from viruses and bacteria (Kawai and Akira, 2010). The cytoplasmic portion of TLRs shows similarity to that of the IL-1 receptor family and is known as the Toll/IL-1 receptor (TIR) domain (Takeda and Akira, 2005). However, despite this similarity, the extracellular regions of IL-1 receptors possess an Ig-like domain, whereas TLRs bear leucine-rich repeats (LRRs) (Akira et al., 2006).

The activation of TLR signalling pathways originates from the cytoplasmic TIR domains and TLR signalling is primarily mediated via the recruitment of different TIR domain-containing adaptor molecules such as myeloid differentiation primary response gene 88 (MyD88), TRIF (TICAM-1), TIRAP (Mal), and TRAM to the TIR domains (Kawai and Akira, 2010). All TLRs, except for TLR3 and TLR4, signal exclusively via MyD88 and, in addition to MyD88, TLR1, TLR2, TLR4 and TLR6 recruit TIRAP to initiate MyD88-dependent signalling (Akira et al., 2006). MyD88 associates with the TIR domain of TLRs and, upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs (Kawai and Akira, 2010). IRAK is activated by phosphorylation and then associates with TRAF6, leading to the activation of JNK and NF- κ B signalling pathways (Kawai and Akira, 2010). MyD88 signal transduction results in the production of pro-inflammatory cytokines such as TNF- α and IL-12 (Kawai and Akira, 2010).

A MyD88-independent signalling pathway exists for TLR3 and TLR4 and the MyD88-independent pathway is mediated by TRIF (Kawai and Akira, 2010). The TLR3-TRIF pathway is initiated through the recruitment of TRAF6 to the distinct

domain of TRIF (Kawai and Akira, 2010). The TLR3 ligand dsRNA has been shown to induce NF- κ B activation and IFN- β production in cells deficient in MyD88 (Alexopoulou et al., 2001) and, in mice deficient in TRIF signalling, TLR3-mediated expression of IFN- β and IFN-inducible genes is impaired (Yamamoto et al., 2003). TLR4 is unique in that this TLR signals via both MyD88-dependent and TRIF-dependent pathways (Kawai and Akira, 2010). TLR4 first recruits TIRAP, which facilitates the recruitment of MyD88 to initiate the first phase of NF- κ B and MAPK activation. For TRIF-dependent signalling, TLR4 is trafficked to the endosome via dynamin-dependent endocytosis and forms a complex with TRAM and TRIF (Kawai and Akira, 2010). LPS-TLR4 signalling has been shown to activate the transcription factor IRF-3 that induces IFN- β and IFN- β subsequently activates STAT1, which then promotes the IFN-inducible gene expression (Kawai and Akira, 2010). In TRIF knockout (KO) mice TLR4-mediated expression of IFN-inducible genes is defective (Hoebe et al., 2003). A summary of the TLR signalling pathways and ligands is shown in Figure 4.1 (adapted from Takeda and Akira, 2005).

Lipopolysaccharide (LPS) is component of Gram negative bacteria found in HDM extracts (Douwes et al., 2000). LPS signals through TLR4, with LPS-binding protein (LBP), CD14 and MD-2 facilitating this pathway (Akira et al., 2006) and low level inhaled LPS signalling through TLR4 is necessary to induce Th2 responses to OVA (Eisenbarth et al., 2002). The major HDM allergen *Der p 2* shares structural homology with MD-2 (Derewenda et al., 2002; Gruber et al., 2004) and Trompette and colleagues have demonstrated that *Der p 2* facilitates TLR4 signalling through direct interactions with the TLR4 complex by reconstituting LPS-driven TLR4 signalling in the absence of MD-2 (Trompette et al., 2009). The authors also illustrated *in vivo* that *Der p 2* drives experimental allergic asthma in a TLR4-dependent manner and that this TLR4 signalling pathway was retained in mice genetically deficient in MD-2 (Trompette et al., 2009). This is of particular importance as human airway epithelial cells express TLR4, but minimal amounts of MD-2 under homeostatic conditions (Jia et al., 2004).

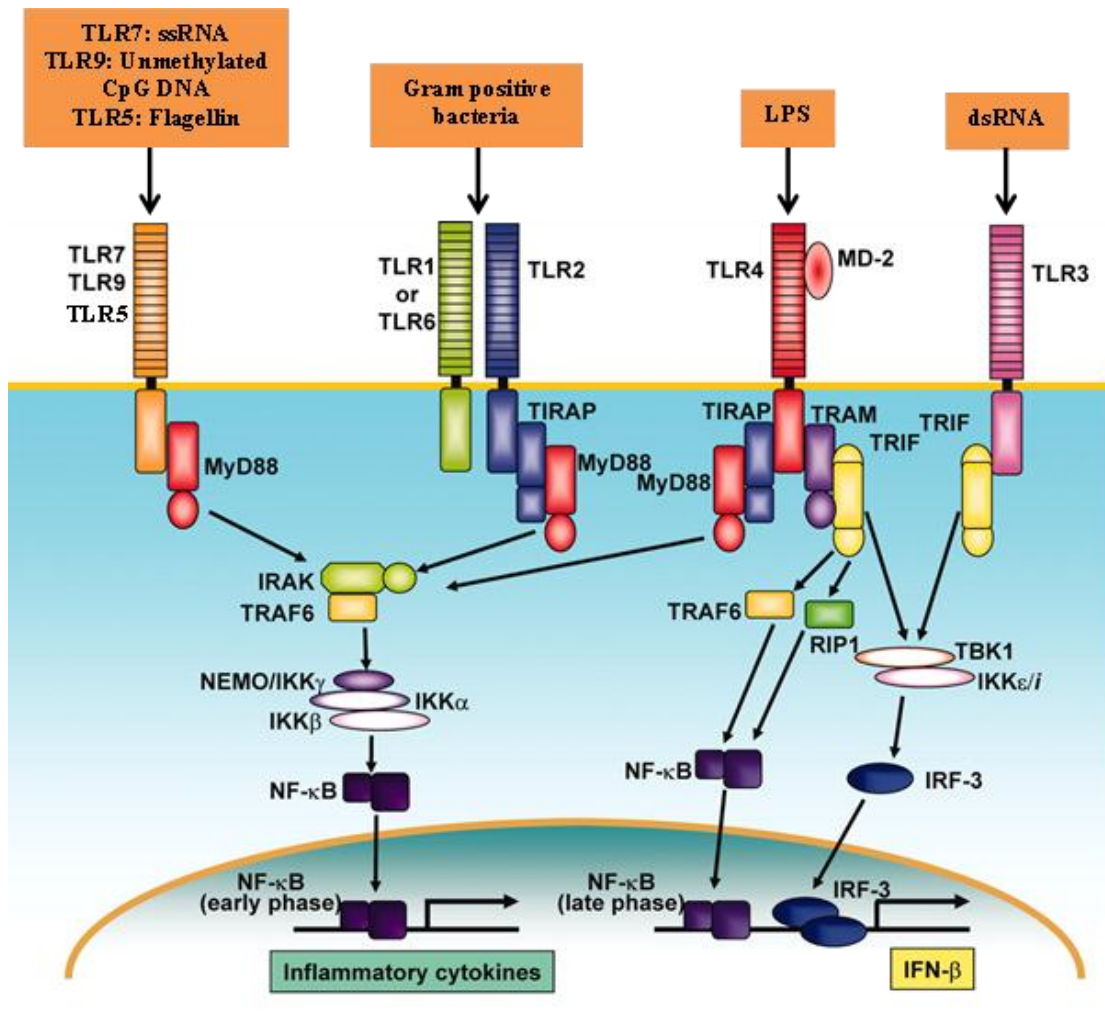


Figure 4.1. TLR signalling pathways.

TLR signalling pathways originate from the cytoplasmic TIR domain. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the I κ B kinase (IKK) complex. The IKK complex phosphorylates I κ B, resulting in nuclear translocation of NF- κ B which induces expression of inflammatory cytokines. A second TIR-domain containing adaptor, TIRAP, is involved in the MyD88-dependent signalling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signalling pathways, activation of IRF-3 and induction of IFN- β are observed in a MyD88-independent manner. The TIR domain-containing adaptor TRIF is essential for the MyD88-independent pathway. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway. The main TLR ligands are also shown. ssRNA, single-stranded RNA; dsRNA, double-stranded RNA (adapted from Takeda and Akira, 2005).

Upon commencement of the current project, the contribution of TLR signalling to the immune response to HDM *in vivo* remained to be elucidated. However, concurrent with the work discussed in this thesis, utilising intermittent HDM exposure protocols, Hammad and colleagues showed that stromal cell TLR4 signalling was sufficient to drive Th2 immune responses to HDM (Hammad et al., 2009) and Phipps and colleagues showed that attenuated HDM-driven Th2 responses occurred in MyD88 KO and TLR4 KO mice (Phipps et al., 2009). The importance of these two reports with the observations from this Chapter will be discussed further in section 4.5.

In this Chapter the contribution of TLR signalling to the immune response to HDM-induced allergic airways disease was investigated using both genetically modified mice and pharmacological approaches. Firstly, mice lacking systemic TLR4 signalling were exposed to HDM in order to determine how a deficiency in this pathway influenced disease pathogenesis. C3H-HeJ mice possess a mutation in the LPS response locus of TLR4 that results in these mice being hyporesponsive to LPS (Poltorak et al., 1998) and cells derived from C3H-HeJ mice are resistant to LPS stimulation *in vitro* (Hoshino et al., 1999). Macrophages fail to develop an activation phenotype or die when exposed to high concentrations of LPS, B cells do not respond to the mitogenic, adjuvant or immunogenic properties of LPS and fibroblasts fail to undergo metabolic activation upon LPS exposure (Hoshino et al., 1999). A pharmacological approach was then undertaken to prevent pulmonary TLR4 signalling, whereby a TLR4 antagonist (an underacylated form of *Rhodobacter sphaeroides* LPS) blocked TLR4 signalling via direct competition for the same binding site as LPS on MD-2 and by inhibiting LPS-MD-2 function at TLR4 (Saitoh et al., 2004; Coats et al., 2005; Teghanemt et al., 2005; Visintin et al., 2005). Consequently, this antagonist abolishes both LPS and *Der p 2* TLR4 signalling.

In order to proceed forward from TLR4 and to investigate the contribution of multiple TLRs to the immune response, two further approaches were utilised. MyD88 KO mice were exposed to HDM as this adaptor protein is vital in signalling of all TLR family members, apart from TLR3 and the TRIF-TRAM signalling pathway of TLR4 (Yamamoto et al., 2002; Yamamoto et al., 2003; Akira et al., 2006). An additional pharmacological approach was carried out, targeting multiple TLRs, using a naturally occurring species of phosphatidyl serine (PS), 1-stearoyl-2-arachidonoyl-*sn*-glycero-

3-[phospho-L-serine] (SAPS) (Parker et al., 2008). SAPS has been shown to block TLR1-4, 7 and 8 signalling by disruption of membrane microdomains and preventing the association of TLR2 and TLR4 with their respective membrane signalling partners (Parker et al., 2008).

Following these *in vivo* experiments, the impact of HDM on the gene expression of TLRs, and associated TLR signalling family members, in the lung was determined by PCR array. This was done in order to investigate what effect HDM had on TLR expression and whether these observations were influenced by either the strain of mouse, the absence of MyD88 or the SAPS inhibitor. Thus through the experiments carried out in this Chapter the contribution of TLR signalling, and associated MyD88-dependent and MyD88-independent TRIF signalling, to the immune response to HDM following continuous respiratory exposure was investigated.

4.2. Hypothesis and Aims

4.2.1. Hypothesis

TLR signalling is vital for the *in vivo* immune response to HDM and disruption of TLR pathways will result in reduced Th2-driven airway inflammation, AHR and airway remodelling.

4.2.2. Aims

Thus, the aims of Chapter 4 are to dissect:

- The specific role of TLR4 in the immune response to HDM
- The contribution of MyD88 to the development of HDM-induced disease pathogenesis
- How a broad TLR antagonist will influence the development of the immune response to HDM.

4.3. Experimental Plan

4.3.1. Protocol A: HDM administration to TLR4-deficient mice

Female wildtype (*wt*) C3H-HeN or TLR4-deficient C3H-HeJ mice were administered either 25 μ l PBS or 25 μ g (in 25 μ l) HDM i.n. 5 times a week for 3 weeks and culled 24h post final challenge.

4.3.2. Protocol B: Prophylactic treatment with a TLR4 antagonist

Female BALB/c mice were given either 25 μ l PBS or 25 μ g (in 25 μ l) HDM i.n. 3 times a week for 3 weeks. Prior to each PBS or HDM challenge, mice received either vehicle (water) or a TLR4 antagonist 1 μ g i.t. The vehicle or TLR4 antagonist were administered in a volume of 20 μ l. Mice were culled 24h post final challenge.

4.3.3. Protocols C: Administration of HDM to MyD88 KO mice

Female wildtype (*wt*) C57BL/6 or MyD88 knockout (KO) mice were given either 25 μ l PBS or 25 μ g (in 25 μ l) HDM i.n. 3 times a week for either 1 week, 3 weeks or 5 weeks. After the respective treatment, mice were culled 24h post final challenge.

4.3.4. Protocol D: Prophylactic treatment with SAPS inhibitor antagonist

Female BALB/c mice were administered either 25 μ l PBS or 25 μ g (in 25 μ l) HDM i.n. 3 times a week for 3 weeks. Prior to each PBS or HDM challenge, mice received either vehicle (PBS) or SAPS inhibitor 1 mg/Kg i.n. The vehicle or SAPS inhibitor were administered in a volume of 50 μ l. Mice were culled 24h post final challenge.

4.4. Results

4.4.1. Protocol A: HDM challenge in TLR4-signalling deficient mice

4.4.1.1. HDM exposure causes AHR in TLR4-signalling deficient mice

To determine the contribution of functional TLR4 signalling to HDM-induced allergic airways disease, wildtype (wt) C3H-HeN and naturally occurring TLR4-signalling deficient C3H-HeJ (TLR4 -ve) mice were exposed to either PBS or HDM for 3 weeks.

HDM exposure resulted in increased airway resistance in both wt and TLR4 -ve mice 24h post-final challenge (Figure 4.2A and B). However, only HDM-treated TLR4 -ve mice showed decreased airway compliance compared to the respective PBS control group (Figure 4.2C and D).

4.4.1.2. HDM promotes inflammatory cell recruitment to the lungs of TLR4 -ve mice

HDM inhalation resulted in elevated numbers of total cells in the lung and BAL of wt and TLR4 -ve mice (Figure 4.3A and B). As expected eosinophils composed a significant component of these populations and the numbers of eosinophils recovered from the lung and BAL in response to HDM were not reduced by the absence of functional TLR4 signalling (Figure 4.3C and D). Indeed the number of total cells and eosinophils recovered from the BAL were greater in HDM-treated TLR4 -ve mice than HDM wt mice (Figure 4.3B and D). HDM-treated wt mice were observed to have increased numbers of neutrophils in the lungs compared to PBS controls, however in contrast, neutrophilia was not observed in the lungs of HDM-treated TLR4 -ve mice (Figure 4.3E). This observation was restricted to the lung, as HDM-induced neutrophilia persisted in the BAL of HDM exposed TLR4 -ve mice (Figure 4.3F). Greater numbers of Th2 cells were recovered from the lung and BAL of HDM-treated mice compared to PBS controls and there was no impact of systemic TLR4 signalling deficiency on the number of these cells (Figure 4.3G - H).

Airway Resistance

Airway Compliance

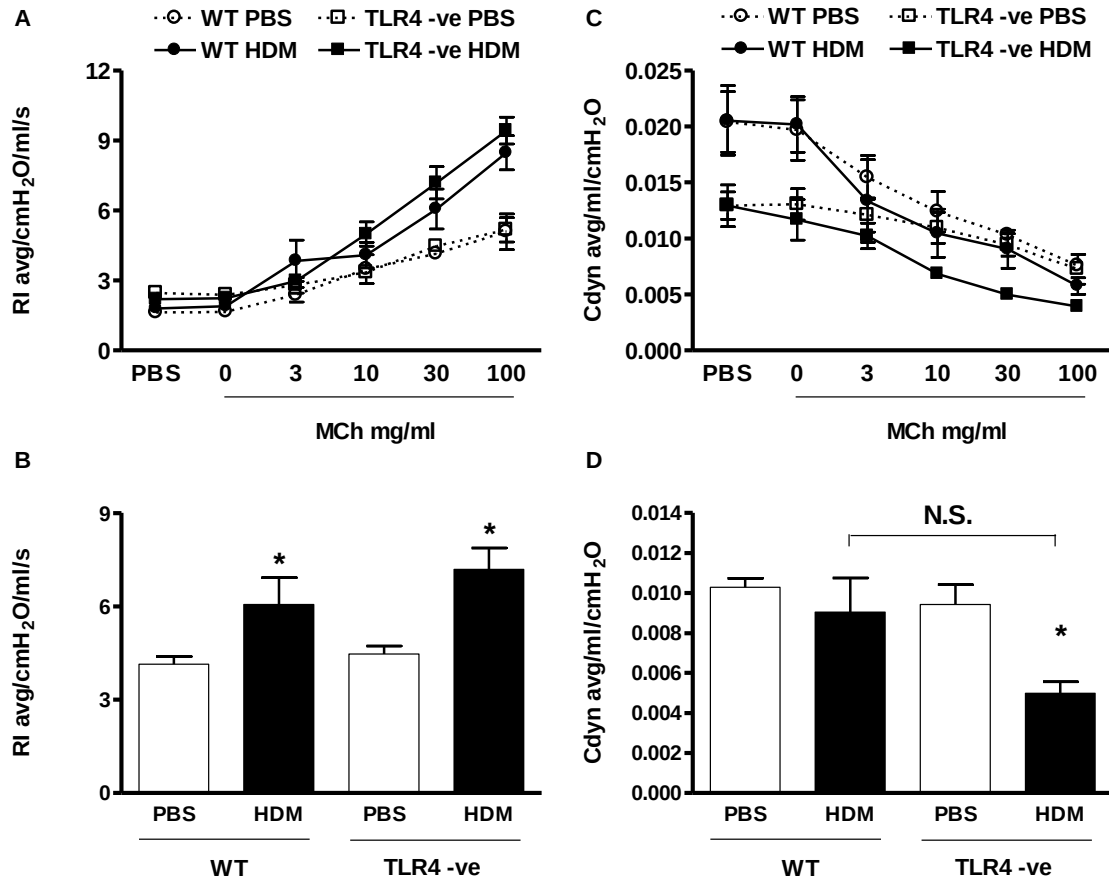


Figure 4.2. AHR in TLR4 -ve HDM-treated mice.

(A) Airway resistance (RI) of PBS and HDM-treated mice at 24h post final challenge. (B) Airway resistance expressed as mean RI $\pm$ SEM for the 30mg/ml dose of methacholine. (C) Airway compliance (Cdyn) of PBS and HDM-treated mice at 24h post final challenge. (D) Airway compliance expressed as mean Cdyn $\pm$ SEM for the 30mg/ml dose of methacholine. (n=4 PBS and n=5-6 HDM). *, P<0.05 (Mann-Whitney *U* test) HDM compared with PBS for the corresponding strain. Data are mean $\pm$ SEM. N.S., not significant. One experiment.

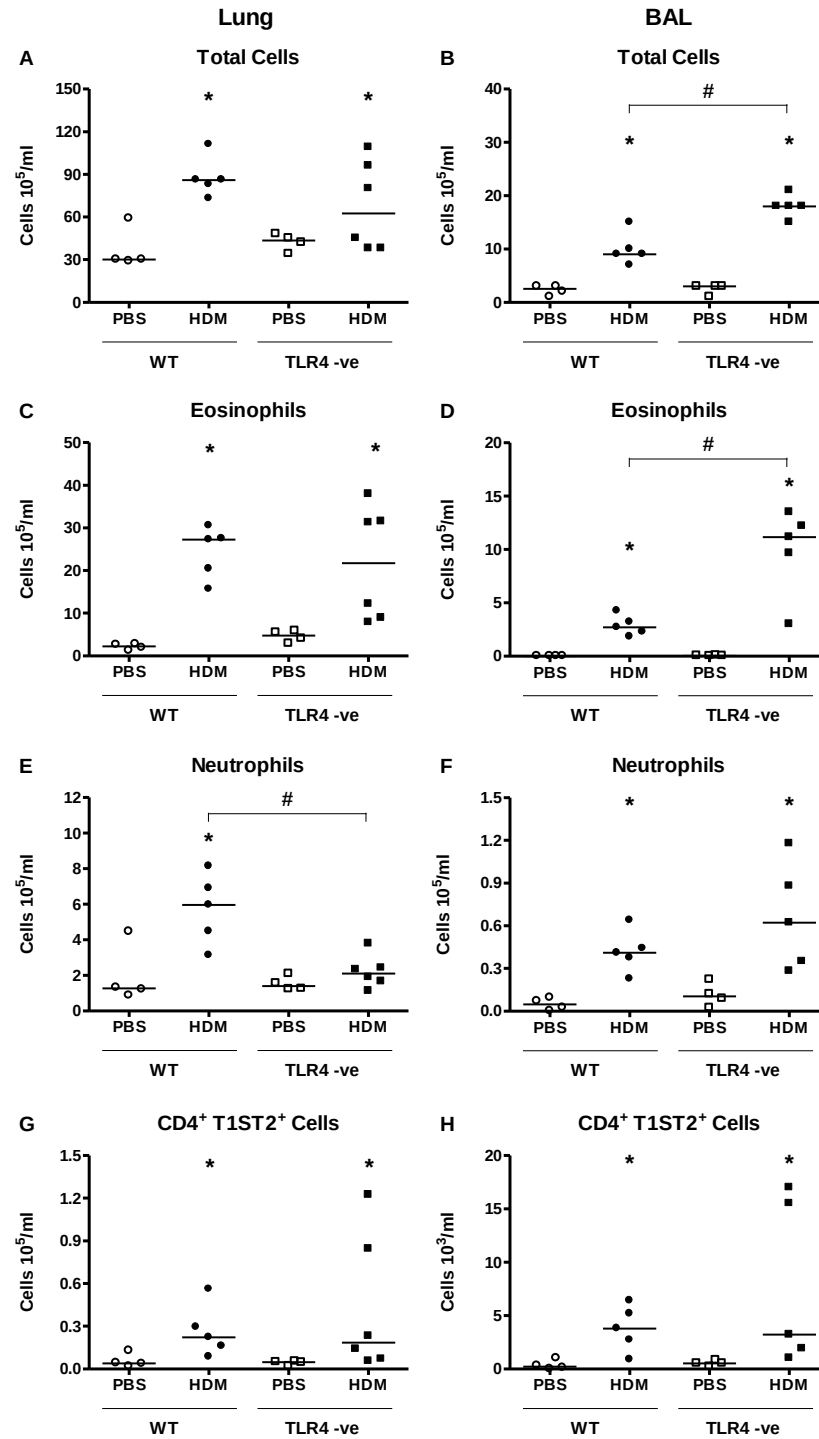


Figure 4.3. Eosinophilia in HDM-treated TLR4 -ve mice.

Total cells, eosinophils, neutrophils and CD4⁺T1S2⁺ cells recovered from the lung (A, C, E and G) and BAL (B, D, F and H) 24h after the final challenge (n=4 PBS and n=5-6 HDM). Total cell counts were determined using a haemocytometer with white blood cell counting fluid, eosinophils and neutrophils were quantified from Wright-Giemsa stained cytopins and CD4⁺T1S2⁺ cells were quantified by flow cytometry, as described in the Materials and Methods. *, P<0.05; (Mann-Whitney *U* test) HDM compared with PBS from corresponding strain. #, P<0.05 (Mann-Whitney *U* test) TLR4 -ve HDM compared with wt HDM. Bars depict median of the groups. One experiment.

4.4.1.3. Inflammatory chemokine and cytokine production in HDM-treated TLR4 -ve mice

The chemokines associated with the eosinophil, neutrophil and Th2 cell recruitment in response to HDM were assessed in both *wt* and TLR4 -ve mice. Eotaxin-1/CCL11 and eotaxin-2/CCL24 levels were elevated in the lungs of both *wt* and TLR4 -ve mice in response to HDM, with no significant differences observed between each HDM-treated group (Figure 4.4A and B). HDM challenged *wt* mice demonstrated increased levels of KC/mCXCL1 in the lung compared to PBS controls. However, in TLR4 -ve mice there was reduced production of KC/mCXCL1 in response to HDM compared to *wt* controls (Figure 4.4C), concomitant with reduced neutrophilia in the lung (Figure 4.3E). HDM-induced production of TARC/CCL17 and RANTES/CCL5 was not seen in the TLR4 -ve mice, due to the high baseline levels in the PBS TLR4 -ve group (Figure 4.4D and E). MCP-1/CCL2 production was unaltered by the absence of TLR4 signalling (Figure 4.4F).

HDM-induced production of IL-4, IL-5 and IL-1 β were not affected by the lack of TLR4 signalling (Figure 4.5A - C). Similarly, TLR4 signalling deficiency did not influence HDM-induced production of the Th1 associated cytokines IFN- γ or IL-12 (Figure 4.5D and E).

IL-33 levels were greater in the lung tissue of HDM TLR4 -ve mice than the *wt* (Figure 4.6A). TLR4 signalling deficiency did not alter IL-25 (IL-17E), TSLP and CCL20/MIP-3 α responses to HDM. The baseline levels of IL-25 (IL-17E), TSLP and CCL20/MIP-3 α in the lung tissue of TLR4 -ve mice were higher than those of the respective *wt* group (Figure 4.6B - D).

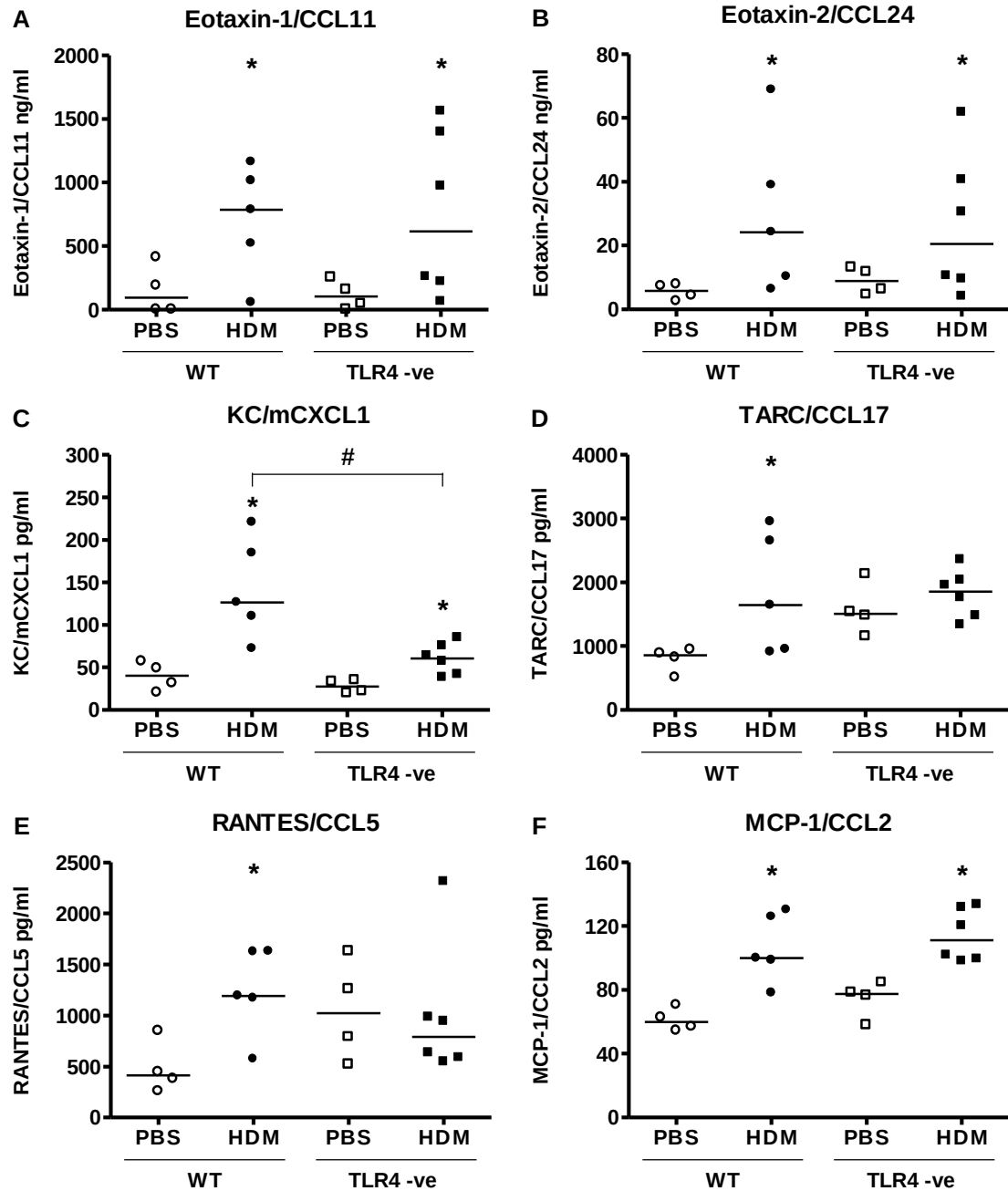


Figure 4.4. Altered chemokine profile of TLR4 -ve mice.

(A) Eotaxin-1/CCL11, (B) Eotaxin-2/CCL24, (C) KC/mCXCL1, (D) TARC/CCL17, (E) RANTES/CCL5 and (F) MCP-1/CCL2 levels measured in the lung 24h after the final challenge. Eotaxin-1/CCL11, eotaxin-2/CCL24, TARC/CCL17, RANTES/CCL5 and MCP-1/CCL2 levels were determined by ELISA and KC/mCXCL1 by MSD. (n=4 PBS and n=5-6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding strain. #, $P < 0.05$ (Mann-Whitney U test) TLR4 -ve HDM compared with wt HDM. Bars depict the median of the group. One experiment.

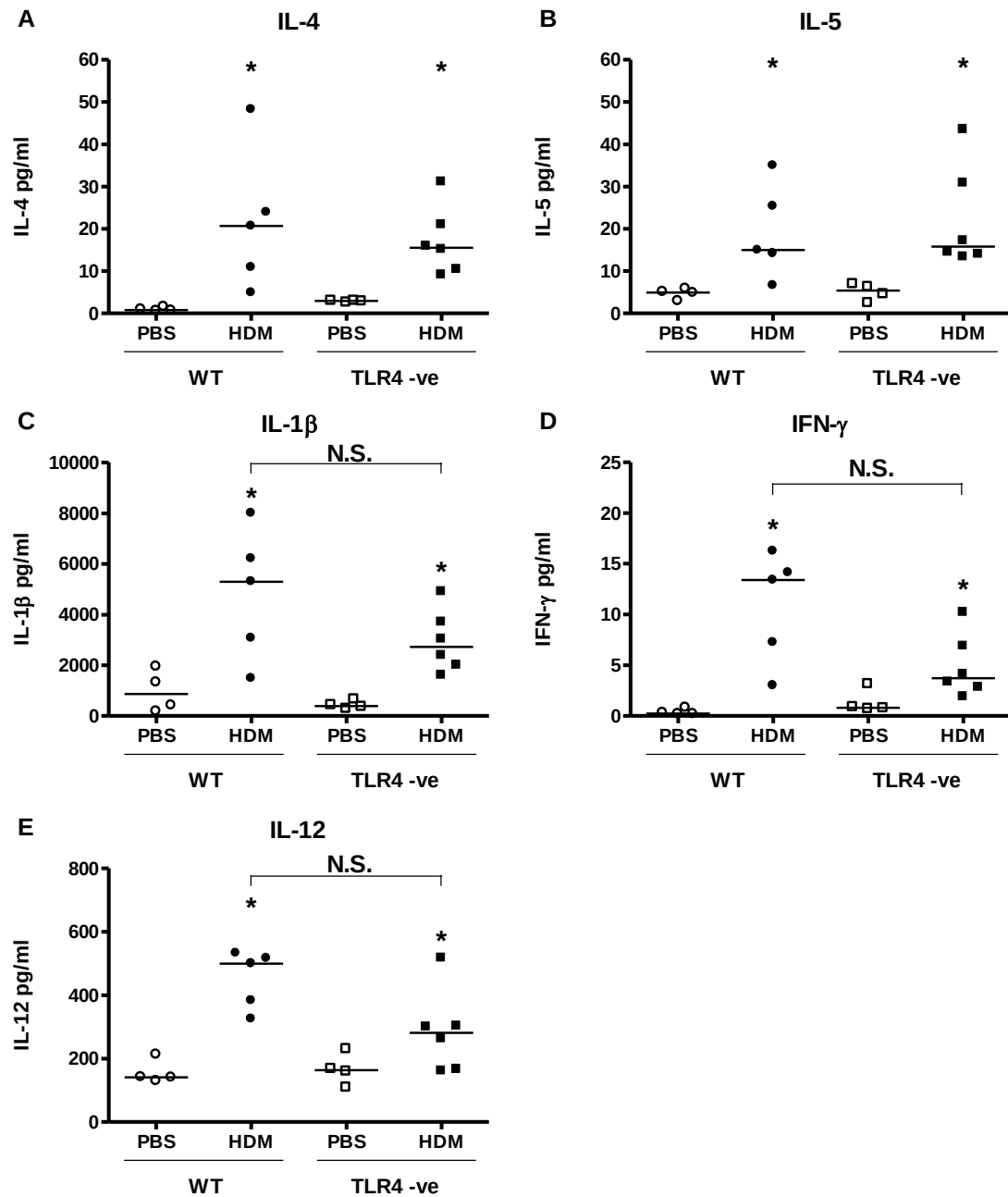


Figure 4.5. Pro-inflammatory cytokine production in HDM-treated mice.

(A) IL-4, (B) IL-5, (C) IL-1 β , (D) IFN- γ and (E) IL-12 were measured in the lung 24h after the final challenge. All mediator levels were determined by MSD. (n=4 PBS and n=5-6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding strain. N.S. Not significant. Bars depict the median of the group. One experiment.

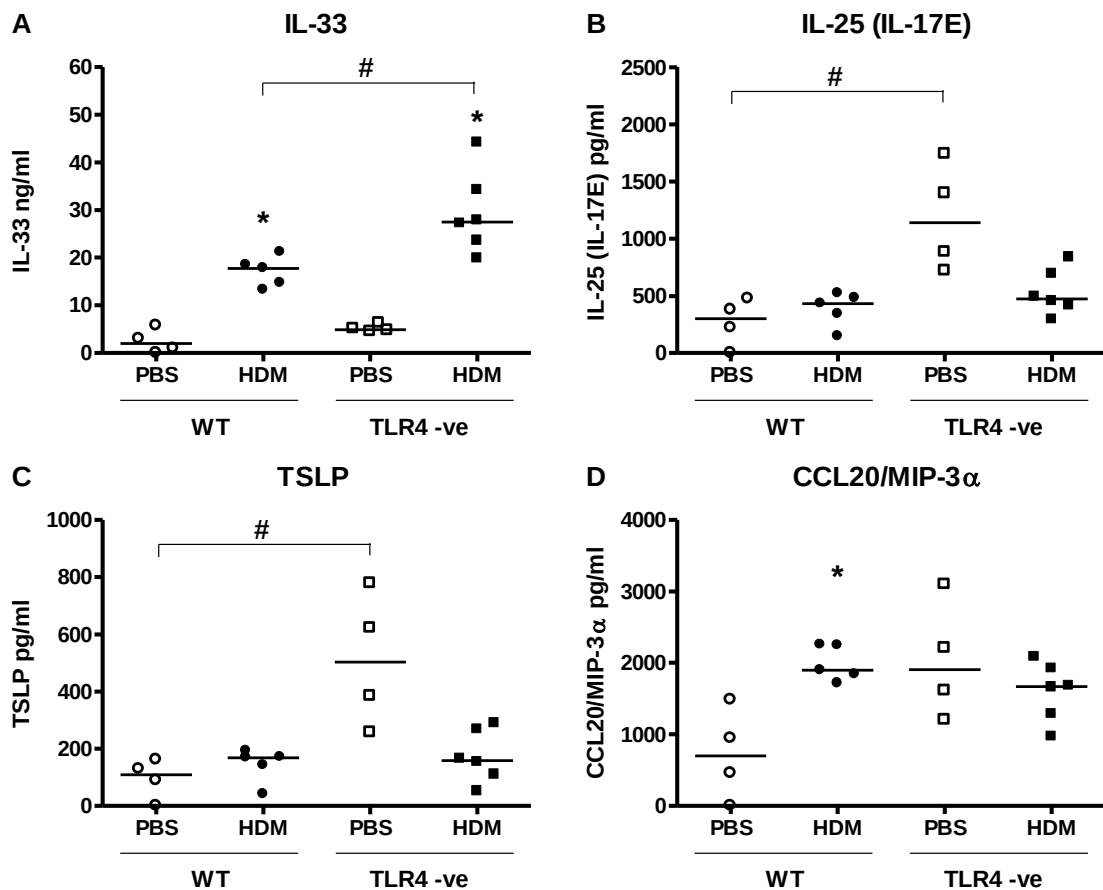


Figure 4.6. Innate cytokine production in HDM-treated mice.

(A) IL-33, (B) IL-25 (IL-17E), (C) TSLP and (D) CCL20/MIP-3 α were measured in the lung 24h after the final i.n. challenge. All mediator levels were determined by ELISA. (n=4 PBS and n=5-6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding strain. #, $P < 0.05$ (Mann-Whitney U test) either TLR4 -ve PBS compared with wt PBS or TLR4 -ve HDM compared with wt HDM. Bars depict the median of the group. One experiment.

4.4.1.4. Pro-inflammatory serum immunoglobulin production in TLR4 -ve mice

As expected, HDM inhalation induced the production of IgE, IgG₁ and IgG_{2a} in the serum of *wt* mice. The total IgE response to HDM was elevated in TLR4 -ve mice compared to HDM exposed *wt* controls (Figure 4.7A), whereas HDM-specific IgE and total IgG₁ and IgG_{2a} production was not altered between strains (Figure 4.7B - D).

4.4.1.5. Goblet cell hyperplasia is not altered by the absence of TLR4

PAS staining of lung sections showed that HDM challenge elicited mucus production in the airways, to similar levels, in both *wt* and TLR4 -ve mice (Figure 4.8A). Representative pictures of PAS stained lung sections from PBS and HDM-treated *wt* and TLR4 -ve mice are shown (Figure 4.8B – E).

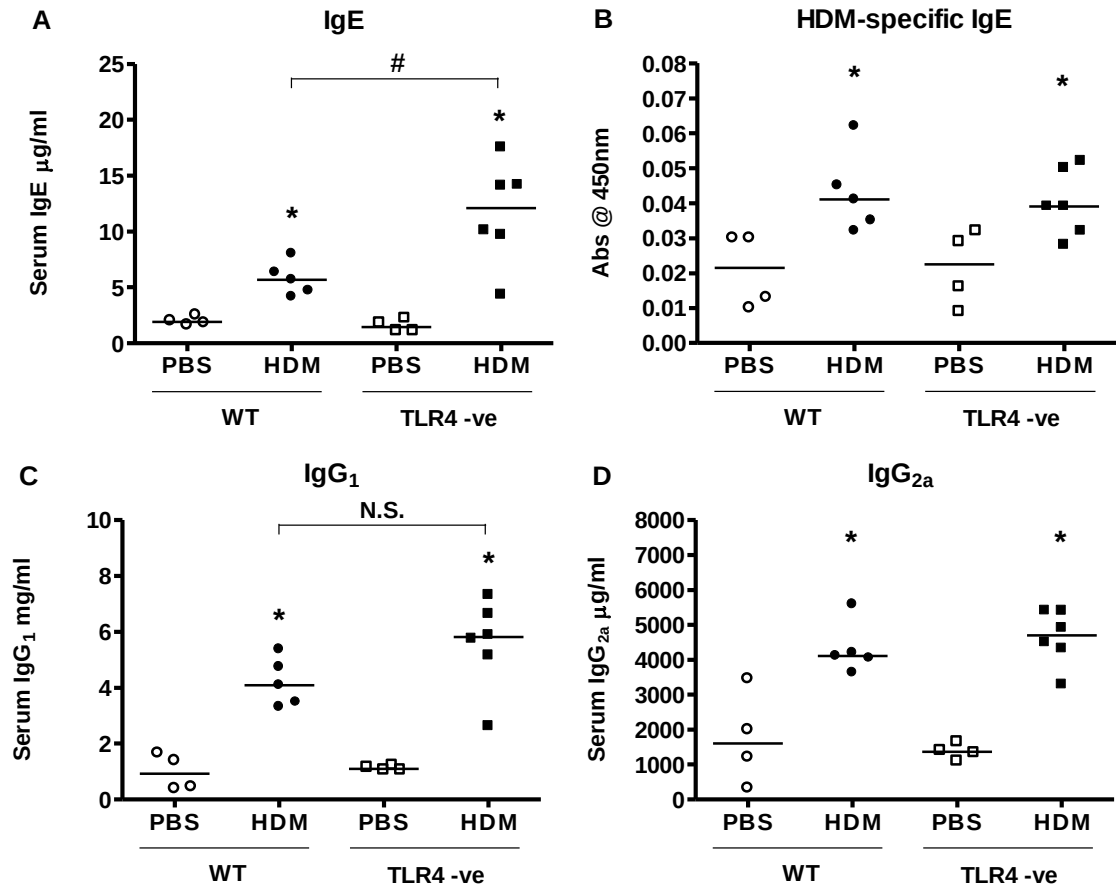
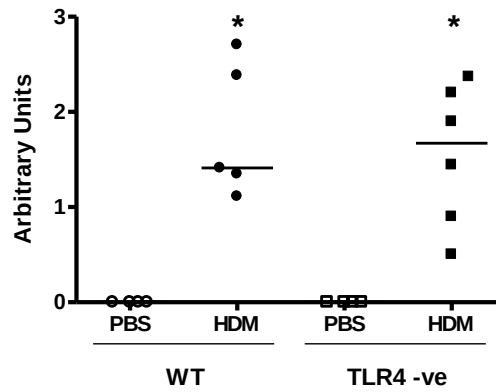


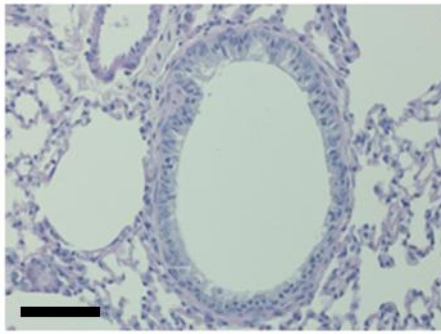
Figure 4.7. Allergic immunoglobulin production in HDM-treated TLR4 -ve mice.

(A) Total IgE, (B) HDM-specific IgE, (C) total IgG₁ and (D) total IgG_{2a} levels measured in the serum 24h after the final i.n. challenge. All levels were determined by ELISA (n=4 PBS and n=5-6 HDM). *, P<0.05; (Mann-Whitney *U* test) HDM compared with PBS from corresponding strain. #, P<0.05 (Mann-Whitney *U* test) TLR4 -ve HDM compared with wt HDM. Bars depict the median of the group. N.S., not significant. One experiment.

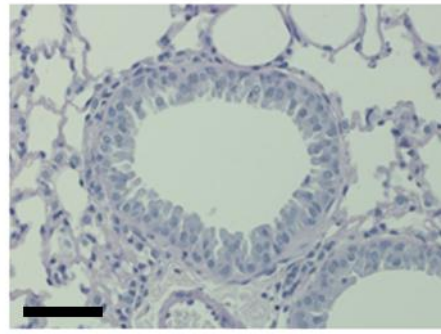
A. Mucus Score



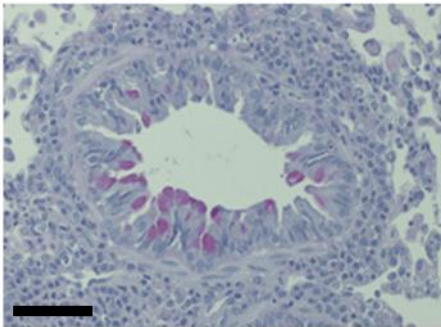
B. wt PBS



C. TLR4 -ve PBS



D. wt HDM



E. TLR4 -ve HDM

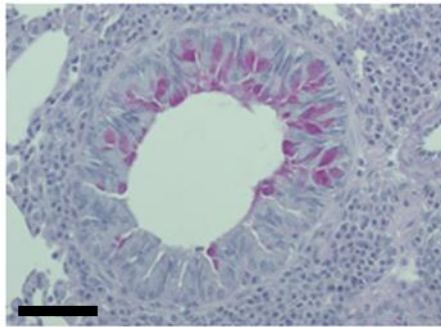


Figure 4.8. Goblet cell hyperplasia in TLR4 -ve mice.

(A) Semi-quantitative scoring of PAS stained lung sections. (n=4 PBS and n=5-6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS. Bars depict the median of the groups. Lung histological sections prepared with Periodic-acid Schiff stain from (B) wt PBS, (C) TLR4 -ve PBS, (D) wt HDM and (E) TLR4 -ve HDM-treated mice (original 20X magnification). Scale bar = 50 μ m. One experiment.

4.4.2. Protocol B: Effect of a TLR4 antagonist on HDM-induced allergic airway inflammation

4.4.2.1. AHR in HDM-treated mice

In order to investigate the specific contribution of TLR4 signalling in the lung to the immune response to inhaled HDM, the effects of prophylactic administration of a TLR4 antagonist on HDM-induced allergic airways disease were assessed. HDM exposure resulted in AHR, as measured by increased airway resistance and decreased airway compliance 24h post-challenge. Administration of a TLR4 antagonist did not affect HDM-induced AHR (Figure 4.9A – D).

4.4.2.2. Reduced inflammatory cell recruitment following TLR4 antagonism

HDM exposure resulted in greater numbers of total cells, eosinophils, neutrophils and Th2 cells recovered from the lung and BAL (Figure 4.10A - H). Following pharmacological antagonism of TLR4, there was a reduction in the numbers of total cells in the lung and BAL, eosinophils in the lung, neutrophils in the lung and BAL and Th2 cells in the BAL (Figure 4.10A, B, C, E, G and H).

Airway Resistance

Airway Compliance

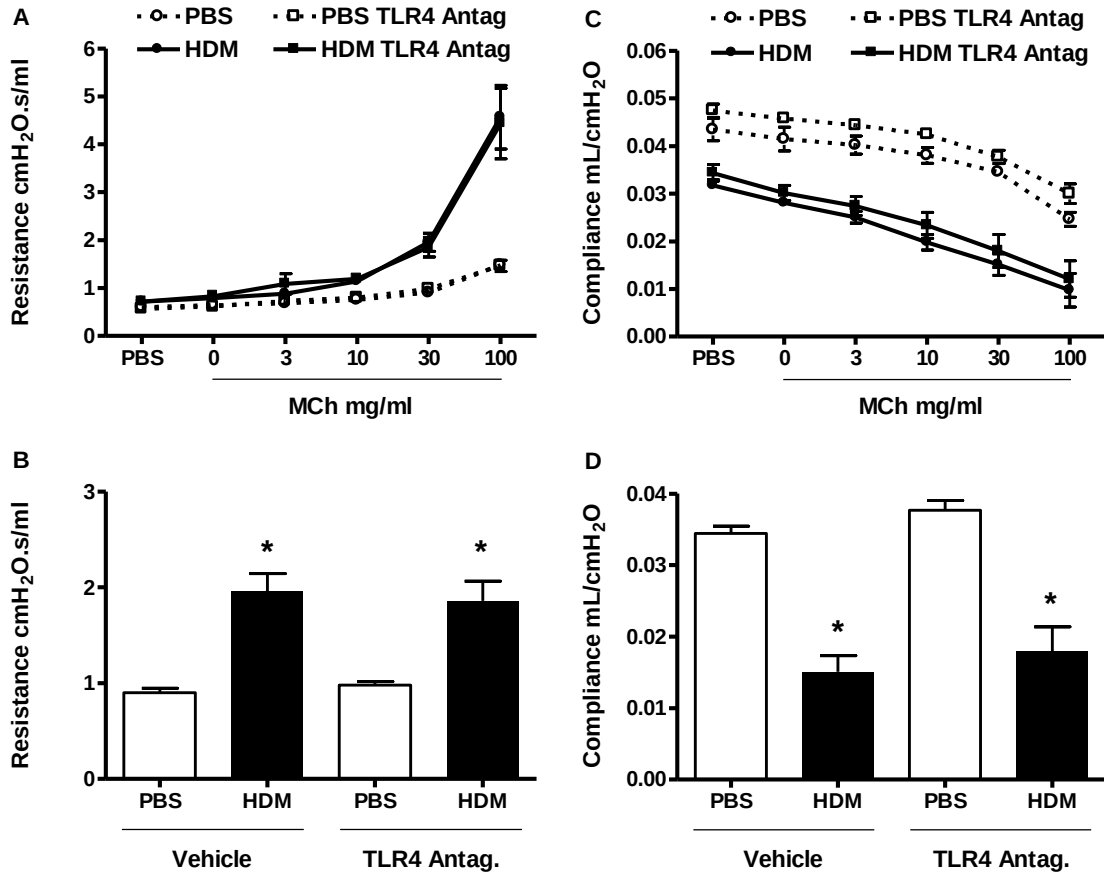


Figure 4.9. TLR4 antagonist does not prevent HDM-induced AHR.

(A) Airway resistance of PBS and HDM-treated mice at 24h post final challenge treated with either vehicle or TLR4 antagonist. (B) Airway resistance is expressed as mean resistance $\pm$ SEM for the 30mg/ml dose of methacholine. (C) Airway compliance of PBS and HDM-treated mice at 24h post final challenge treated with either vehicle or TLR4 antagonist. (D) Airway compliance is expressed as mean $\pm$ SEM for the 30mg/ml dose of methacholine. (n=4 PBS and n=6 HDM). *, $P < 0.05$ (Mann-Whitney U test) HDM compared with PBS from corresponding treatment group. Data are mean $\pm$ SEM. One experiment.

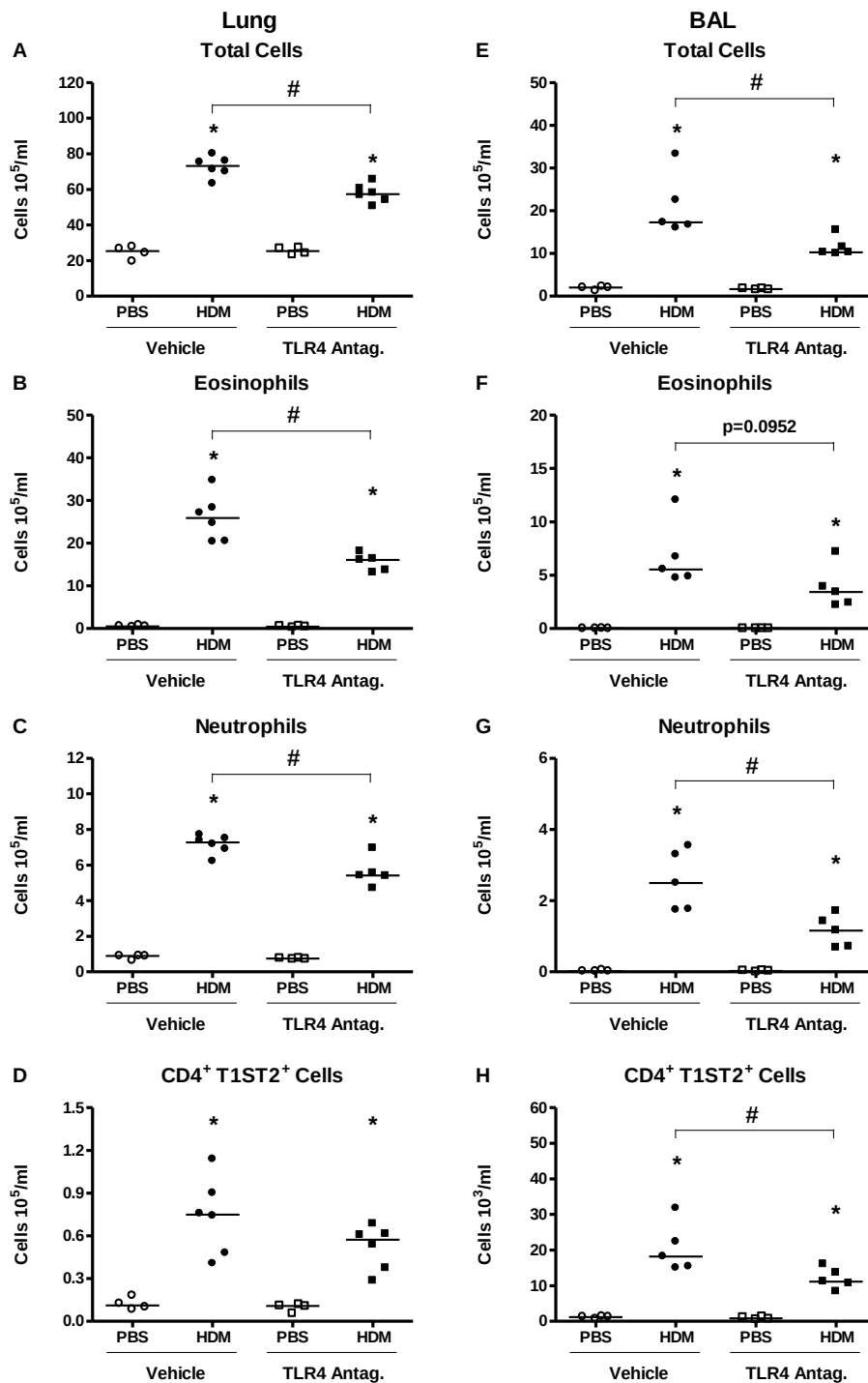


Figure 4.10. Inflammatory cell profile of HDM-treated mice.

Total cells, eosinophils, neutrophils and CD4⁺T1ST2⁺ cells recovered from the lung (A - D) and BAL (E - H) 24h after the final challenge (n=4 PBS and n=5-6 HDM). Total cell counts were determined using a haemocytometer with white blood cell counting fluid, eosinophils and neutrophils were quantified from Wright-Giemsa stained cytopins and CD4⁺T1ST2⁺ cells were quantified by flow cytometry, as described in the Materials and Methods. *, P<0.05; (Mann-Whitney *U* test) HDM compared with PBS from corresponding treatment. #, P<0.05 (Mann-Whitney *U* test) TLR4 antagonist HDM compared with vehicle HDM. Bars depict median of the groups. One experiment.

4.4.2.3. No alteration of chemokine production with a TLR4 antagonist

Eotaxin-1/CCL11, KC/mCXCL1, MDC/CCL22, MCP-1/CCL2, TARC/CCL17 and RANTES/CCL5 which are recruitment mediators of eosinophils, neutrophils and Th2 cells, were quantified in the lung tissue. HDM exposure resulted in increased levels of each of these chemokines as compared to PBS controls. The administration of a TLR4 antagonist did not alter HDM-induced production of these mediators (Figure 4.11A – F).

4.4.2.4. Abrogated IL-25 (IL-17E) production following TLR4 antagonism

HDM-induced production of IL-4 and IL-1 β was not altered by the administration of a TLR4 antagonist (Figure 4.12A and B). IL-25 (IL-17E), TSLP, CCL20/MIP-3 α and IL-33 were quantified in the lungs following the respective treatments. HDM-treated mice showed elevated levels of IL-25 (IL-17E) in the lung tissue as compared to PBS controls and this was completely abrogated following the administration of the TLR4 antagonist (Figure 4.12C). Increased levels of TSLP, CCL20/MIP-3 α and IL-33 were observed in the lung tissue of HDM-treated mice as compared to PBS controls and these levels were not altered by the TLR4 antagonist (Figure 4.12D - F).

4.4.2.5. IgE production in HDM-treated mice

Elevated levels of total and HDM-specific IgE were observed in the serum of HDM-treated mice. The TLR4 antagonist did not alter the levels of HDM-induced production of IgE (Figure 4.13A and B).

4.4.2.6. Goblet cell hyperplasia in HDM-treated mice

Goblet cell hyperplasia was observed in HDM-treated mice. Despite the administration of a TLR4 antagonist, no effect was seen on the mucus production (Figure 4.14A). Representative PAS stained lung sections are shown (Figure 4.14B – E).

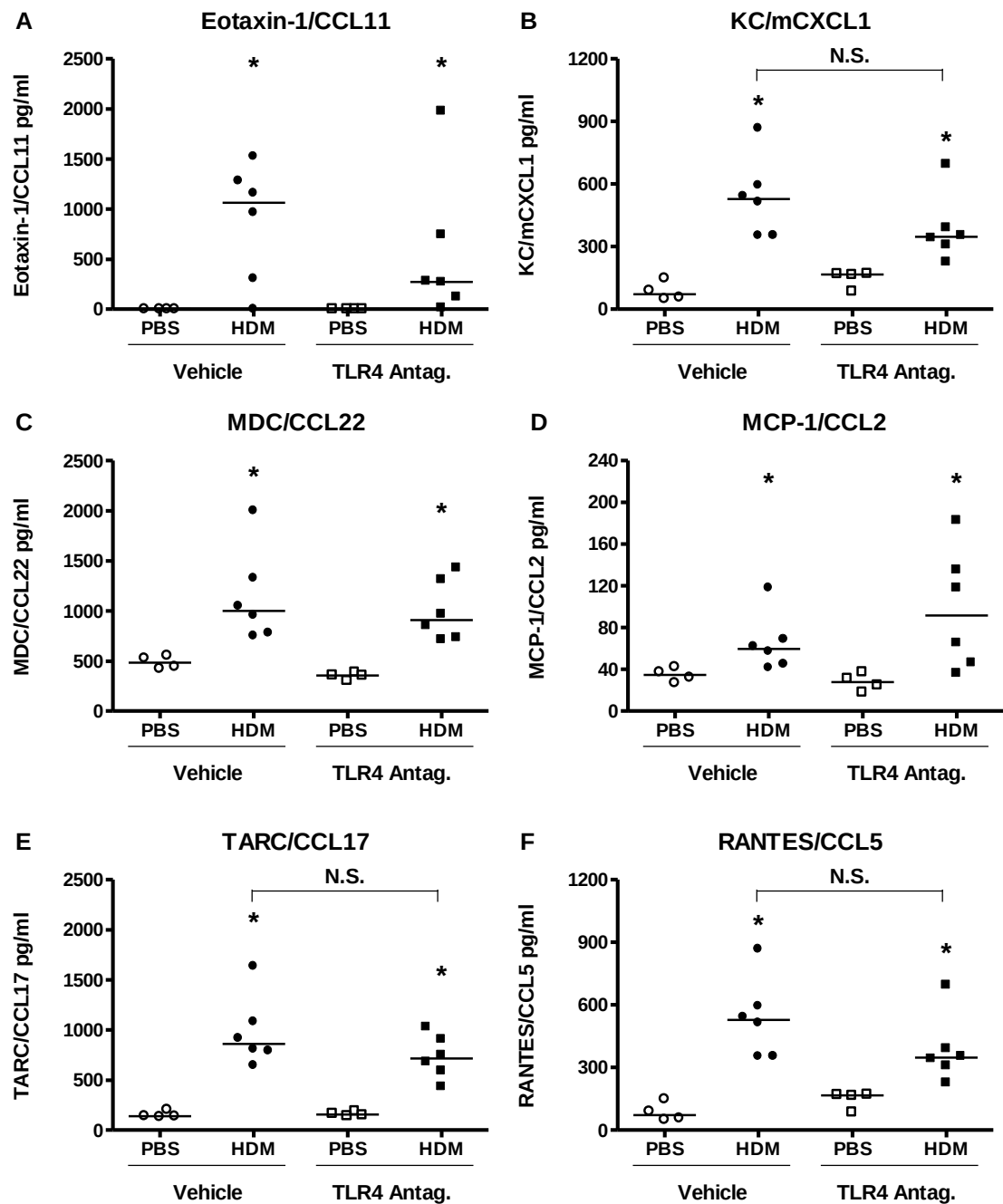


Figure 4.11. TLR4 antagonist does not prevent inflammatory chemokine production.

(A) Eotaxin-1/CCL11, (B) KC/mCXCL1, (C) MDC/CCL22, (D) MCP-1/CCL2, (E) TARC/CCL17 and (F) RANTES/CCL5 levels measured in the lung 24h after the final challenge. All mediator levels were determined by ELISA. (n=4 PBS and n=6 HDM). *, $P<0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding treatment. Bars depict the median of the group. N.S., not significant. One experiment.

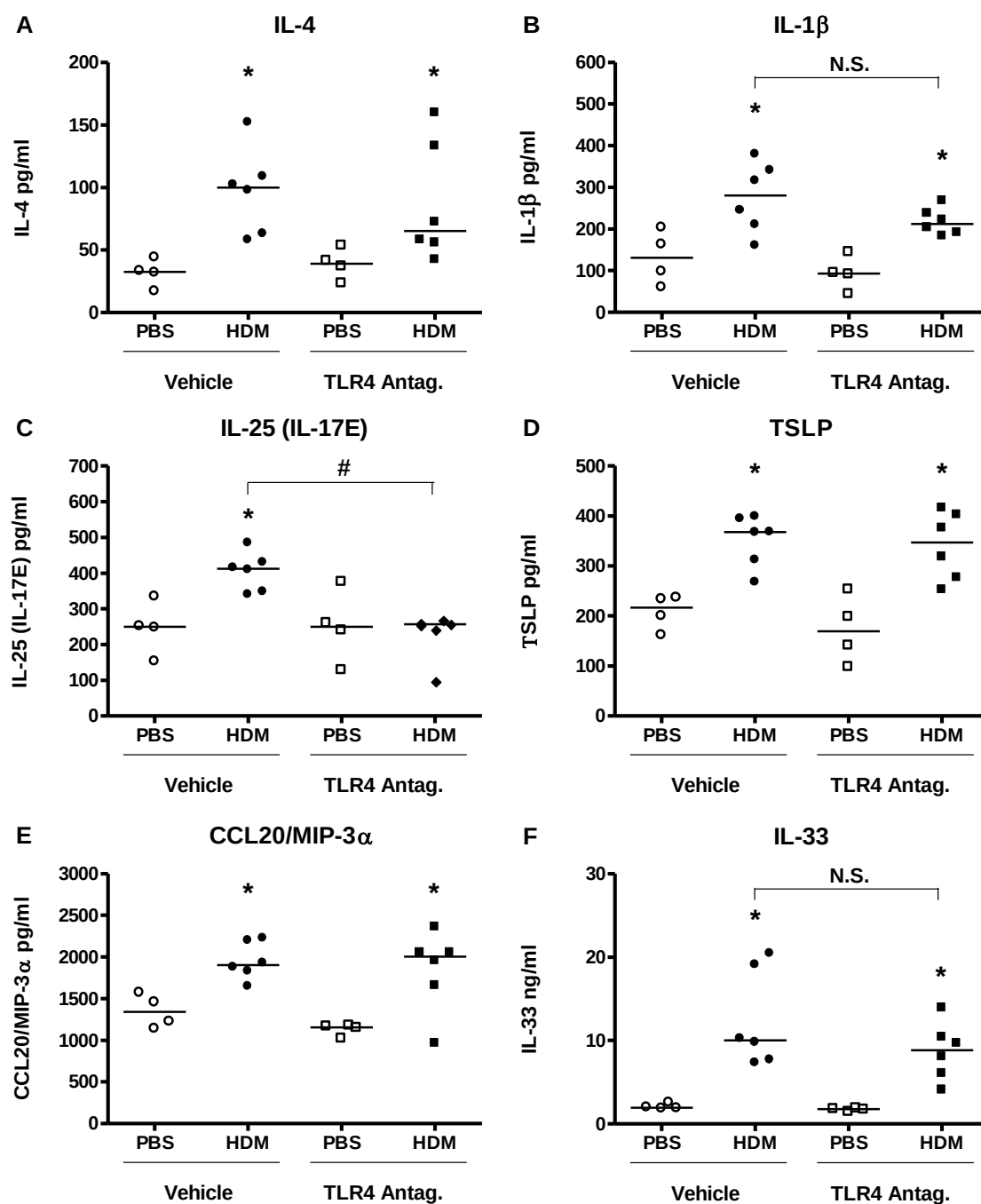


Figure 4.12. TLR4 antagonist blocks HDM-induced IL-25 (IL-17E) production. (A) IL-4, (B) IL-1 β (C) IL-25 (IL-17E), (D) TSLP, (E) CCL20/MIP-3 α and (F) IL-33 measured in the lung 24h after the final challenge. All mediator levels were determined by ELISA. (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding treatment. #, $P < 0.05$ (Mann-Whitney U test) TLR4 antagonist HDM compared with vehicle HDM. Bars depict the median of the group. N.S., not significant. One experiment.

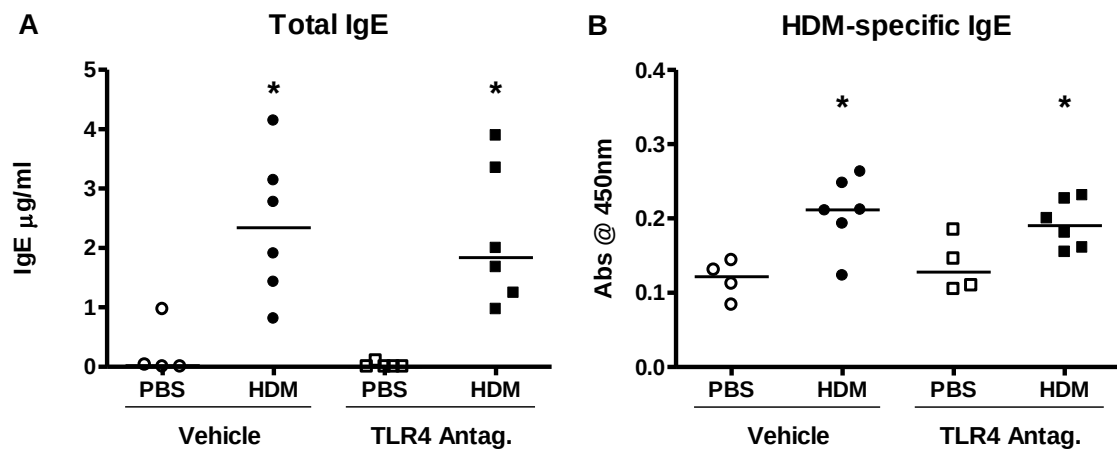
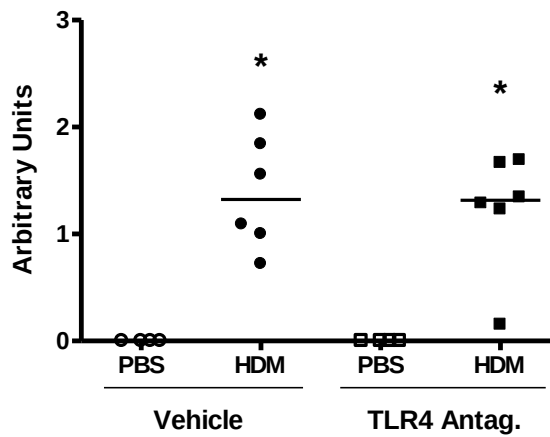


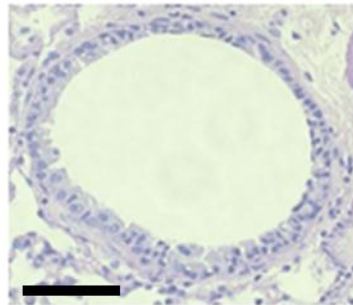
Figure 4.13. Allergic immunoglobulin production in TLR4 antagonist-treated mice.

(A) Total IgE and (B) HDM-specific IgE levels measured in the serum 24h after the final challenge. All levels were determined by ELISA (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding treatment. Bars depict the median of the groups. One experiment.

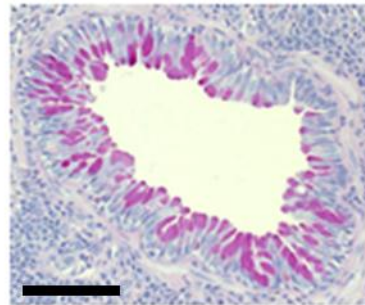
A. Mucus Score



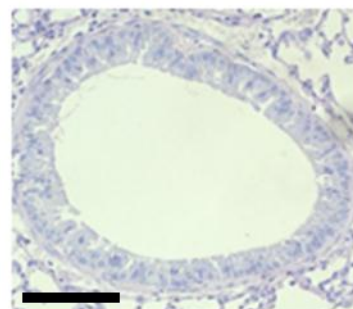
B. PBS Vehicle



C. HDM Vehicle



D. PBS TLR4 Antagonist



E. HDM TLR4 Antagonist

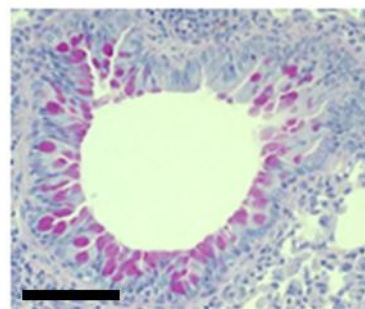


Figure 4.14. TLR4 antagonist does not prevent HDM-induced goblet cell hyperplasia.

(A) Semi-quantitative scoring of PAS stained lung sections. (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS. Bars depict the median of the group. Lung histological sections prepared with Periodic-acid Schiff stain from (B) vehicle PBS, (C) vehicle HDM, (D) TLR4 antagonist PBS and (E) TLR4 antagonist HDM-treated mice (original 20X magnification). Scale bar = 50 μ m. One experiment.

4.4.3. Protocol C: Impact of MyD88 deficiency on HDM-induced allergic airways disease

Thus far it has been established that neither the absence of systemic TLR4 signalling nor the pharmacological antagonism of TLR4 prevented the development of several key features of HDM-induced allergic airways disease, including AHR and HDM-specific IgE production. To investigate the contribution of multiple TLRs to the immune response, MyD88 knockout (KO) mice were exposed to HDM, for either 1, 3 or 5 weeks, in order to determine the role of this adaptor protein in the development of Th2 pathology. MyD88 signalling contributes to all TLR signalling pathways except for TLR3 and the TLR4 TRIF-TRAM-dependent signalling cascade (Kawai and Akira, 2006).

4.4.3.1. AHR in HDM-treated MyD88 KO mice

To investigate the contribution of MyD88 to HDM-induced AHR, *wt* C57BL/6 and MyD88 KO mice were dosed for 3 or 5 weeks. No increased airway resistance was observed in HDM-treated *wt* mice, whereas MyD88 KO mice showed increased airway resistance at week 3 (Figure 4.15A – D). HDM-treated MyD88 KO mice also showed decreased airway compliance compared to the respective PBS-treated My88 KO mice at weeks 3 and 5 (Figure 4.15E – H).

4.4.3.2. Inflammatory cell recruitment in MyD88 KO mice

Following 1 week of HDM exposure, there was no elevation in the number of total cells, eosinophils or neutrophils in the lung tissue in either *wt* or MyD88 KO mice (Figure 4.16A - C). After HDM exposure for either 3 or 5 weeks, the number of total cells and inflammatory cell numbers in the lung were not reduced by the absence of MyD88 (Figure 4.16A – D), with lung eosinophilia greater in the MyD88 KO mice than *wt* mice at week 3 (Figure 4.16B). HDM exposure resulted in elevated numbers of total cells and inflammatory cell recruitment to the BAL from as early as 1 week and this was not reduced in the absence of MyD88 (Figure 4.17A - D).

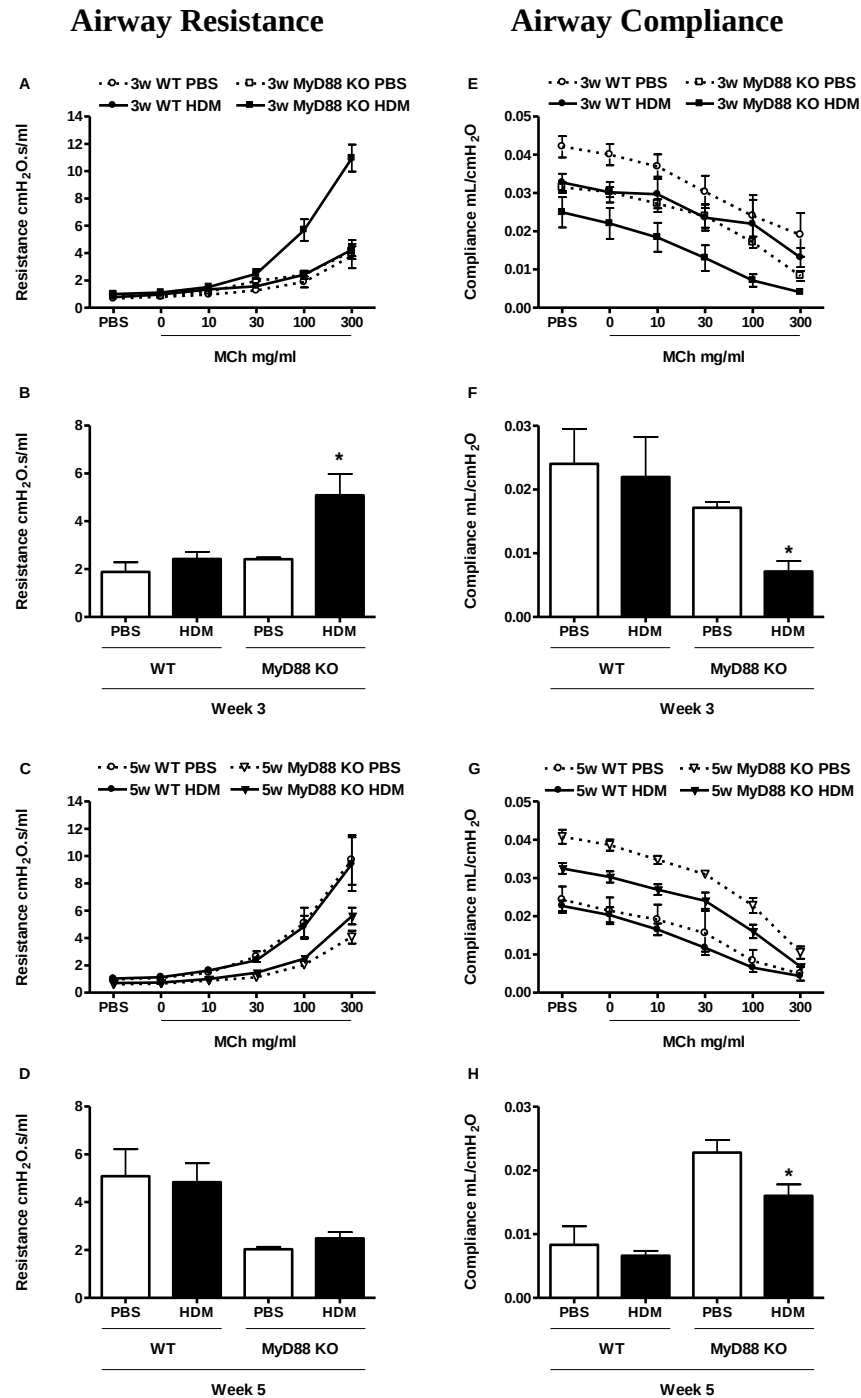


Figure 4.15. AHR in HDM-treated MyD88 KO mice.

(A) Airway resistance of PBS and HDM-treated mice 24h post final challenge following 3 weeks of exposure. (B) Airway resistance is expressed as mean resistance $\pm$ SEM for the 100mg/ml dose of methacholine. (C) Airway resistance of PBS and HDM-treated mice 24h post final challenge following 5 weeks of exposure. (D) Airway resistance is expressed as mean resistance $\pm$ SEM for the 100mg/ml dose of methacholine. (E) Airway compliance of PBS and HDM-treated mice 24h post final challenge following 3 weeks of exposure. (F) Airway compliance is expressed as mean $\pm$ SEM for the 100mg/ml dose of methacholine. (G) Airway compliance of PBS and HDM-treated mice 24h post final challenge following 5 weeks of exposure. (H) Airway compliance is expressed as mean $\pm$ SEM for the 100mg/ml dose of methacholine. (n=4 PBS and n=6 HDM). *, P<0.05 (Mann-Whitney U test) HDM compared with PBS from WT from corresponding strain. Data are mean $\pm$ SEM. One experiment.

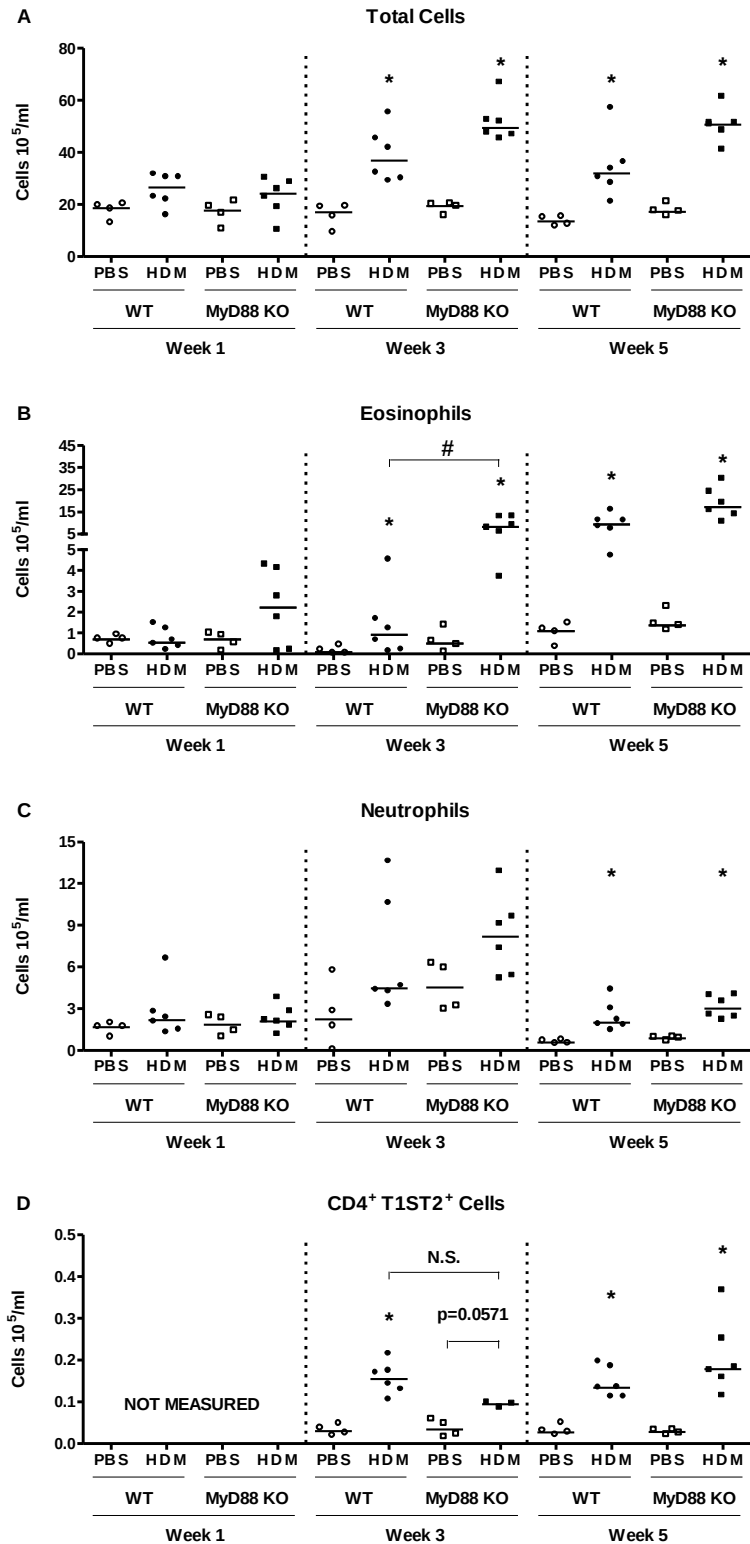


Figure 4.16. Inflammatory cell recruitment to the lungs of MyD88 KO mice.

(A) Total cells, (B) eosinophils, (C) neutrophils and (D) CD4⁺T1ST2⁺ cells recovered from the lung 24h after the final i.n. challenge, following either 1, 3 or 5 weeks of exposure. (n=4 PBS and n=3-6 HDM). Total cell counts were determined using a haemocytometer with white blood cell counting fluid, eosinophils and neutrophils were quantified from Wright-Giemsa stained cytopins and CD4⁺T1ST2⁺ cells were quantified by flow cytometry, as described in the Materials and Methods. *, P<0.05; (Mann-Whitney *U* test) HDM compared with PBS from corresponding strain and time point. Bars depict median of the groups. One experiment.

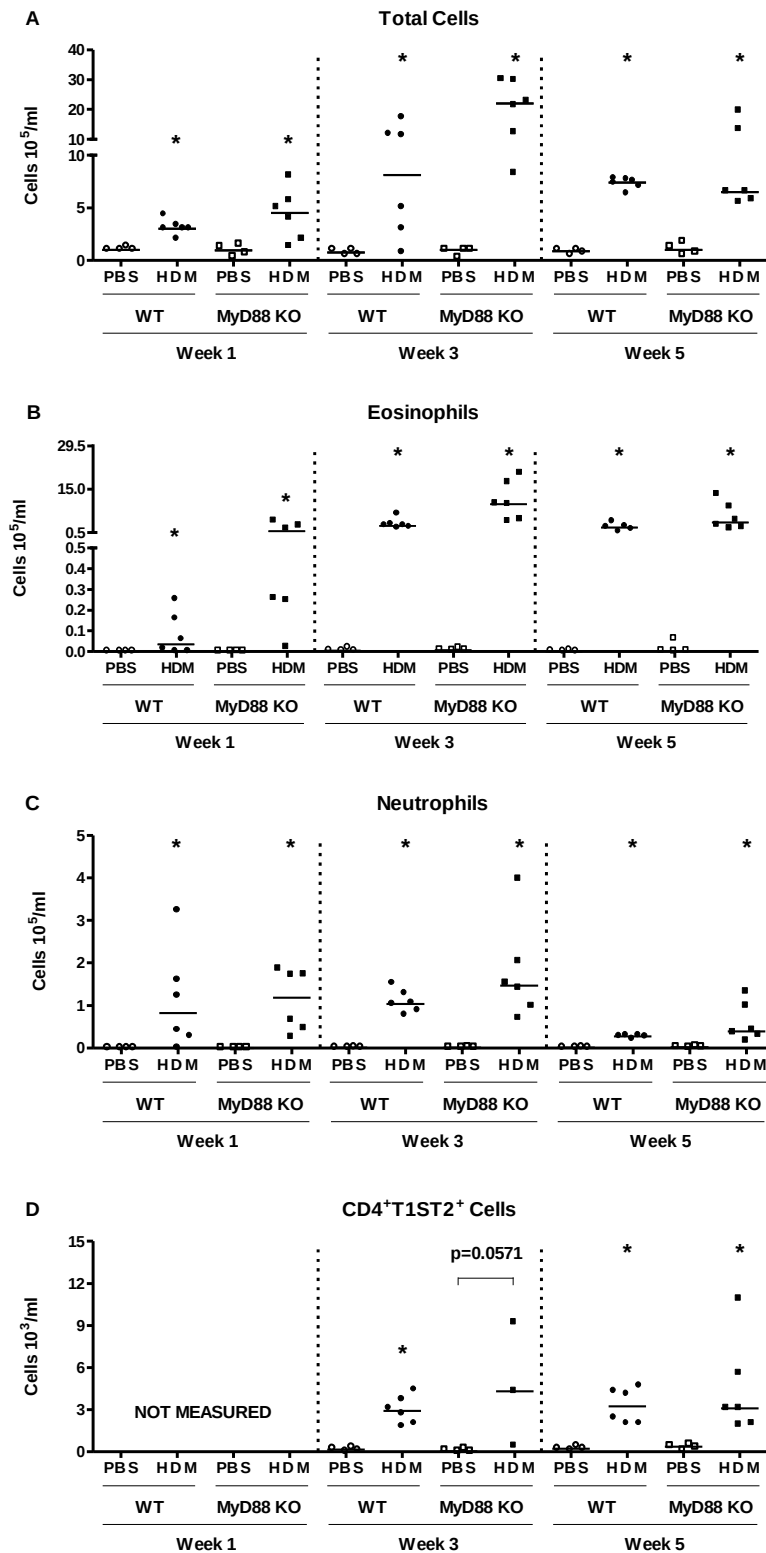


Figure 4.17. Inflammatory cell recruitment to the BAL of MyD88 KO mice.

(A) Total cells, (B) eosinophils, (C) neutrophils and (D) CD4⁺T1ST2⁺ cells recovered from the BAL 24h after the final i.n. challenge, following either 1, 3 or 5 weeks of exposure. (n=4 PBS and n=3-6 HDM). Total cell counts were determined using a haemocytometer with white blood cell counting fluid, eosinophils and neutrophils were quantified from Wright-Giemsa stained cytopins and CD4⁺T1ST2⁺ cells were quantified by flow cytometry, as described in the Materials and Methods. *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding strain and time point. Bars depict median of the groups. One experiment.

4.4.3.3. Pro-inflammatory mediator production in MyD88 KO mice

HDM-induced production of eotaxin-1/CCL11 and KC/mCXCL1 was not altered by the absence of MyD88 (Figure 4.18A and B). TARC/CCL17, RANTES/CCL5 and MCP-1/CCL2 production were not reduced in MyD88 KO mice (Figure 4.18C - E). Indeed, TARC/CCL17 (at week 1) and MCP-1/CCL2 (week 3) levels were higher in HDM-treated MyD88 KO mice than *wt* controls (Figure 4.18C and E).

Less IL-1 β production was seen in HDM-treated MyD88 KO mice than the *wt* controls at week 3. However, the levels observed in HDM-treated mice were equivalent at week 5 (Figure 4.19A). IL-33 production was observed in both *wt* and MyD88 KO mice exposed to HDM from as early as week 1, which then persisted to week 5 (Figure 4.19B). HDM did not induce the production of IL-25 (IL-17E) in either the C57BL/6 *wt* controls or the MyD88 KO mice (Figure 4.19C). CCL20/MIP-3 α was elevated in HDM-treated *wt* and MyD88 KO mice from week 1 compared to PBS controls and this continued through the 5 week time course (Figure 4.19D).

4.4.3.4. Allergic immunoglobulin production in MyD88 KO exposed mice

The absence of MyD88 did not abrogate allergic immunoglobulin production in response to HDM, with total and HDM-specific IgE, as well as the Th2-associated IgG₁ levels elevated from PBS controls in MyD88 KO mice (Figure 4.20A – C). Indeed, at week 5 total IgE levels were higher in HDM-treated MyD88 KO mice than the respective *wt* (Figure 4.20A).

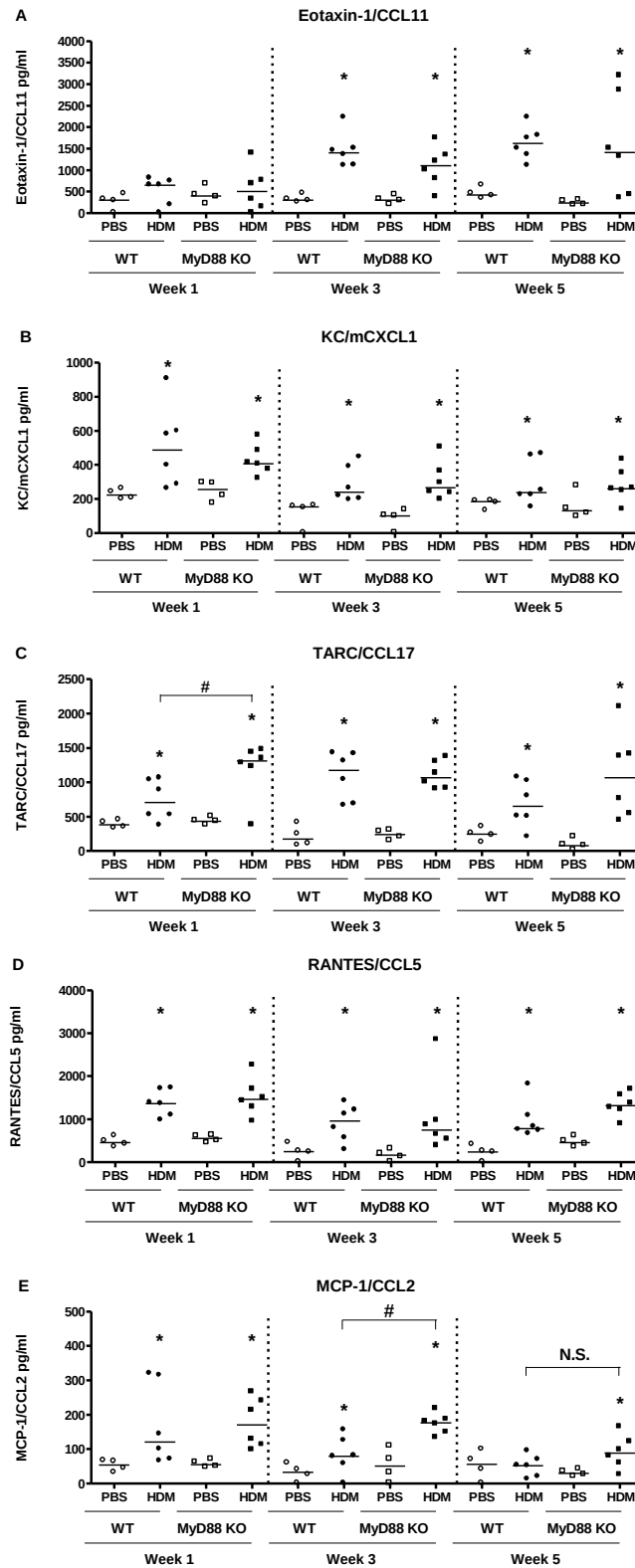


Figure 4.18. Pro-inflammatory chemokine production in MyD88 KO mice.

(A) Eotaxin-1/CCL11, (B) KC/mCXCL1, (C) TARC/CCL17, (D) RANTES/CCL5 and (E) MCP-1/CCL2 measured in the lung 24h after the final i.n. challenge. All mediator levels were determined by ELISA. (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding strain and time point. #, $P < 0.05$ (Mann-Whitney U test) HDM MyD88 KO compared with HDM wt from corresponding time point. Bars depict the median of the groups. N.S., not significant. N.S., not significant. One experiment.

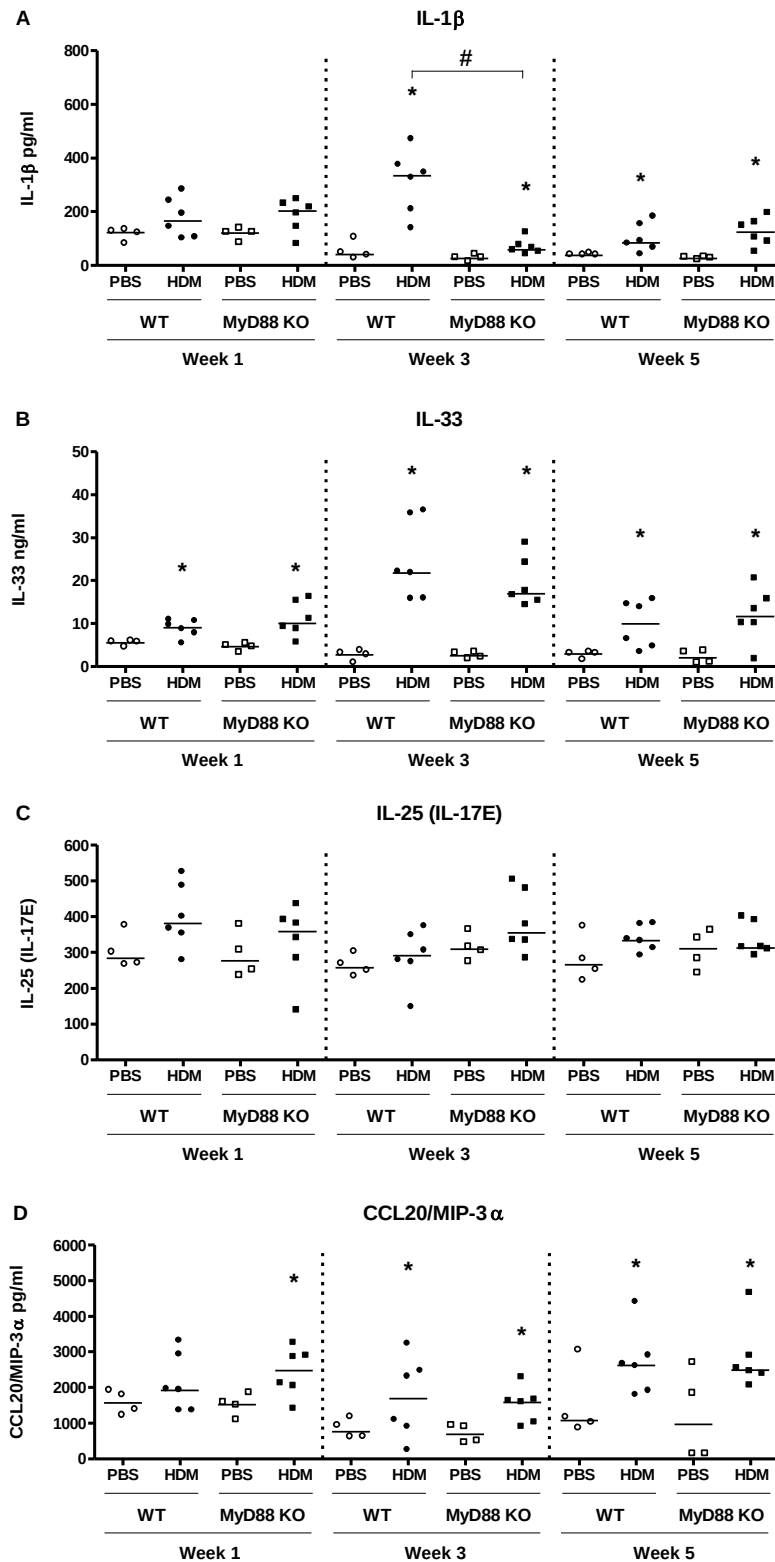


Figure 4.19. Pro-inflammatory mediator release in MyD88 KO mice.

(A) IL-1 β , (B) IL-33, (C) IL-25 (IL-17E) and (D) CCL20/MIP-3 α measured in the lung 24h after the final i.n. challenge. All mediator levels were determined by ELISA. (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding strain and time point. #, $P < 0.05$; (Mann-Whitney U test) HDM MyD88 KO compared with HDM wt from corresponding time point. Bars depict the median of the groups. One experiment.

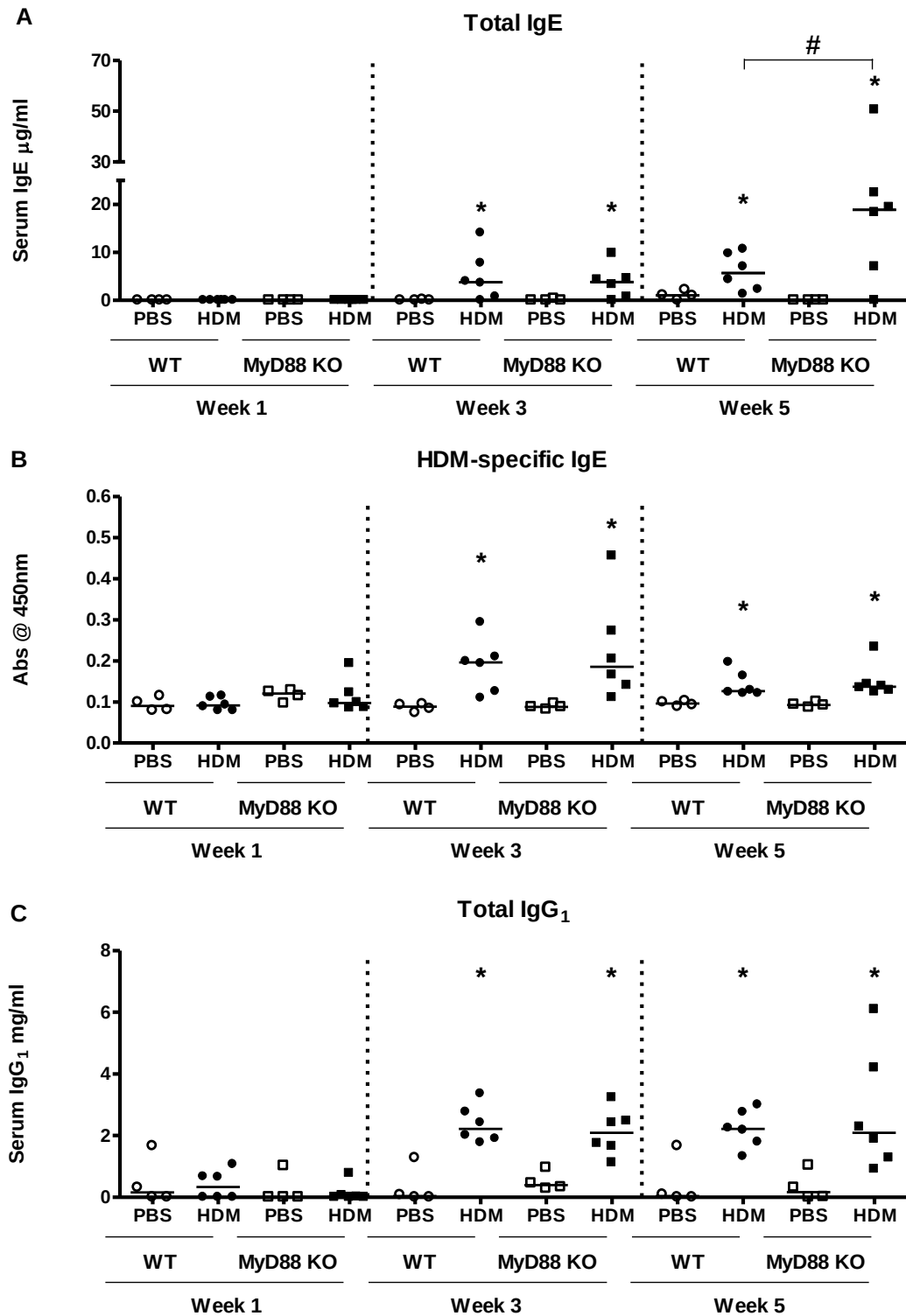


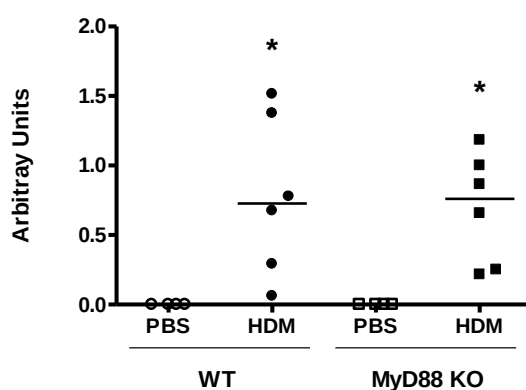
Figure 4.20. Allergic immunoglobulin production in MyD88 KO mice.

(A) Total IgE, (B) HDM-specific IgE and (C) total IgG₁ measured in the serum 24h after the final i.n. challenge. All mediator levels were determined by ELISA. (n=4 PBS and n=6 HDM). *, P<0.05; (Mann-Whitney *U* test) HDM compared with PBS from corresponding strain and time point. #, P<0.05 (Mann-Whitney *U* test) HDM MyD88 KO compared with HDM wt from corresponding time point. Bars depict the median of the groups. N.D., not detected. One experiment.

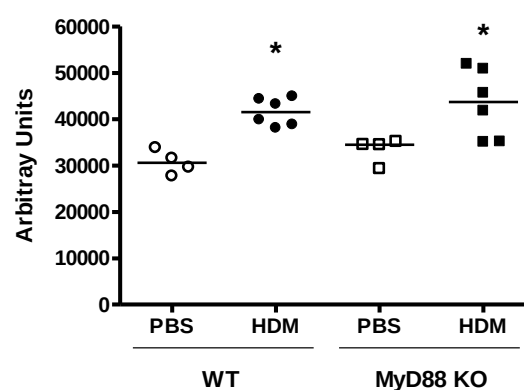
4.4.3.5. Goblet cell hyperplasia and peri-bronchial collagen deposition in HDM-treated MyD88 KO mice

Goblet cell hyperplasia was observed in both *wt* and MyD88 KO mice following 5 weeks of HDM exposure. HDM-induced mucus secretion was not altered by the absence of MyD88 (Figure 4.21A). Peri-bronchial collagen deposition is a feature of airway remodelling and following 5 weeks of HDM exposure, peri-bronchial collagen deposition was observed in both *wt* and MyD88 KO mice (Figure 4.21B). Representative photos from PAS and sirius red stained lung sections are shown (Figure 4.21C – F).

A. Mucus Score



B. Mean Density of Peri-bronchial Collagen Deposition



C. wt PBS

D. wt HDM

E. MyD88 KO PBS

F. MyD88 KO HDM

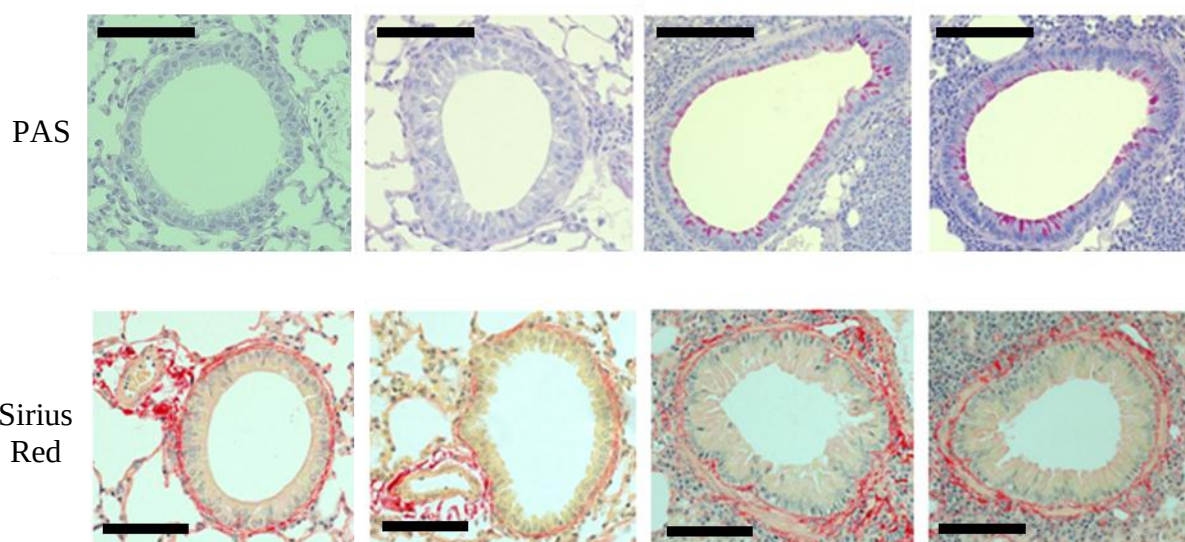


Figure 4.21. Goblet cell hyperplasia and peri-bronchial collagen deposition in MyD88 KO mice.

(A) Semi-quantitative scoring of PAS stained lung sections. (B) Quantification of peri-bronchial collagen deposition, expressed as mean intensity. (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding strain. Bars depict the median of the groups. Representative pictures of PAS and sirius red stained lung sections from (C) wt PBS, (D) wt HDM, (E) MyD88 KO PBS and (F) MyD88 KO HDM-treated mice, following 5 weeks of either PBS or HDM exposure (original 20X magnification). Scale bar = 50 μ m. One experiment.

4.4.4. Protocol D: Effect of a SAPS inhibitor on HDM-induced allergic airway inflammation

4.4.4.1. AHR in HDM-treated mice

In order to further dissect the role of TLR signalling to HDM-induced allergic airways disease, the effects of prophylactic administration of a SAPS inhibitor on HDM-induced allergic airways disease was investigated. *In vitro* SAPS has been shown to inhibit TLR1-4, 7 and 8 signalling via disruption of membrane microdomains (Parker et al., 2008). Administration of a SAPS inhibitor prevented HDM-induced increase in airway resistance however there was no effect on airway compliance (Figure 4.22A – D).

4.4.4.2. Reduced inflammatory cell recruitment following TLR antagonism

Following inhibition of TLR signalling with the SAPS inhibitor, there was a reduction in the number of total cells and eosinophils and Th2 cells in the lung and BAL and neutrophils in the BAL (Figure 4.23A - H).

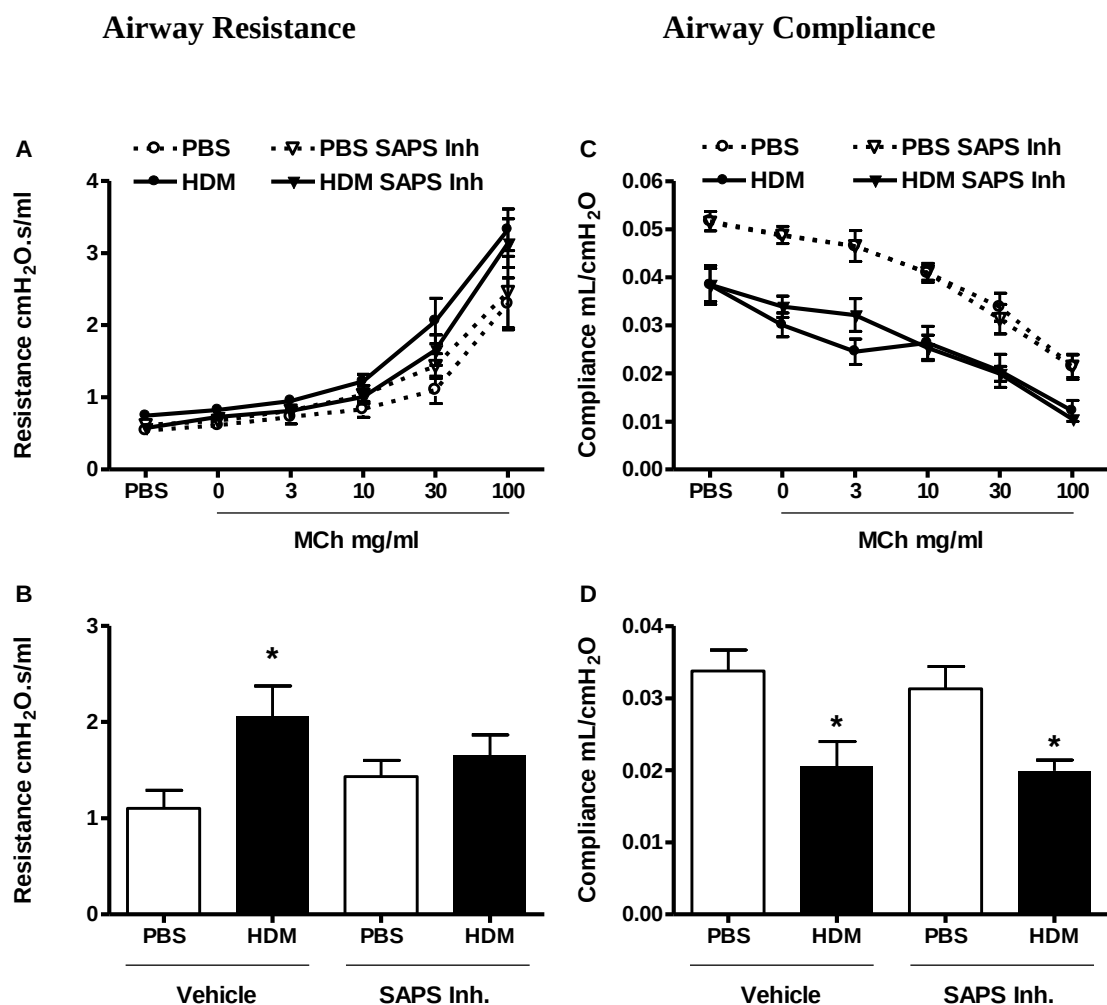


Figure 4.22. SAPS inhibitor prevents HDM-induced increase in airway resistance.

(A) Airway resistance of PBS and HDM-treated mice at 24h post final challenge treated with either vehicle or SAPS inhibitor. (B) Airway resistance is expressed as mean resistance $\pm$ SEM for the 30mg/ml dose of methacholine. (C) Airway compliance of PBS and HDM-treated mice at 24h post final challenge treated with either vehicle or SAPS inhibitor. (D) Airway compliance is expressed as mean $\pm$ SEM for the 30mg/ml dose of methacholine. (n=4 PBS and n=6 HDM). *, P<0.05 (Mann-Whitney *U* test) HDM compared with PBS from corresponding treatment group. Data are mean $\pm$ SEM. One experiment.

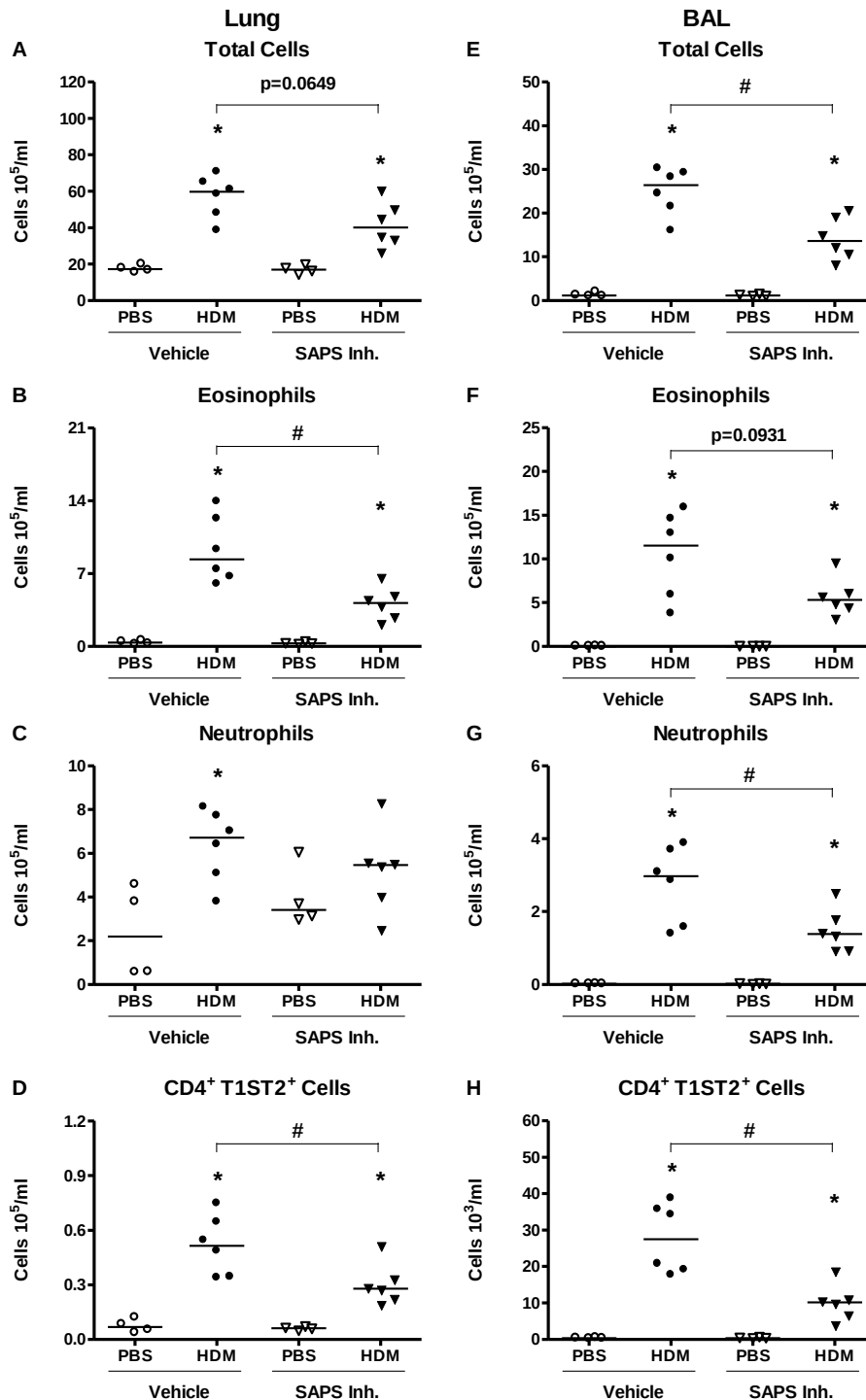


Figure 4.23. Inflammatory cell profile of HDM-treated mice.

Total cells, eosinophils, neutrophils and $\text{CD4}^+ \text{T1ST2}^+$ cells recovered from the lung (A - D) and BAL (E - H) 24h after the final challenge ($n=4$ PBS and $n=6$ HDM). Total cell counts were determined using a haemocytometer with white blood cell counting fluid, eosinophils and neutrophils were quantified from Wright-Giemsa stained cytopspins and $\text{CD4}^+ \text{T1ST2}^+$ cells were quantified by flow cytometry, as described in the Materials and Methods. *, $P<0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding treatment. #, $P<0.05$ (Mann-Whitney U test) SAPS inhibitor HDM compared with vehicle HDM. Bars depict median of the groups. One experiment.

4.4.4.3. Reduction in chemokine production with SAPS inhibitor

The pro-inflammatory chemokines eotaxin-1/CCL11, KC/mCXCL1, TARC/CCL17, RANTES/CCL5, MDC/CCL22 and MCP-1/CCL2 were quantified in the lung tissue. The administration of the SAPS inhibitor reduced the levels of eotaxin-1/CCL11 and TARC/CCL17 and completely abrogated RANTES/CCL5, MDC/CCL22 and MCP-1/CCL2 production. However, the neutrophil chemoattractant KC/mCXCL1 production was unaltered (Figure 4.24A – F).

4.4.4.4. Abrogated IL-25 (IL-17E) production following TLR antagonism

Neither IL-4 nor IL-5 production was influenced by the SAPS inhibitor (Figure 4.25A and B). However, SAPS-treated HDM exposed mice showed reduced levels of IL-13 in the lung (Figure 4.25C). HDM-induced IL-1 β production was not altered by the SAPS inhibitor (Figure 4.25D). HDM-treated mice showed elevated levels of IL-25 (IL-17E) in the lung tissue as compared to PBS controls, which was completely abrogated following the administration of the SAPS inhibitor (Figure 4.25E). Greater levels of IL-33 and CCL20/MIP-3 α were observed in the lung tissue of HDM-treated mice as compared to PBS controls, which were not altered by the SAPS inhibitor (Figure 4.25F and G).

4.4.4.5. IgE production in HDM-treated mice

The SAPS inhibitor did not alter the levels of HDM-induced production of serum IgE (Figure 4.26A and B).

4.4.4.6. Goblet cell hyperplasia in HDM-treated mice

Goblet cell hyperplasia was observed in HDM-treated mice compared to PBS controls. Administration of the SAPS inhibitor had no effect on the mucus production in HDM-treated mice (Figure 4.27A). Representative PAS stained lung sections are shown (Figure 4.27B – E).

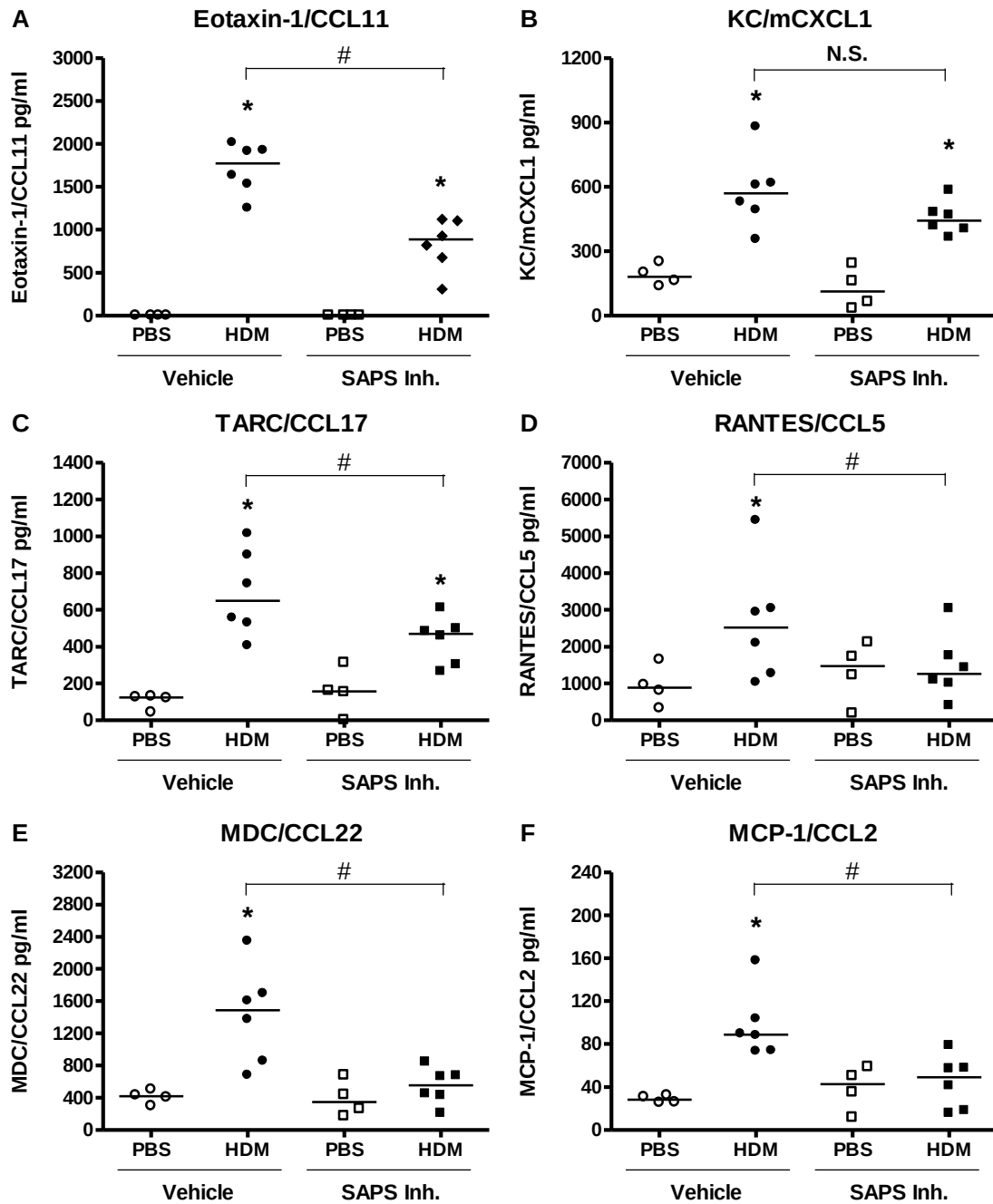


Figure 4.24. SAPS inhibitor reduces inflammatory chemokine production.

(A) Eotaxin-1/CCL11, (B) KC/mCXCL1, (C) TARC/CCL17, (D) RANTES/CCL5, (E) MDC/CCL22 and (F) MCP-1/CCL2 levels measured in the lung 24h after the final challenge. All mediator levels were determined by ELISA. (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding treatment. #, $P < 0.05$ (Mann-Whitney U test) SAPS inhibitor HDM compared with vehicle HDM. Bars depict the median of the groups. N.S., not significant. One experiment.

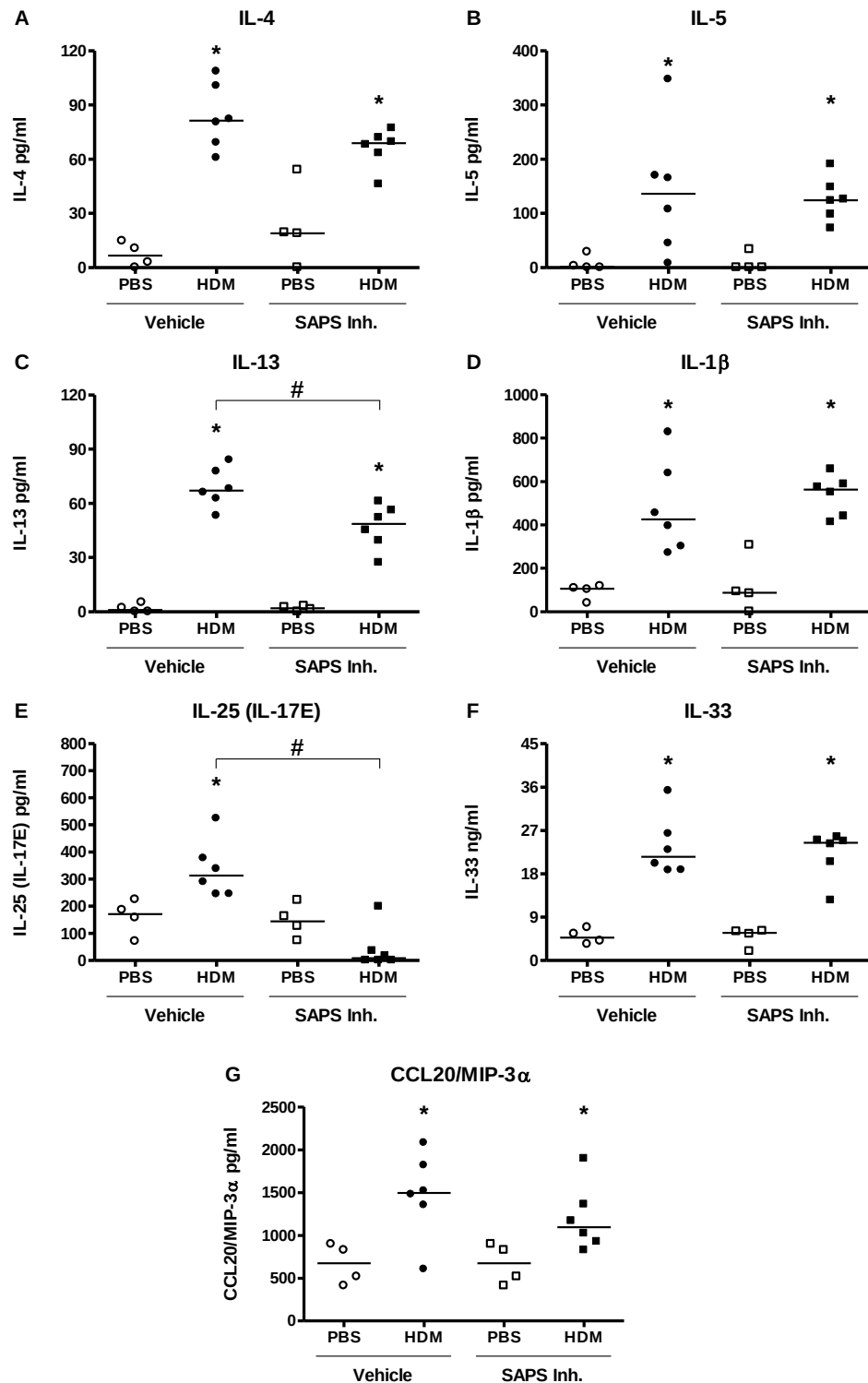


Figure 4.25. SAPS inhibitor abrogates HDM-induced production of IL-25 (IL-17E).

(A) IL-4, (B) IL-5, (C) IL-13, (D) IL-1 β , (E) IL-25 (IL-17E), (F) IL-33 and (G) CCL20/MIP-3 α measured in the lung 24h after the final challenge. All mediator levels were determined by ELISA. (n=4 PBS and n=6 HDM). *, P<0.05; (Mann-Whitney *U* test) HDM compared with PBS from corresponding treatment. #, P<0.05 (Mann-Whitney *U* test) SAPS inhibitor HDM compared with vehicle HDM. Bars depict the median of the groups. One experiment.

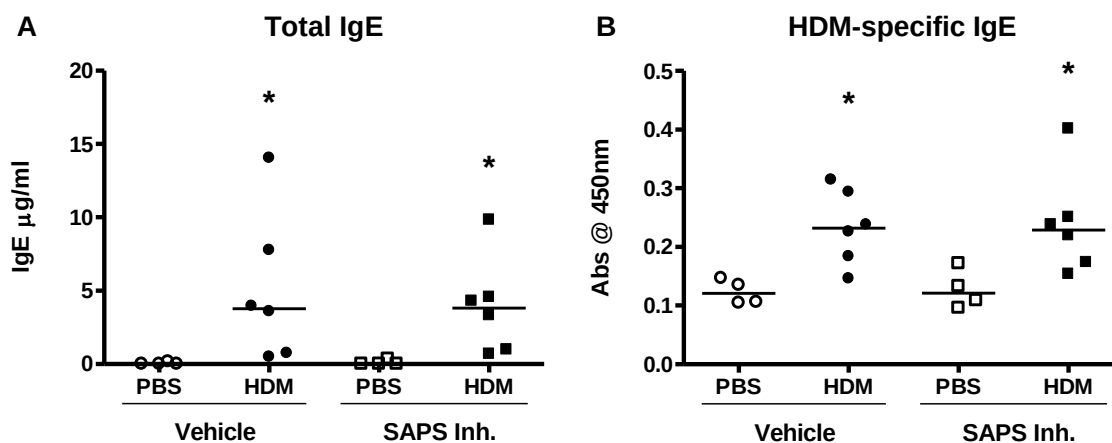
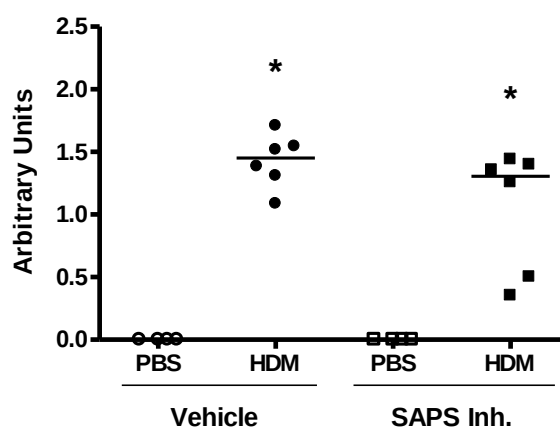


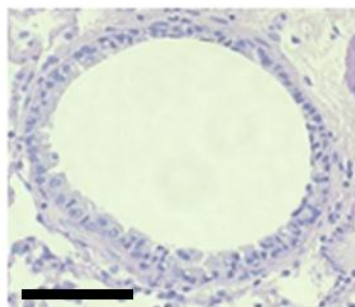
Figure 4.26. SAPS inhibitor does not prevent allergic immunoglobulin production.

(A) Total IgE and (B) HDM-specific IgE levels measured in the serum 24h after the final challenge. All levels were determined by ELISA (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding treatment. Bars depict the medians of the groups. One experiment.

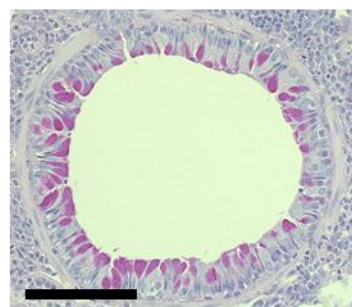
A. Mucus Score



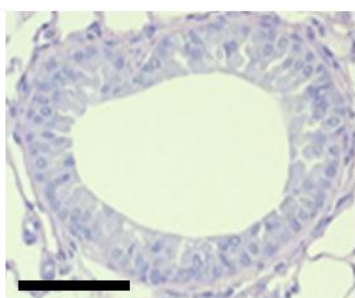
B. PBS Vehicle



C. HDM Vehicle



D. PBS SAPS Inhibitor



E. HDM SAPS Inhibitor

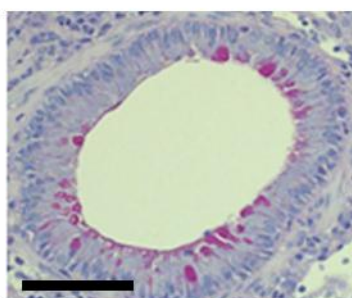


Figure 4.27. SAPS inhibitor does not prevent goblet cell hyperplasia.

(A) Semi-quantitative scoring of PAS stained lung sections. (n=4 PBS and n=6 HDM). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS from corresponding treatment group. Bars depict the median of the groups. Lung histological sections prepared with Periodic-acid Schiff stain from (B) vehicle PBS, (C) vehicle HDM, (D) SAPS inhibitor PBS and (E) SAPS inhibitor HDM-treated mice (original 20X magnification). Scale bar = 50 μm . One experiment.

4.4.5. Results Summary

A summary of the airway inflammatory and remodelling parameters and how these were influenced in each of the *in vivo* experiments is shown below. The table shows which parameters were unaltered between the HDM control and HDM-treatment groups ($\leftrightarrow$), which parameters were elevated in the HDM-treatment group compared to HDM controls ($\uparrow$) and which parameters were reduced in the HDM-treatment group compared to HDM controls ($\downarrow$) (Table 4.1).

	TLR4 -ve	TLR4 Antagonist	MyD88 KO	SAPS Inhibitor
AHR	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
Lung: Total Cells	$\leftrightarrow$	$\downarrow$	$\leftrightarrow$	$\leftrightarrow$
Lung: Eosinophils	$\leftrightarrow$	$\downarrow$	$\leftrightarrow$	$\downarrow$
Lung: Neutrophils	$\downarrow$	$\downarrow$	$\leftrightarrow$	$\leftrightarrow$
Lung: Th2 Cells	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\downarrow$
Eotaxin-1/CCL11	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\downarrow$
KC/mCXCL1	$\downarrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
TARC/CCL17	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\downarrow$
RANTES/CCL5	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\downarrow$
MCP-1/CCL2	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\downarrow$
IL-4	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
IL-1 β	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
IL-33	$\uparrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
IL-25 (IL-17E)	$\leftrightarrow$	$\downarrow$	$\leftrightarrow$	$\downarrow$
CCL20/MIP-3 α	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
Serum IgE	$\uparrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
Goblet Cell Hyperplasia	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
Collagen Deposition	Not Done	Not Done	$\leftrightarrow$	Not Done

Table 4.1. A summary of the inflammatory and remodelling parameters and how these were influenced by either systemic TLR4 signalling deficiency, a TLR4 antagonist, the absence of MyD88 or the SAPS inhibitor.

Key:

- $\leftrightarrow$ = No change between HDM and HDM-treatment groups.
- $\uparrow$ = Increase in HDM-treatment group compared to HDM.
- $\downarrow$ = Decrease in HDM-treatment group compared to HDM.

4.4.6. TLR gene expression in the lung

4.4.6.1. Impact of HDM and MyD88 deficiency on TLR gene expression in the lung

To investigate how HDM altered the levels of gene expression of TLRs and associated TLR signalling transduction molecules in the lung, the RNA was extracted from the lung tissue of C57BL/6 mice exposed to the allergen for three weeks. The extracted RNA was then converted to cDNA and the gene expression was assessed by PCR array. In HDM-treated mice, high fold increases in gene expression of MCP-1/CCL2, IP-10/CXCL10 and IFN- β expression and a small fold increase in IL-1 β were observed (Figure 4.28A). MyD88 KO mice showed higher expression levels of TLR1, TLR6, TLR9 and the MyD88-independent signalling molecules TRAM, MCP-1/CCL2, IP-10/CXCL10 and IFN- β compared to the C57BL/6 wt controls (Figure 4.28B). The impact of MyD88 deficiency on HDM-induced changes in gene expression was then investigated. The absence of MyD88 had minimal effect on the gene expression alterations caused by HDM, apart from causing a large decrease in TLR6 expression (Figure 4.28C).

4.4.6.2. Effects of HDM and the SAPS inhibitor on TLR gene expression in the lung

BALB/c mice have been shown to be more susceptible to allergen-induced AHR than C57BL/6 mice (Whitehead et al., 2003; Kelada et al., 2011). It was therefore investigated whether HDM-induced changes in TLR gene expression differed between C57BL/6 and BALB/c mice and also whether the SAPS inhibitor altered HDM-induced changes in gene expression in BALB/c mice. In BALB/c mice, HDM treatment increased the level of gene expression for MyD88, MCP-1/CCL2 and IP-10/CXCL10 compared to PBS controls (Figure 4.29A). The SAPS inhibitor alone decreased the expression of IL-1 β , IL-12 and IFN- β in naive mice (Figure 4.29B) and the SAPS inhibitor markedly reduced HDM-induced expression of MyD88 in BALB/c mice (Figure 4.29C).

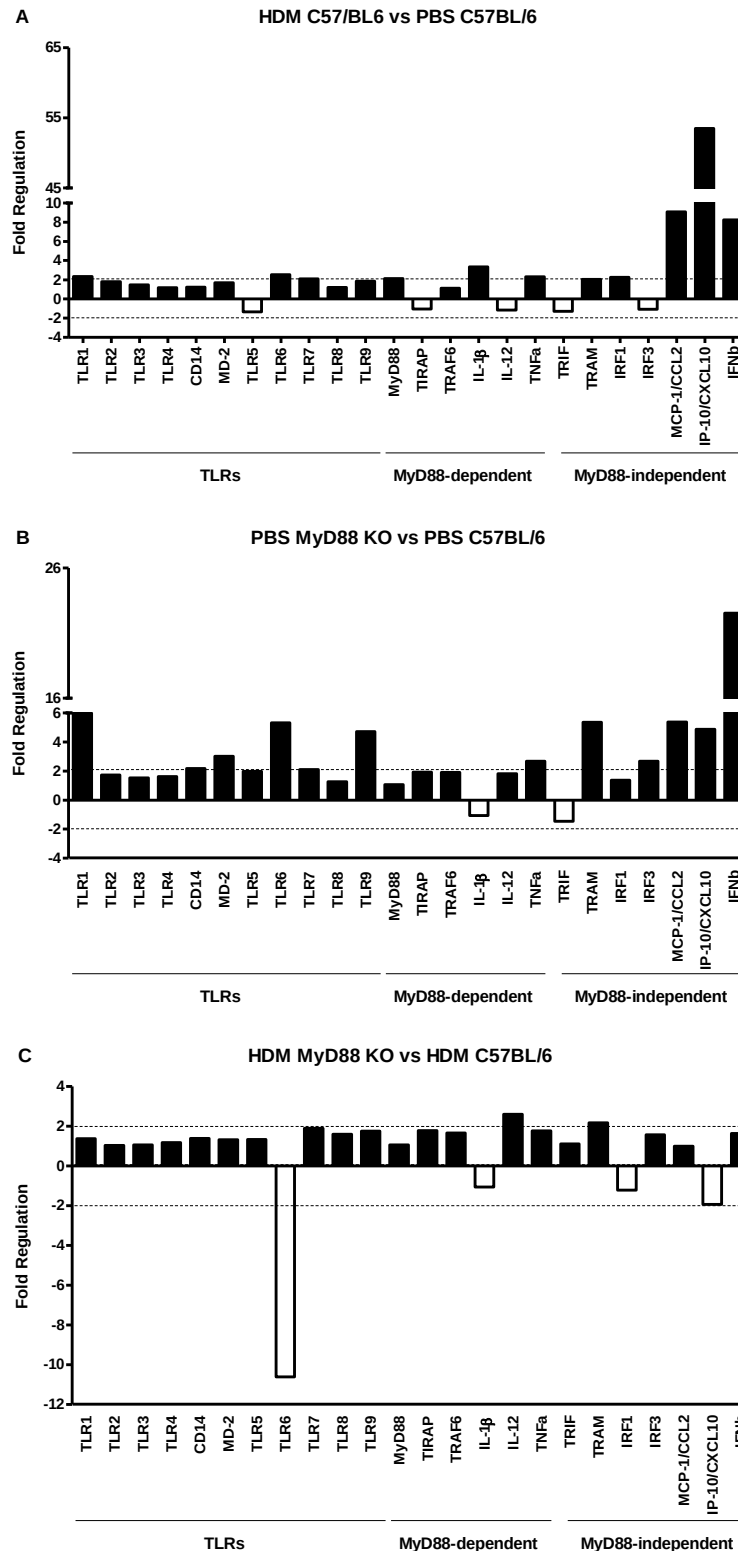


Figure 4.28. Impact of HDM and MyD88 deficiency on TLR gene expression in the lung. RNA was extracted from the lung tissue and assessment of gene expression was carried out by PCR array. (A) Fold regulation of gene expression from HDM-treated C57BL/6 mice, compared to PBS-treated C57BL/6 mice. (B) Fold regulation of gene expression from MyD88 KO mice compared to C57BL/6 mice. (C) Fold regulation of gene expression from HDM-treated MyD88 KO mice compared to HDM-treated C57BL/6 mice. Data is expressed as change in fold regulation, with a fold regulation of +1 indicative of no change. (n=2 mice for each gene).

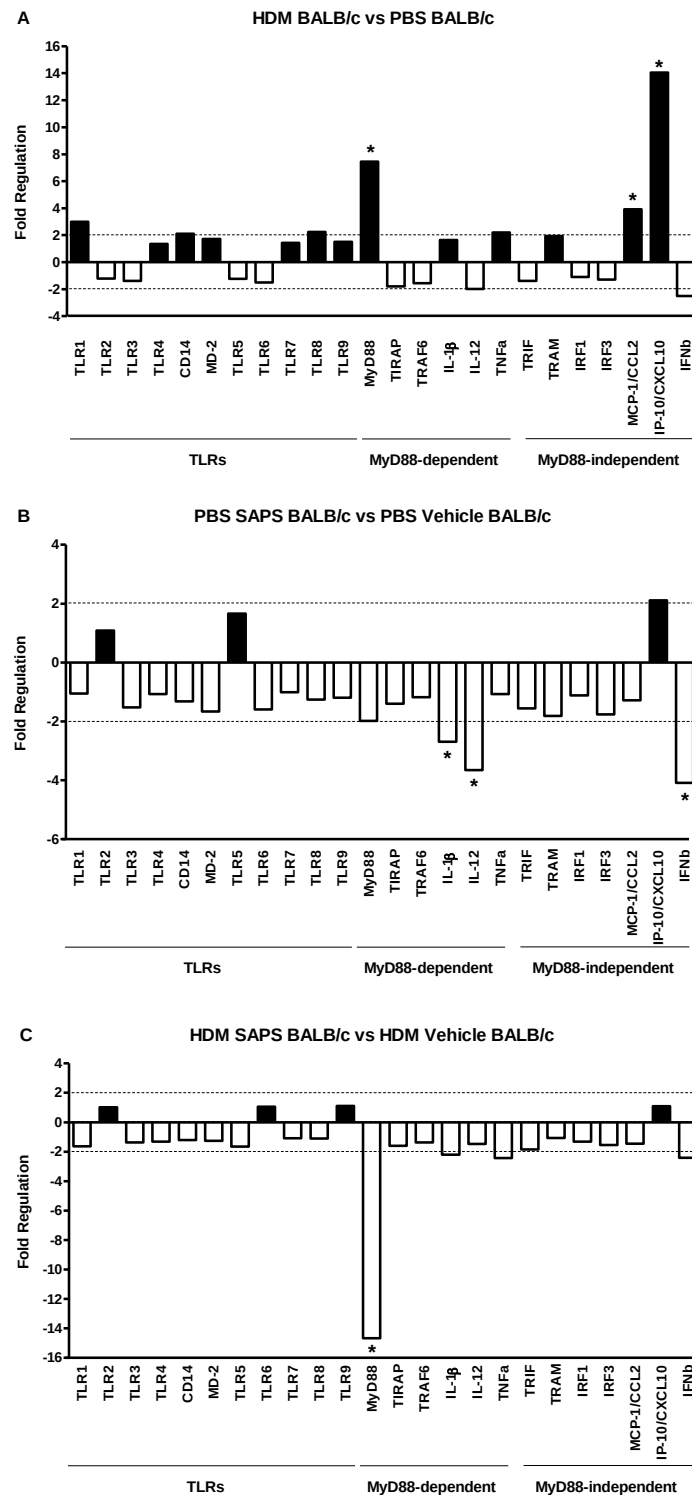


Figure 4.29. Impact of HDM and SAPS inhibitor on the TLR gene expression in the lung.

RNA was extracted from the lung tissue of mice and assessment of gene expression was carried out by PCR array. (A) Fold regulation of gene expression from HDM-treated BALB/c mice compared to PBS-treated BALB/c mice. (B) Fold regulation of gene expression from SAPS-treated BALB/c mice compared to vehicle-treated BALB/c mice. (C) Fold regulation of gene expression from SAPS-treated HDM exposed mice compared to vehicle-treated HDM exposed mice. Data is expressed as change in fold regulation, with a fold regulation of +1 indicative of no change. (n=3 mice for each gene). *, P<0.05 (Student's t-test).

4.5. Discussion

The focus of this Chapter was to dissect the role of TLR signalling in the development of HDM-induced allergic airways disease. Pharmacological approaches were carried out to block TLR4 and then multiple TLR activation, with a TLR4 antagonist and SAPS inhibitor respectively, to investigate the impact of TLR ligation on the development of Th2 responses following HDM exposure. Mice lacking systemic TLR4 and MyD88 signalling were also exposed to HDM to investigate the contribution of downstream TLR signalling pathways to the immune response. From these experiments it can be concluded that the MyD88-independent TRIF signalling pathway is the key contributor to the development of the immune response to HDM *in vivo*.

C3H-HeJ mice were utilised to dissect the role of systemic TLR4 signalling in HDM-induced allergic airways disease. C3H-HeJ mice have been shown to develop a less severe asthma phenotype in response to OVA than BALB/c mice (Whitehead et al., 2003) and C3H-HeJ mice do not develop AHR following intermittent exposure to HDM (Lewkowich et al., 2008). However, pulmonary eosinophilia and Th2 cytokine production are not completely absent in C3H-HeJ mice upon either OVA (Whitehead et al., 2003) or HDM exposure (Lewkowich et al., 2008). In the current study in this Chapter, C3H-HeJ mice upon continuous exposure to HDM for 3 weeks developed AHR and showed robust Th2 cytokine, eosinophil and IgE responses. The only aspect of the inflammatory response to HDM that was impaired by systemic TLR4 signalling deficiency was neutrophil recruitment to the lung. Lajoie and colleagues have recently shown that C3H-HeJ mice exposed to HDM produce the Th2-associated cytokines IL-4, IL-5 and IL-13 but fail to mount a Th17 response (Lajoie et al., 2010), which may explain the lack of neutrophils recovered from the lung observed here in the current study (Figure 4.2E).

The exact mechanism by which KC/mCXCL1 production in response to HDM is reduced in C3H-HeJ mice is unclear as macrophages derived from C3H-HeJ mice are not activated upon LPS stimulation (Hoshino et al., 1999). The reduced production of this chemokine could occur as a result of either the absence of TLR4 signalling on airway epithelial cells or as a result of impaired macrophage function, since both

these cells produce KC/mCXCL1 or the human equivalent IL-8 (Koch AE et al., 1992; Adam et al., 2006). In the current study it was observed that C3H-HeJ mice had higher baseline levels of TARC/CCL17, RANTES/CCL5, IL-25 (IL-17E), TSLP and CCL20/MIP-3 α compared to the C3H-HeN *wt* mice. Therefore, the administration of the TLR4 antagonist via the i.t. route allowed for a more specific tissue targeting experiment by blocking TLR4 activation on airway macrophages, DCs and epithelial cells. Administration of the TLR4 antagonist reduced eosinophil numbers recovered from the lung and BAL and completely abrogated IL-25 (IL-17E) production, suggesting that TLR4 signalling in the airways is responsible for IL-25 (IL-17E) production. The observations regarding HDM-driven inflammation in mice lacking systemic TLR4 signalling are not mirrored in mice treated with the TLR4 antagonist. This is likely due to strain differences between C3H and BALB/c mice and the impact of deficiency of systemic TLR4 signalling on basal levels of Th2 mediators and cell functions (Hoshino et al., 1999). In contrast, the TLR4 antagonist had no impact on the baseline levels of TARC/CCL17, RANTES/CCL5, IL-25 (IL-17E), TSLP and CCL20/MIP-3 α in BALB/c mice.

Results from the TLR4 targeting experiments discussed in this Chapter conflict with a recent study that proposes a vital role for TLR4 in promoting HDM sensitisation and Th2 responses (Hammad et al., 2009). Hammad and colleagues showed that TLR4 present on lung structural cells was required for mucosal DC activation and for the production of GM-CSF, IL-25 (IL-17E) and IL-33 (Hammad et al., 2009). The authors also showed that the inhalation of a TLR4 antagonist reduced eosinophil numbers in the BAL and HDM-induced AHR (Hammad et al., 2009), thus showing a vital role for TLR4 activation in the immune response to HDM. The observations from Hammad and colleagues differ from those in the current study whereby mice lacking systemic TLR4 signalling or mice treated with a TLR4 antagonist developed HDM-induced AHR. The observations from the Hammad study and the current study are likely to differ because of a number of factors, including differing dosage regimens, time points of analyses and techniques utilised to analyse the role of TLR4. The dosing protocols utilised in this Chapter involve exposing mice to a more frequent dose of HDM than the Hammad study. Thus a dependence on TLR4 may be overridden in the more continuous dosing protocols because of more frequent exposure to allergenic epitopes within HDM that do not signal via TLR4, such as β -

glucans (Nathan et al., 2009) or proteases that can degrade epithelial tight junctions (Wan et al., 1999; Wan et al., 2001). The mice used in the TLR4 antagonist experiment in the current study were of the BALB/c strain, whereas in the Hammad study the HDM TLR4 antagonist-treated mice were of the C57BL/6 strain (Hammad et al., 2009). BALB/c mice are more susceptible to allergen-induced AHR (Whitehead et al., 2003; Kelada et al., 2011) and differences in gene expression of molecules involved in TLR signalling in response to HDM were observed between BALB/c and C57BL/6 mice. Specifically, BALB/c mice displayed an upregulation of MyD88 gene expression in the lung in response to HDM, whereas C57BL/6 mice did not. Therefore, strain differences may also contribute to the differing observations between the current work and that of Hammad and colleagues.

A mechanism by which the observations in the current study may differ from those of Hammad and colleagues is that other TLR family members are more important in driving the *in vivo* immune response following continuous, as opposed to intermittent, HDM exposure. One such TLR family member that may be important in driving the immune response to HDM is TLR2 because in addition to TLR4, LPS and *Der p 2* have also been shown to signal via TLR2 (Kirschning et al., 1998; Chiou and Lin, 2009). Thus, interfering with TLR2 signalling may reduce the inflammatory response to HDM as this receptor is located on DCs (Asai et al., 2007), macrophages (Zhang et al., 2006) and airway epithelial cells (Bailey et al., 2008) and TLR2-MyD88 signalling results in IL-13, GM-CSF and IL-1 β production (Ritz et al., 2002; Wynn, 2003; Takeda and Akira, 2005). TLR2 has also been associated with asthma and polymorphisms in TLR2 are linked to the development of asthma and allergies in children (Eder et al., 2004). TLR2 activation also promotes Th2 responses from DCs *in vitro* (Agrawal et al., 2003) and *in vivo* (Dillon et al., 2004) and TLR2 activation also heightens OVA-induced allergic airways disease (Redecke et al., 2004). Therefore, the first approach to investigate the contribution of multiple TLRs, including TLR2, to the immune response to HDM *in vivo*, was to expose MyD88 KO mice to the allergen.

Following exposure to HDM for either 1, 3 or 5 weeks, MyD88 KO mice developed Th2-driven, eosinophilic inflammation, IgE responses, AHR and features of airway remodelling. These results strongly suggest that MyD88 is not required for the

development of Th2 inflammatory responses to HDM and that the MyD88-independent TRIF pathways of TLR3 and TLR4 are the key contributors to driving HDM-induced Th2 inflammation. Indeed, the TLR4 antagonist, which blocked both MyD88-dependent and MyD88-independent TLR4 signalling, had a greater impact on dampening the features of allergic airways disease than MyD88 deficiency. This non-requirement of MyD88 also conflicts with a recent study, where Phipps and colleagues reported that the induction of the features of allergic airways disease in response to inhaled HDM was dependent on MyD88 (Phipps et al., 2009). The authors showed that the absence of either TLR4 or MyD88 attenuated Th2-driven eosinophilia and AHR and the diminished Th2 responses were associated with fewer OX40L positive mDCs in the draining LNs (Phipps et al., 2009). As with the Hammad study, the HDM exposure protocols carried out by Phipps and colleagues are more intermittent than the continuous, uninterrupted inhalation protocols carried out in Chapter 4. Therefore, it is plausible that a certain dependency on TLR4 and MyD88 may be overridden in more continuous dosage regimens. Importantly, increased epithelial permeability and impaired airway barrier function are not observed following a single dose of HDM *in vivo* (Turi et al., 2011) and are most likely a consequence of continuous exposure. It is possible that the intermittent dosing protocols used in the Phipps and Hammad studies allow for the epithelium to repair, whereas there is no such lag in exposure to HDM for the epithelium in the continuous dosing protocols used in Chapter 4.

A key mechanism by which the results in Chapter 4 differ from the Phipps publication is that the MyD88 KO mice used in the current study were bred on a C57BL/6 background, whereas in the Phipps study the adaptor protein deficient mice were bred on a BALB/c background (Phipps et al., 2009). This is important with regards to AHR, as C57BL/6 mice are less susceptible to OVA and HDM-induced AHR than BALB/c mice (Whitehead et al., 2003; Kelada et al., 2011). The array data in this Chapter showed that C57BL/6 mice had no increase in MyD88 gene expression in the lung following HDM exposure, whereas there is a very clear upregulation in BALB/c mice. Therefore, in BALB/c mice MyD88 may be more important in the immune response to HDM and thus explain why MyD88 KO mice on a BALB/c background show resistance to HDM-induced airway inflammation, whereas MyD88 KO mice bred on a C57BL/6 background do not. Data from the gene

array also suggests that MyD88 KO mice are predisposed to TRIF signalling as there are basal increases in TRAM, MCP-1/CCL2, IP-10/CXCL10 and IFN- β gene expression compared to C57BL/6 mice. Indeed, non-allergic MyD88 KO mice showed increased IRF3, MCP-1/CCL2 and IP-10/CXCL10 expression, which was also seen in C57BL/6 mice in response to HDM.

The production of IgE in MyD88 KO mice is also a key observation, since there is conflicting data regarding the role of MyD88 in the generation of IgE *in vivo*, with Piggott and colleagues proposing a vital role for MyD88 in the generation of IgE in OVA-LPS driven inflammation (Piggott et al., 2005), whereas Schnare and colleagues have shown that MyD88 is dispensable in OVA-alum sensitisation models (Schnare et al., 2001). With regards to HDM there are antigenic epitopes within the extract that can promote IgE responses independently of MyD88 signalling, including *Der p 1* that can cleave the low affinity IgE receptor CD23 (Hewitt et al., 1995;Schulz et al., 1995). Schnare and colleagues and Phipps and colleagues have also reported that baseline levels of IgE in the serum of MyD88 KO mice are higher than the respective *wt* controls (Schnare et al., 2001;Phipps et al., 2009), however this was not observed in the experiments discussed in this Chapter.

In view of the fact that MyD88 KO mice developed AHR and Th2-driven airway inflammation, another approach was undertaken to target multiple TLR pathways. The next experiment was designed to assess the impact of the TRIF signalling pathways, in addition to certain MyD88 signalling cascades, on disease pathology. The administration of a SAPS inhibitor blocked TLR1-4, 7 and 8 signalling by disruption of membrane microdomains and preventing the association of TLR2 and TLR4 with their respective membrane partners (Parker et al., 2008). Therefore, the absence of HDM-induced increase in MyD88 gene expression in SAPS-treated mice is likely a consequence of SAPS-mediated TLR blockade preventing MyD88 signalling. The SAPS inhibitor reduced the numbers of eosinophils and Th2 cells recovered from the lung, concomitant with reduced levels of eotaxin-1/CCL11 and TARC/CCL17 respectively, and ablated IL-25 (IL-17E) production. Indeed, RANTES/CCL5 and MCP-1/CCL2 production which can be induced via TRIF signalling (Akira et al., 2006) was abrogated with the SAPS inhibitor. Therefore, these results show that blockade of the TLR-TRIF signalling pathways, as well as the

MyD88 pathways of TLR1, 2, 4, 7 and 8, has the most significant impact on the development of inflammation as opposed to targeting either TLR4 or MyD88. In order to test the precise role of the TRIF pathway, TRIF KO mice should be exposed to HDM to specifically determine the contribution of this signalling pathway to the immune response. Further to this to determine the combined contribution of both MyD88 and TRIF signalling pathways to the immune response to HDM, MyD88 KO mice should be treated with the SAPS inhibitor prior to exposure to HDM, as this would inhibit all TLR MyD88 and TRIF signalling. Alternatively, available MyD88 and TRIF double KO mice could also be used, as these mice have no functional signalling TLRs and have been used to show that alum-induced Th2 responses are not attenuated in mice deficient in both MyD88 and TRIF (Gavin et al., 2006; Eisenbarth et al., 2008).

The results discussed in this Chapter suggest that the TRIF signalling pathway is the key contributor from TLR signalling cascades to HDM-induced allergic airways disease and this is highlighted with three key observations. Firstly, the SAPS inhibitor blocking TLR1-4, 7 and 8 had the greatest impact on reducing the number of eosinophils and Th2 cells in the lung following HDM exposure. Secondly, the TLR4 antagonist blocking the TRIF and MyD88 pathways of this receptor had a greater anti-inflammatory effect compared to when MyD88 alone was deficient. Thirdly, MyD88 KO mice developed features of allergic airways disease and were found to have elevated gene expression levels of TRIF signalling molecules in the lung. Indeed, the elevations in gene expression of TRIF signalling molecules in MyD88 KO mice mirrored the gene expression changes seen in HDM-treated C57BL/6 mice.

TLR-TRIF signalling has been shown to promote Th2 inflammatory responses, whereby LPS *in vitro* can induce MyD88-deficient DC maturation and upregulation of costimulatory molecules to support Th2 cell differentiation (Kaisho et al., 2002). TLR3, which signals exclusively via TRIF, has also been associated with the promotion of Th2 responses. Signalling via TLR3, which is expressed on innate immune cells, including epithelial cells (Ritter M et al., 2005; Akira et al., 2006), leads to the production of IL-1 β , GM-CSF, IL-6, TNF- α , IP-10/CXCL10, RANTES/CCL5, CCL3/MIP-1 α and IL-8 (Ritter et al., 2005; Matsukura et al., 2006; Wang et al., 2007). TLR3 mRNA is upregulated in OVA-sensitised rats and

blockade of TLR3 reduces IgE production and IL-4 mRNA expression levels (Meng et al., 2011). dsRNA signalling via TLR3 promotes eotaxin-1/CCL11, TARC/CCL17 and TSLP release from airway epithelial cells (Kato et al., 2007; Bogiatzi et al., 2007; Torres et al., 2010). TLR3 can also recognise endogenous danger signals, such as heterologous mRNA, which are released from necrotic cells (Kariko et al., 2004a; Kariko et al., 2004b). TLR3 activation has also been shown to activate mast cells (Orinska et al., 2005) and, as a result, TLR3 signalling could enhance Th2-mediated inflammation (Kinoshita et al., 2009).

This Chapter has focused specifically on the contribution of the TLR family to the immune response to inhaled HDM. Although the SAPS inhibitor had the greatest impact on reducing HDM-induced disease pathology, HDM-specific IgE and IL-4 production were unaltered, suggesting that other PRRs could contribute to Th2-driven inflammation and direct HDM-induced airway inflammation. One such PRR family that has been shown to promote Th2 responses are the C-type lectin receptor (CLR) family and carbohydrate moieties, signalling via CLRs, are strong Th2 promoting PAMPs that include β -glucans that are present in HDM extracts (Douwes et al., 2000). *In vitro*, β -glucans have been shown to recruit immature DCs via the release of CCL20/MIP-3 α from airway epithelial cells (Nathan et al., 2009). In the current study, HDM-induced production CCL20/MIP-3 α was not reduced by either the TLR4 antagonist, the SAPS inhibitor or MyD88 deficiency, suggesting that the production of this chemokine and recruitment of immature DCs occurs independently of TLR activation and MyD88 signalling *in vivo*. DCs have also been shown to express the mannose receptor (MR), which has a crucial role in DC maturation (Li et al., 2010; Royer et al., 2010), and Dectin-2, which mediates HDM-driven Th2 inflammation via the generation of cysteinyl leukotrienes from DCs (Barrett et al., 2009; Barrett et al., 2011). Thus, in addition to TLRs there are other PRRs which may contribute to the development of Th2 immunity following continuous exposure to HDM. Although mainly associated with anti-viral immunity, IRF3 KO mice have also been shown to be resistant to HDM-induced goblet cell hyperplasia, AHR and BAL inflammation, with DCs derived from these mice having impaired maturation and migration (Marichal et al., 2010). Marichal and colleagues also rule out a role for TLR4 in the IRF3-dependent effects of HDM on the pro-allergic functions of DCs and postulate that other PRRs, such as RIG-I-like receptors (RLRs), can also activate

IRF3 (Marichal et al., 2010). It is therefore possible that in the continuous HDM exposure protocols utilised in the current study that CLRs and RLRs play a prominent role in promoting the immune response to HDM.

From the experiments undertaken in this Chapter it can be concluded that the MyD88-independent TRIF signalling pathway is the key contributing TLR signalling pathway in promoting the immune response to HDM *in vivo*. However, HDM contains multiple antigenic epitopes which can promote the development of Th2 inflammation, independently of TLR signalling. One such feature of HDM that has been shown to promote Th2 responses is the intrinsic protease activity and the impact of proteases on the immune response was then investigated in Chapter 5.

Chapter 5 - Contribution of protease activity to HDM-induced allergic airways disease

5.1. Introduction

The intrinsic protease activity of HDM allergens is believed to be central to allergic sensitisation and atopic asthma (Chapman et al., 2007). The major HDM allergen *Der p 1* is an active cysteine protease (Chua et al., 1988;Gough et al., 1999), while *Der p 3*, 6 and 9 have been shown to possess serine protease activity (Nishiyama et al., 1995;Bennett and Thomas., 1996;King et al., 1996;Flores et al., 2003). There is a significant amount of *in vitro* data linking these enzymatic epitopes of HDM with the development of Th2-mediated inflammation and IgE responses, summarised in Table 5.1.

Together these data show that the activities of HDM-associated proteases promote the development of Th2 immunity and IgE production, acting via multiple sites on the airway epithelium and various cells. For example, *Der p 1* has been shown to increase IgE synthesis directly from B cells via cleavage of the low affinity IgE receptor CD23 (Schulz et al., 1995;Hewitt et al., 1995). In addition to this, *Der p 1* has also been shown to cleave components of epithelial tight junctions (TJs) (Wan et al., 1999), thereby resulting in greater epithelial permeability and accessibility to antigen presenting cells (APCs) residing in the airway mucosa. Airway epithelial cells exposed to *Der p 1* release a vast assortment of pro-inflammatory mediators, which promote DC, eosinophil and Th2 cell recruitment. These mediators include IL-6, IL-8, MCP-1/CCL2, GM-CSF, RANTES/CCL5, eotaxin-1/CCL11 (King et al., 1998;Asokanathan et al., 2002a;Adam et al., 2006;Kauffman et al., 2006), TSLP (Pichavant et al., 2005;Ying et al., 2005) and IL-25 (IL-17E) (Yu et al., 2010). Mast cells pulsed with *Der f 1* produce IL-4, IL-6, and TNF- α (Yu and Chen, 2003) and basophils exposed to *Der p 1* release IL-4 and IL-13 (Phillips et al., 2003). In addition, *Der p 1* promotes further Th2 inflammatory bias by activating T cells via the cleavage of CD25 (Shakib et al., 1998;Schulz et al., 1998;Ghaemmaghami et al., 2001;Harris et al., 2004), directly activating DCs which increases Th2 cell recruitment (Hammad et al., 2001;Hammad H et al., 2003) and reducing IL-12 (Ghaemmaghami et al., 2002) and IL-10 responses (Furmonaviciene et al., 2007). *Der p 3*, 6 and 9 also further polarise the Th2 response by promoting the release of GM-CSF, eotaxin-1/CCL11 and IL-8 from airway epithelial cells (Tomee et al., 1998;King et al., 1998;Sun et al., 2001;Adam et al., 2006) and by cleaving

components of both epithelial TJs (Wan et al., 2001) and the complement system (Maruo et al., 1997).

Thus, there is substantial evidence linking the intrinsic protease activity of HDM to the development of allergy and Th2 responses, with many of the pro-inflammatory effects occurring at the airway epithelium (as summarised in Figure 5.1.). However, precisely how the protease activity of HDM contributes to the immune response *in vivo* remains to be determined. In order to investigate this, HDM was boiled to interfere with the protease activity of the extract and the impact on disease pathogenesis assessed. In addition, the effects of boiling on the antigenicity of HDM and the recognition of HDM by APCs in the airways were also investigated.

Protease Epitope	Function	Consequence	Reference
<i>Der p 1</i>	Cleavage of tight junction components	Increased epithelial permeability	(Herbert et al., 1995;Wan et al., 1999;Roche et al., 2000)
	PAR2-dependent and PAR2-independent activation	Epithelial activation and release of pro-inflammatory mediators	(King et al., 1998;Asokanathan et al., 2002a;Gough et al., 2003;Ying et al., 2005Adam et al., 2006;Kauffman et al., 2006;Pichavant et al., 2005;Heijink et al., 2010a)
	Cleave CD25	T cell activation	(Shakib et al., 1998;Schulz et al., 1998;Ghaemmaghami et al., 2001;Harris et al., 2004)
	Activate DCs	Th2 polarisation	(Hammad et al., 2001)
	Induce production of TARC/CCL17 and MDC/CCL22	Activate and recruit Th2 cells	(Hammad et al., 2003)
	Cleave CD40	Failure to induce IL-12	(Ghaemmaghami et al., 2002)
	Cleave DC-SIGN and DC-SIGN-R	Failure to induce regulatory IL-10	(Furmonaviciene et al., 2007)
	Cleave CD23	Stimulate IgE production by B cells	(Hewitt et al., 1995;Schulz et al., 1995)
	Cleave SP-A and SP-D	Reduce innate immune defence	(Deb et al., 2007)
<i>Der p 3</i>	Degrade α 1-anti-trypsin	Enhanced tissue damage	(Kalsheker et al., 1996;Takai et al., 2005)
	Cleavage of tight junction components	Increased epithelial permeability	(Wan et al., 2001)
	Activation of PAR2	Epithelial activation and release of pro-inflammatory mediators	(Sun et al., 2001;Adam et al., 2006)
<i>Der p 6</i>	Cleave complement components C3 and C5	Recruitment of innate immune cells	(Maruo et al., 1997)
	Cleavage of tight junction components	Increased epithelial permeability	(Wan et al., 2001)
<i>Der p 9</i>	Activation of PAR2	Epithelial activation and release of pro-inflammatory mediators	(Tomee et al., 1998)
	Cleavage of tight junction components	Increased epithelial permeability	(Wan et al., 2001)
<i>Der p 9</i>	Activation of PAR2	Epithelial activation and release of pro-inflammatory mediators	(King et al., 1998;Tomee et al., 1998;Sun et al., 2001)

Table 5.1. Summary of the interactions of protease active epitopes within HDM extracts with the host defence.

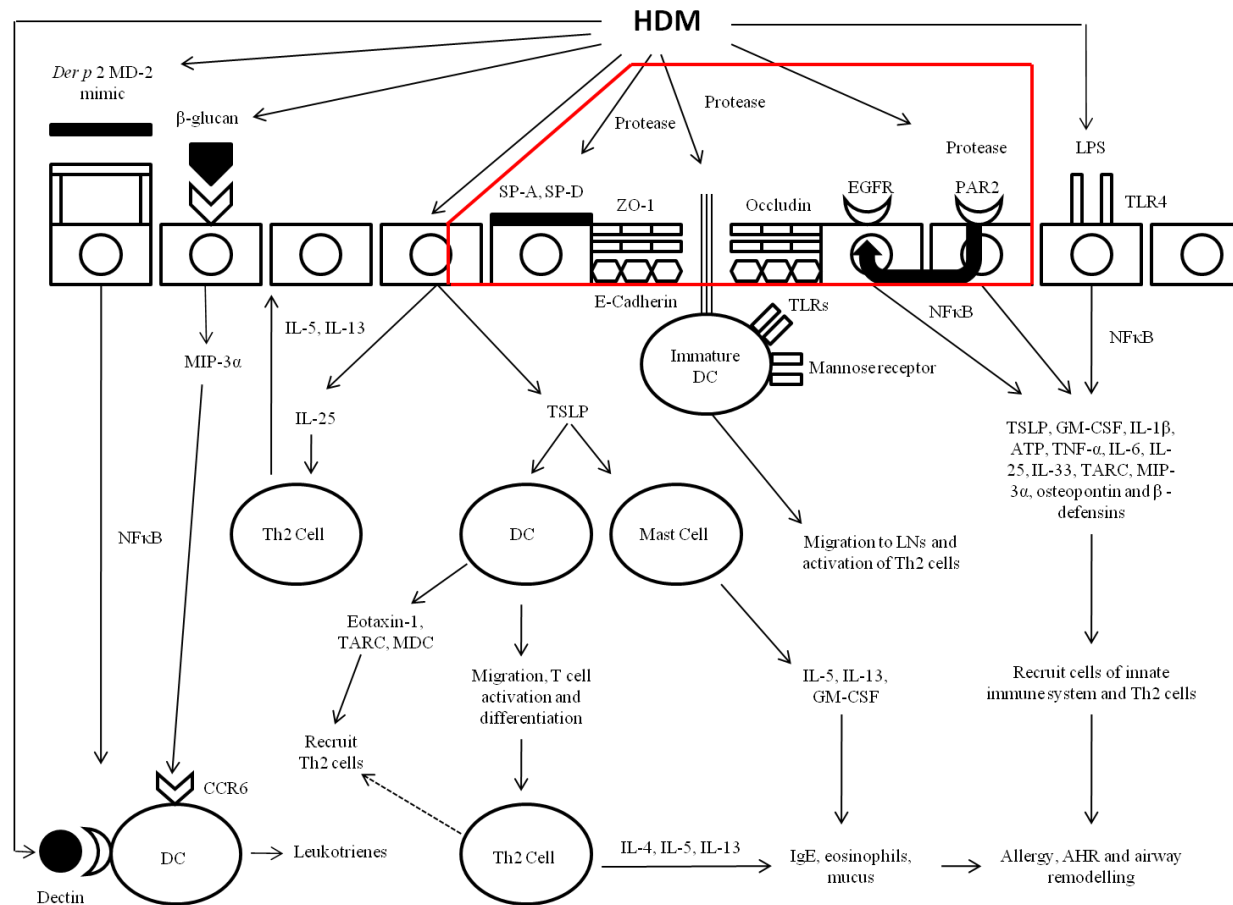


Figure 5.1. Summary of the interactions of proteases within HDM with the airway epithelium (highlighted in red).

Protease active epitopes within HDM have been shown to promote pro-inflammatory mediator release from the airway epithelium, via PAR-2-dependent and -independent mechanisms. Protease active HDM allergens can also degrade components of epithelial tight junctions and surfactant proteins that make up crucial components of the innate host defence.

5.2. Hypothesis and Aims

5.2.1. Hypothesis

Intrinsic protease activity of HDM promotes the immune response to HDM *in vivo* and boiling of HDM will inactivate serine and cysteine proteases, thereby reducing the magnitude of the Th2 inflammatory response. In addition to disrupting the protease activity of the extract, boiling HDM will also result in reduced recognition by APCs in the airways.

5.2.2. Aims

The aims of Chapter 5 are to determine:

- The impact of HDM on the integrity and function of the airway epithelium
- How boiling of HDM effects the development of airway inflammation, AHR and airway remodelling *in vivo*
- How boiling impacts the antigenicity of HDM and the process by which the immune system is activated.

5.3. Experimental Plan

5.3.1. Protocol A: Induction of HDM-induced allergic airways disease and investigation into effect of boiling on disease development

In order to investigate how boiling impacted the immune response to inhaled HDM *in vivo*, HDM was boiled for 10 minutes at 100°C, prior to challenge, to disrupt enzymatic activity (Lombardero et al., 1990). This heat treatment has been shown to reduce the binding of *Der p* 1 to both IgE antibodies and to non-overlapping epitopes by approximately 100-fold, whereas *Der p* 2 allergens are heat stable (Lombardero et al., 1990). The migration of immunoreactive *Der p* 1 across Madin-Darby canine kidney (MDCK) epithelial cell monolayers has been shown to depend upon enzymatic activity and is abolished upon boiling (Wan et al., 1999).

Female BALB/c mice were administered either 25 µl PBS, 25 µg (in 25 µl) HDM or 25 µg (in 25 µl) boiled HDM (bHDM) i.n. 5 times a week for 5 weeks and culled 24h post final challenge.

5.3.2. Protocol B: Effects of HDM administration on epithelial permeability

To investigate and quantify epithelial permeability in response to acute HDM exposure, mice were exposed to either 25 µl PBS, 25 µg (in 25 µl) HDM or 25 µg (in 25 µl) bHDM i.n. 5 times a week for one week. 24h after the final PBS, HDM or bHDM challenge, each mouse was administered 20 µl of 50 mg/ml FITC-Dextran i.n. and culled 1h later. The FITC-Dextran is 4 kDa in size and normally resides in the airway lumen in a naive mouse. The presence of FITC-Dextran in the lung tissue is indicative of increased airway epithelial permeability.

5.3.3. Protocol C: Effects of boiling on the identification of HDM by APCs

To determine how boiling influenced HDM recognition by APCs, mice were given either 25 µl PBS, 25 µg (in 25 µl) HDM or 25 µg (in 25 µl) bHDM i.n. 3 times a week for 1 week. All mice were then administered 25 µg (in 25 µl) of Alexa-488-labelled HDM. A group of PBS-treated mice did not receive the Alexa-488-labelled HDM. Mice were culled 24h post Alexa-488-labelled HDM administration.

5.4. Results

5.4.1. Protocol A: Influence of boiling on HDM-induced allergic airways disease

5.4.1.1. Protease activity of HDM reduced by boiling

In order to determine the protease activity of HDM and to investigate the effects of boiling on the degree of protease activity, an *in vitro* colorimetric protease assay was developed. N-Benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (NBP-VANA) is a substrate that is broken down by both serine and cysteine proteases leading to an increase in absorbance (Lottenberg et al., 1981). For normalisation, the rate of breakdown is represented as a percentage increase from baseline. PBS did not breakdown NBP-VANA, whereas both trypsin (a control serine protease) and papain activated with DTT (a control cysteine protease) were observed to breakdown the substrate. When trypsin was incubated with the serine protease inhibitor AEBSF and papain and DDT were incubated with the cysteine protease inhibitor E-64, breakdown of NBP-VANA was abrogated (Figure 5.2A).

HDM degraded NBP-VANA throughout the 120 minute time course. When bHDM was assessed, there was a significant 5-fold reduction in the breakdown of NBP-VANA compared to HDM. However, the breakdown of NBP-VANA was not ablated with boiling, suggesting that boiling does not completely destroy all the protease activity, and that there are thermostable proteases within the HDM extract. To determine the specific contribution of serine and cysteine proteases, HDM was incubated with both AEBSF and E-64. A 40% reduction in the protease activity was observed compared to HDM alone (Figure 5.2B). Thus, this assay showed that with boiling the protease activity of the HDM extract was reduced.

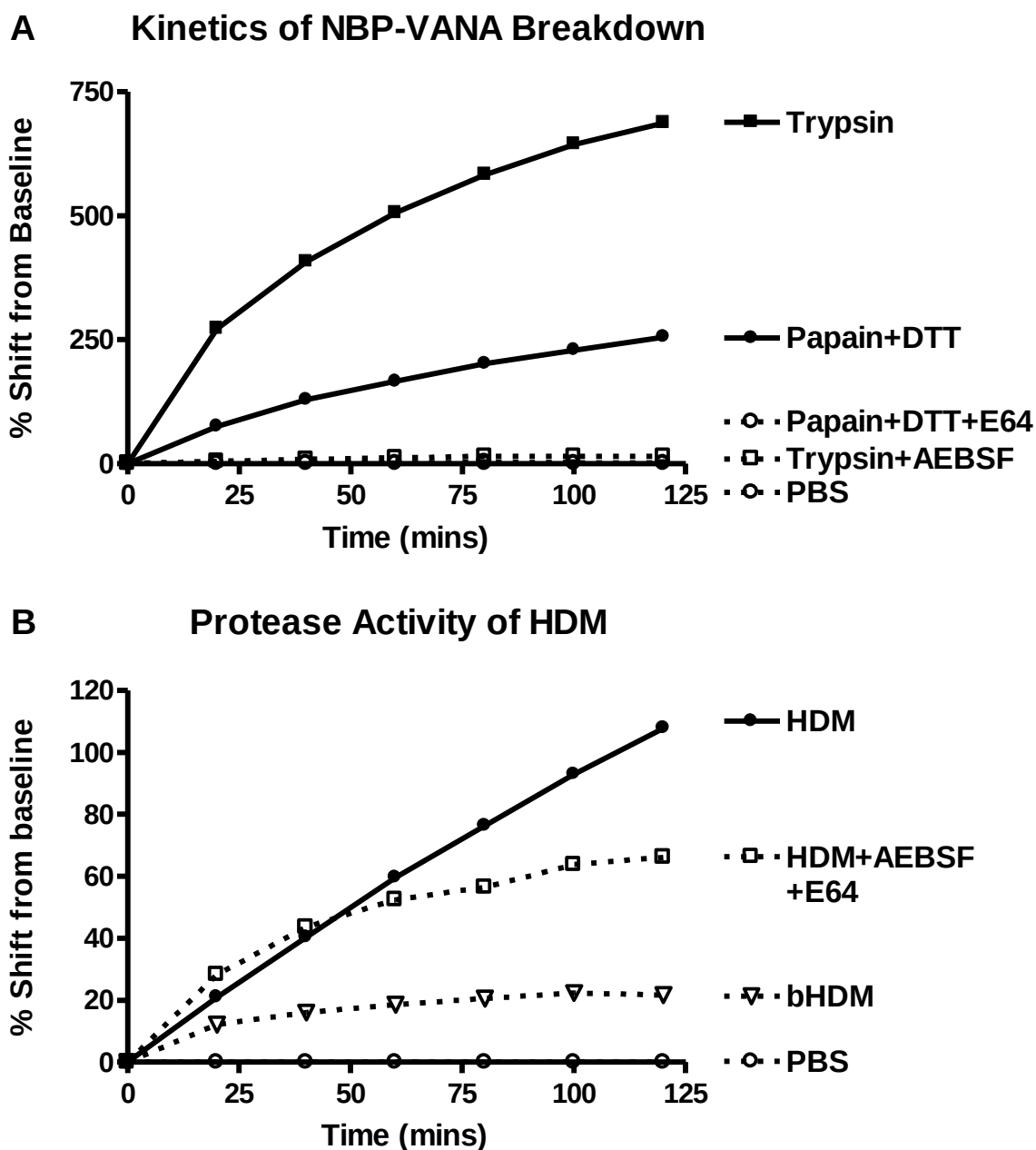


Figure 5.2. Assessment of protease activity of HDM.

Percentage increase from baseline of NBP-VANA breakdown following incubation with (A) PBS, trypsin, trypsin and AEBSF, papain activated with DTT and papain activated with DTT and E-64 and (B) PBS, HDM, bHDM and HDM with E-64 and AEBSF.

5.4.1.2. Onset of AHR is delayed in bHDM-treated mice - Whole Body Plethysmography

To investigate the effects of boiling on the immune response to inhaled HDM *in vivo*, mice were challenged i.n. with either PBS, HDM or bHDM for 5 weeks. AHR was measured weekly in each mouse, non-invasively by whole body plethysmography, as this technique can be used to monitor the development of AHR. At each time point, HDM was observed to induce AHR. However, there was a delay in onset of AHR development following bHDM exposure, as these mice had significantly reduced AHR from HDM at week 1 and only showed increased Penh compared to PBS at weeks 3 and 4 (Figure 5.3).

5.4.1.3. bHDM-treated mice do not develop increased airway resistance – Flexivent

Airway resistance and compliance were measured invasively in anaesthetised mice at week 5, 24h post final challenge. HDM significantly increased airway resistance in response to methacholine as expected. In contrast, no increase in airway resistance was observed after bHDM treatment compared to PBS controls (Figure 5.4A and B). Decreased compliance of the airways was observed in HDM and bHDM-treated mice. However, boiling attenuated allergen-induced decrease in airway compliance (Figure 5.4C and D).

Whole Body Plethysmography

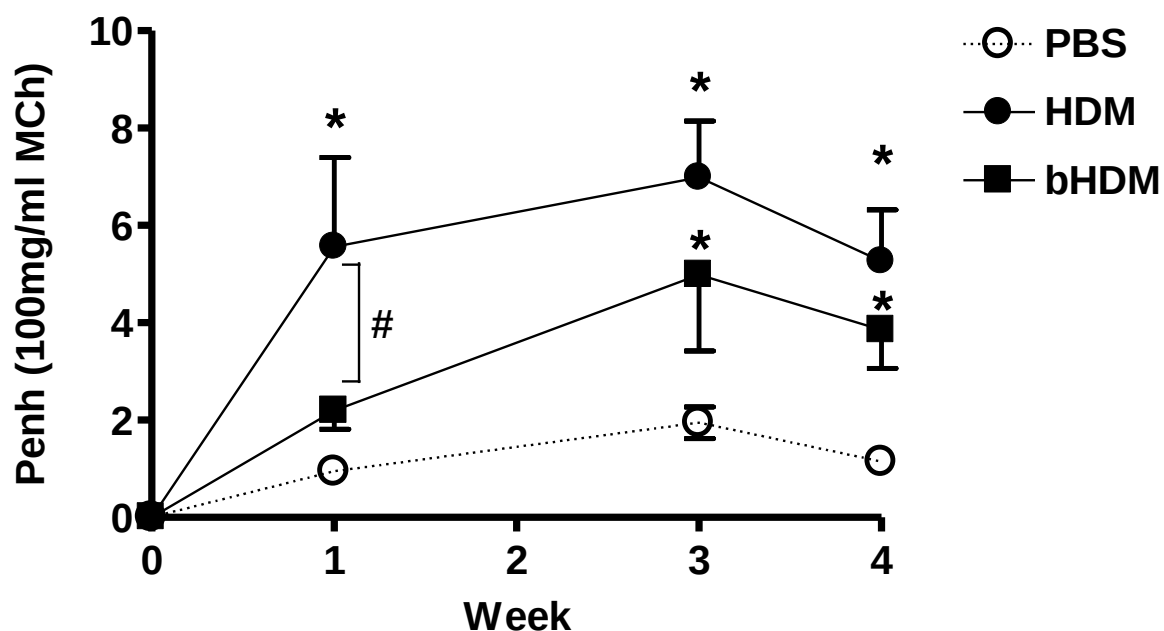


Figure 5.3. Development of AHR after HDM exposure.

AHR was measured at weekly time points using an EMMS Penh system. Results are expressed as Penh for the 100mg/ml dose. (n=5 PBS and n=7 HDM or bHDM at each time point). *, $P < 0.05$ (Mann-Whitney U test) HDM or bHDM compared with PBS. #, $P < 0.05$ (Mann-Whitney U test) bHDM compared with HDM. Data are mean $\pm$ SEM. Representative of two experiments.

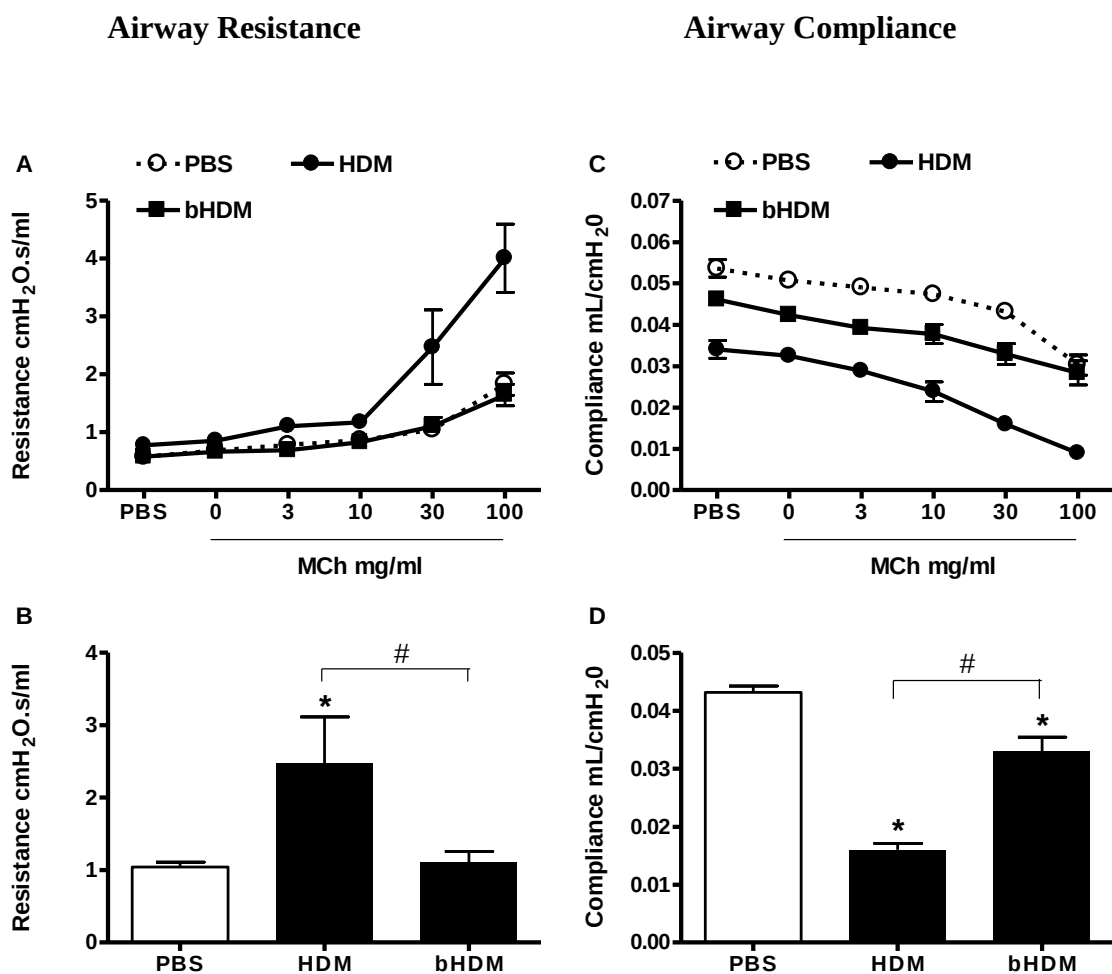


Figure 5.4. Absence of increased airway resistance in bHDM-treated mice.

(A) Airway resistance of PBS, HDM and bHDM-treated mice at 5 weeks 24h post final challenge. (B) Airway resistance is expressed as mean $\pm$ SEM for the 30mg/ml dose of methacholine. (C) Airway compliance of PBS, HDM and bHDM-treated mice at 5 weeks 24h post final challenge. (D) Airway compliance is expressed as mean $\pm$ SEM for the 30mg/ml dose of methacholine. (n=5 PBS and n=7 HDM or bHDM). *, $P < 0.05$ (Mann-Whitney U test) HDM or bHDM compared with PBS. #, $P < 0.05$ (Mann-Whitney U test) bHDM compared with HDM. Data are mean $\pm$ SEM. Representative of two experiments.

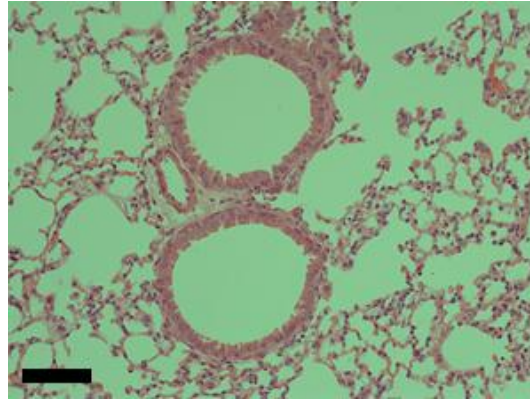
5.4.1.4. Reduction in the recruitment of inflammatory cells following bHDM treatment

Both HDM and bHDM lead to the recruitment of inflammatory cells in the airways as shown in H and E stained lung sections. However, the degree of inflammation was far more severe in HDM-treated mice compared to bHDM-treated mice (Figure 5.5A - C). The total number of cells present in the lung and BAL were attenuated following boiling of HDM (Figure 5.6A and B). In addition, the number of eosinophils was reduced, with approximately a 4-fold reduction in the lung and 10-fold reduction in the BAL (Figure 5.6C and D), as was the number of neutrophils and Th2 cells present in the lung and BAL (Figure 5.6E – H). Whilst the cellular recruitment was reduced in bHDM-treated mice, the number of eosinophils, neutrophils and Th2 cells recovered from the lung and BAL remained significantly higher compared to PBS controls. This suggests that despite boiling of HDM and dramatically reducing enzymatic activity, bHDM is still able to induce an inflammatory response.

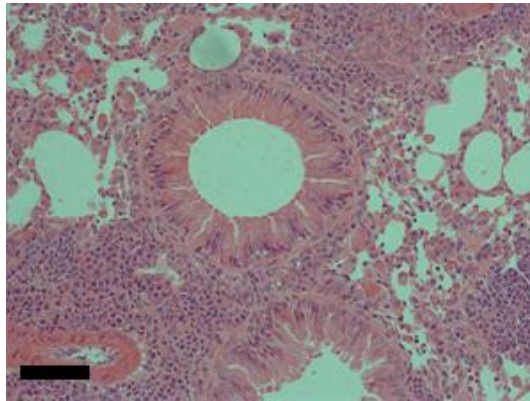
5.4.1.5. Altered chemokine profile of boiled HDM-treated mice

HDM increased the levels of eotaxin-1/CCL11, KC/mCXCL1 and TARC/CCL17 in the lung and BAL (Figure 5.7A - F). The production of these chemokines was reduced in response to bHDM with the levels of eotaxin-1/CCL11 and TARC/CCL17 in the BAL reduced back to baseline (Figure 5.7D and F).

A. PBS



B. HDM



C. bHDM

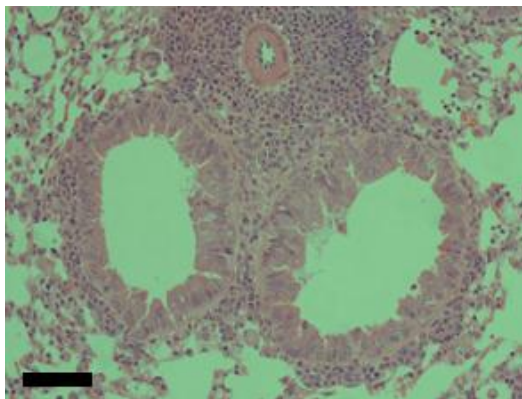


Figure 5.5. Reduction in cellular inflammation in bHDM-treated mice.

Representative pictures from paraffin embedded lung sections from (A) PBS, (B) HDM and (C) bHDM-treated mice stained with Haematoxylin and Eosin (original 20X magnification). *Scale bar* = 50 μm ..

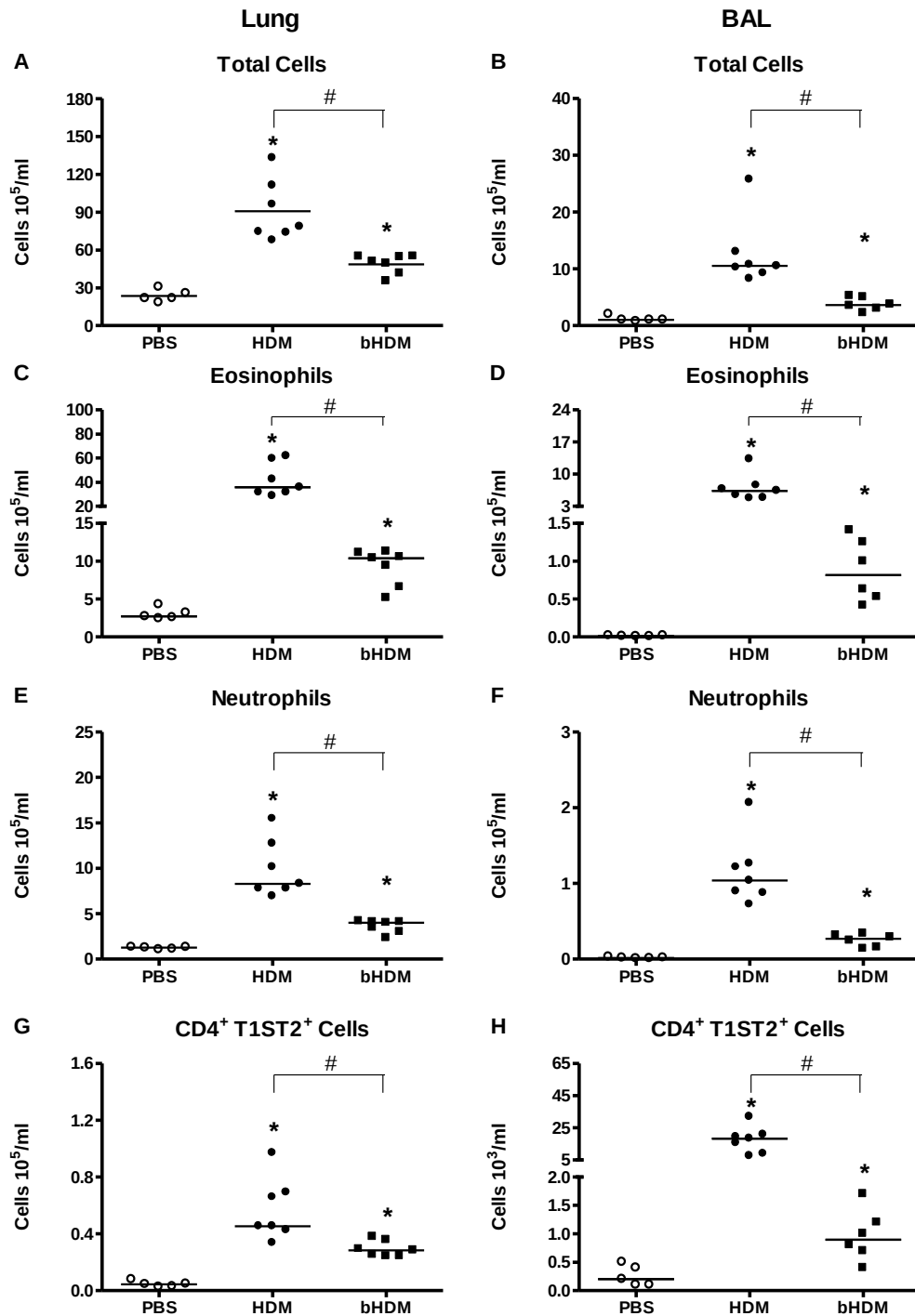


Figure 5.6. Attenuated cellular inflammation in response to bHDM.

Total cells, eosinophils, neutrophils and CD4⁺T1ST2⁺ recovered from the lung (A, C, E and G) and BAL (B, D, F and H) at 5 weeks 24h after the final i.n. challenge. (n=5 PBS and n=6-7 mice treated with HDM or bHDM). Total cell counts were determined using a haemocytometer with white blood cell counting fluid, eosinophils and neutrophils were quantified from Wright-Giemsa stained cytopins and CD4⁺T1ST2⁺ cells were quantified by flow cytometry, as described in the Materials and Methods. *, P<0.05 (Mann-Whitney *U* test) HDM or bHDM compared with PBS. #, P<0.05 (Mann-Whitney *U* test) bHDM compared with HDM. Bars depict median of the groups. Representative of two experiments.

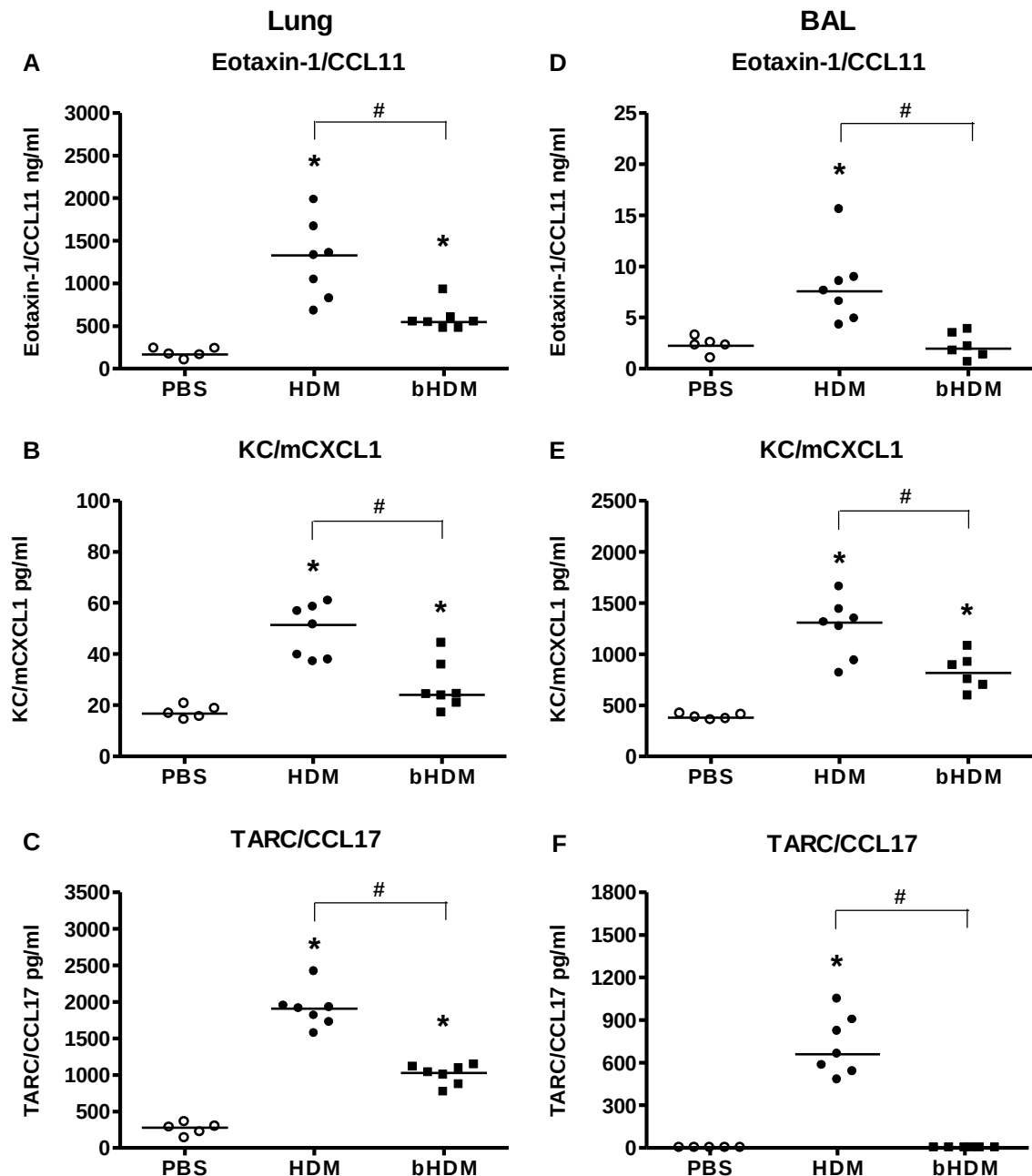


Figure 5.7. Reduced inflammatory chemokine production in response to bHDM. Eotaxin-1/CCL11, KC/mCXCL1 and TARC/CCL17 were measured in the lung (A - C) and BAL (D - F) at 5 weeks, 24h after the final i.n. challenge. Eotaxin-1/CCL11 and TARC/CCL17 levels were determined by ELISA and KC/mCXCL1 levels were determined by MSD. (n=5 PBS and n=6-7 HDM or bHDM). *, $P < 0.05$ (Mann-Whitney U test) HDM or bHDM compared with PBS. #, $P < 0.05$ (Mann-Whitney U test) bHDM compared with HDM. Bars depict the median of the group. Representative of two experiments.

5.4.1.6. Reduction in Th2 cytokine production following boiling of HDM

Th2 cytokines IL-4, IL-5 and IL-13 were quantified in the lung and BAL at the end of week 5. As expected, HDM induced an increase in IL-4 production in the lung compared to PBS controls. Treatment with bHDM led to a 4-fold decrease in IL-4 production when compared to HDM alone (Figure 5.8A). HDM-induced production of IL-4 in the BAL was abrogated in response to bHDM exposure (Figure 5.8B). There was also a 50% reduction in HDM-induced IL-5 production in the lung and BAL following bHDM administration (Figure 5.8C and D), whilst IL-13 levels in the lung and BAL were reduced back to baseline (Figure 5.8E and F). A decrease in Th2 cytokine production in response to bHDM, correlates with a reduction in the number of Th2 cells recruited to the lung and BAL (Figure 5.6G and H).

5.4.1.7. Altered innate cytokine profile of boiled HDM-treated mice

IL-25 (IL-17E), TSLP, IL-33 and CCL20/MIP-3 α were quantified in the lung tissue of PBS, HDM and bHDM-treated mice in order to investigate the effect of boiling on the release of these pro-allergic mediators. HDM exposure resulted in the production of all 4 cytokines in the lung and this was significantly greater than PBS controls (Figure 5.9A – D). Following boiling, the increases in IL-25 (IL-17E) and TSLP were completely abolished (Figure 5.9A and B) and IL-33 production was reduced in response to bHDM (Figure 5.9C). In contrast, levels of CCL20/MIP-3 α remained unaltered by boiling (Figure 5.9D).

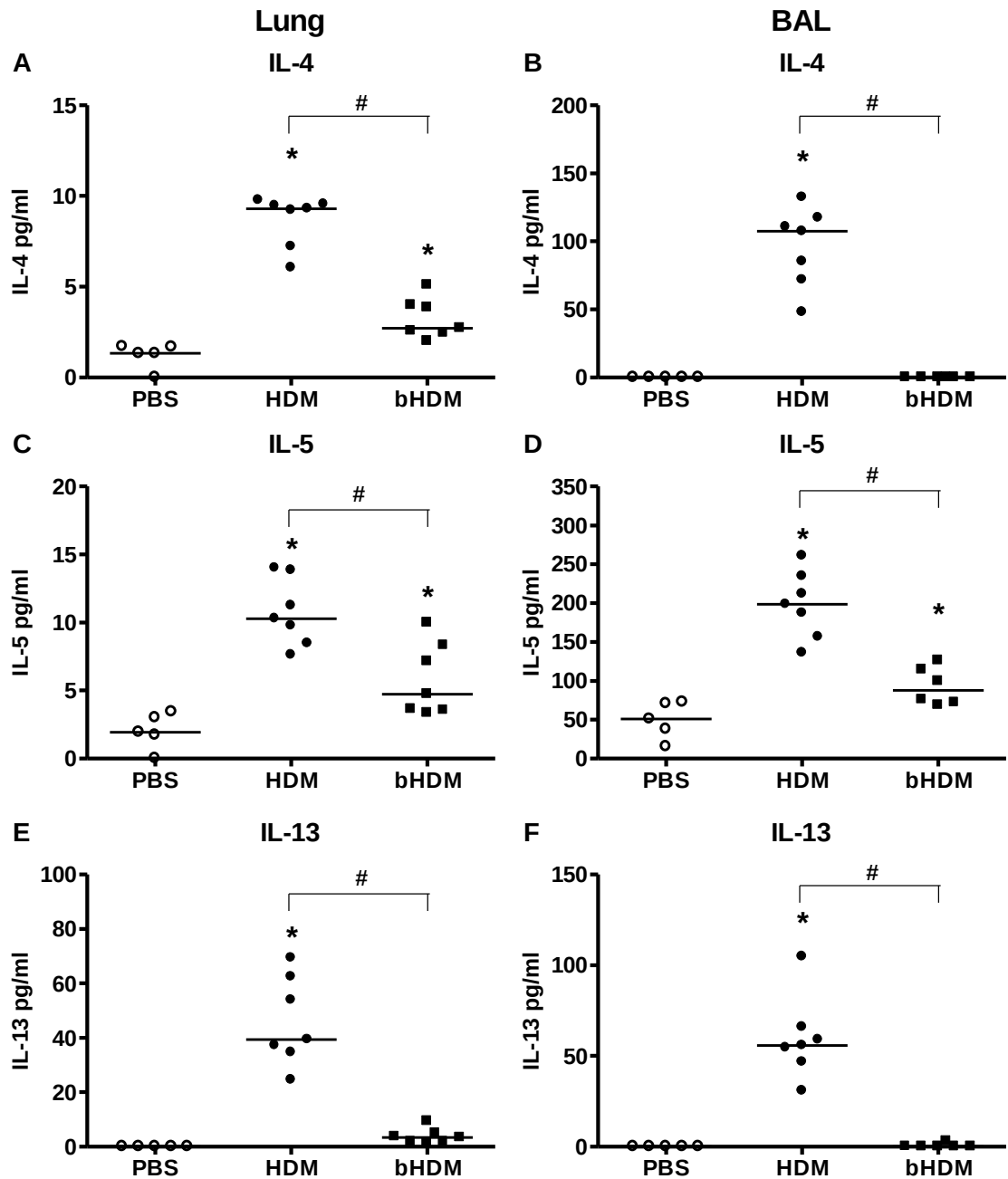


Figure 5.8. Attenuated in Th2 cytokine production in response to bHDM.

IL-4, IL-5 and IL-13 were measured in the lung (A, C and E) and BAL (B, D and F) at 5 weeks 24h after the final i.n. challenge. IL-4 and IL-5 levels were determined by MSD and IL-13 by ELISA. (n=5 PBS and n=6-7 HDM or bHDM). *, $P < 0.05$ (Mann-Whitney U test) HDM or bHDM compared with PBS. #, $P < 0.05$ (Mann-Whitney U test) bHDM compared with HDM. Bars depict the median of the group. Representative of two experiments.

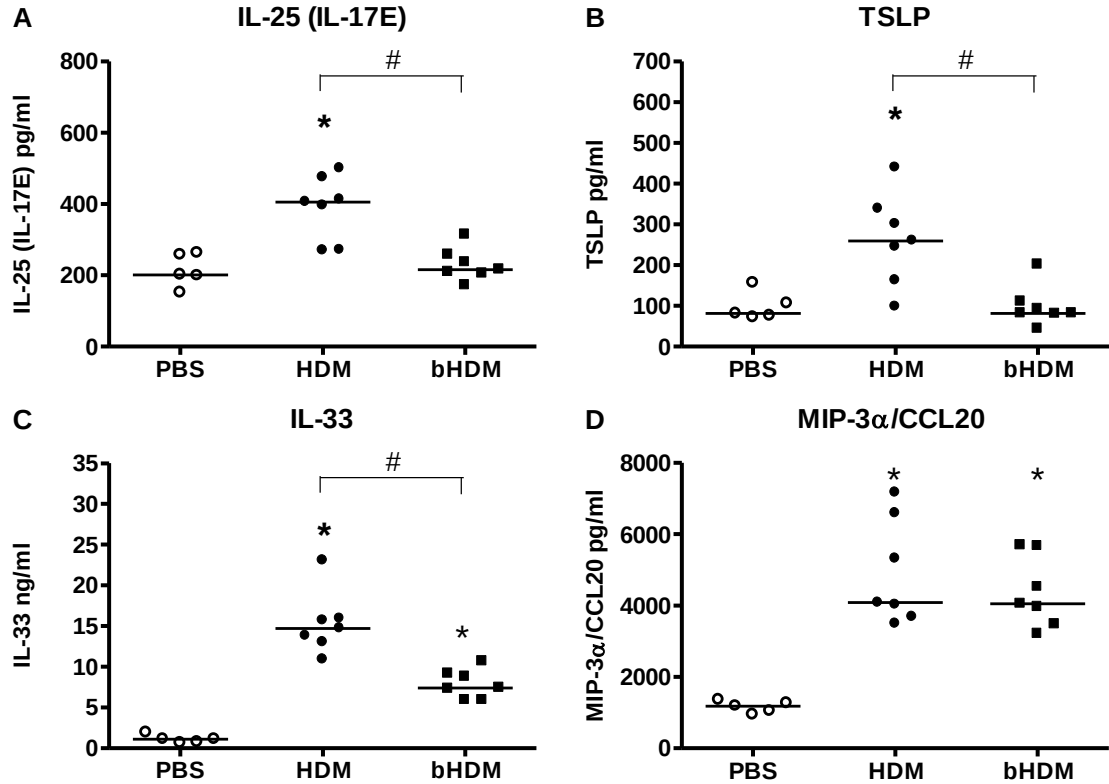


Figure 5.9. Altered pro-allergic cytokine production in response to bHDM.

(A) IL-25 (IL-17E), (B) TSLP, (C) IL-33 and (D) CCL20/MIP-3 α were measured in the lung at 5 weeks 24h after the final i.n. challenge. Mediator levels were determined by ELISA. (n=5 PBS and n=7 HDM or bHDM). *, $P < 0.05$ (Mann-Whitney U test) HDM or bHDM compared with PBS. #, $P < 0.05$ (Mann-Whitney U test) bHDM compared with HDM. Bars depict the median of the group. Representative of two experiments.

5.4.1.8. Absence of allergic immunoglobulin class switching in bHDM-treated mice

Tail bleeds were performed at the end of each week throughout the 5 week time course in order to monitor the onset of immunoglobulin class switching in response to allergen. To determine how boiling impacted the antigenic properties of HDM and the immunoglobulin response to HDM, the levels of total IgM, IgA, IgE, IgG₁ and IgG_{2a} in the serum were quantified. In HDM-treated mice there was a steady increase in the production of total IgM throughout the time course. From week 2 onwards the levels of IgM were significantly greater than those detected in PBS-treated mice. The levels of IgM were reduced following boiling of HDM, but still remained elevated compared to PBS-treated mice (Figure 5.10A). Both HDM and bHDM were observed to induce the production of serum IgA and levels increased throughout the time course. From week 3 onwards, the levels of IgA were significantly greater in HDM and bHDM-treated mice compared to PBS controls. However, there were no differences in IgA production at any time point between HDM and bHDM-treated mice (Figure 5.10B). As expected a steady increase in production of IgE was seen in response to HDM throughout the time course. In contrast, IgE was not detected in the serum of bHDM-treated mice (Figure 5.10C). Levels of IgG₁, the Th2-associated immunoglobulin, in bHDM-treated mice were similar to PBS levels and reduced compared to HDM treatment (Figure 5.10D). Both HDM and bHDM-induced the production of total IgG_{2a}, which is associated with Th1 inflammation, throughout the time course and, from week 3 onwards, these levels were greater than those detected in PBS-treated mice. There were no differences in the IgG_{2a} levels quantified at any time point between HDM and bHDM-treated mice (Figure 5.10E).

In addition to total immunoglobulin levels, HDM-specific IgA, IgE, IgG₁ and IgG_{2a} from the treated mice were also measured throughout the time course. HDM-specific IgA was significantly elevated in both HDM and bHDM-treated mice at weeks 4 and 5 compared to PBS controls. However, HDM-specific IgA production was greater in HDM-treated mice compared to bHDM-treated animals (Figure 5.11A). HDM-specific IgE and IgG₁ were detected at greater levels than PBS controls at weeks 4 and 5. HDM-specific IgE and IgG₁ production was completely ablated following boiling (Figure 5.11B and C). The levels of HDM-specific IgG_{2a} were elevated in

response to both HDM and bHDM and these levels were greater than those of PBS controls at weeks 4 and 5. However, there was no difference in HDM-specific IgG_{2a} production between HDM and bHDM-treated mice (Figure 5.11D).

5.4.1.9. Reduction in mast cell numbers and activation with removal of protease activity

Since IgE production was attenuated in response to bHDM, the numbers of mast cells in the lung tissue and the degree of mast cell degranulation and activation, as quantified by mMCP-1 in the serum, were assessed. Paraffin embedded lung sections were stained using an antibody which recognises mast cell tryptase 7 (MCPT7) by immunohistochemistry. Representative pictures are shown from PBS, HDM and bHDM-treated mice (Figure 5.12A - C). There was a dramatic 15-fold reduction in mast cells recruited in mice which received bHDM. No intraepithelial mast cells were present in PBS controls (Figure 5.12D). mMCP-1 is released following mast cell degranulation and is therefore, an indicator of mast cell activation. mMCP-1 was quantified in the serum and HDM-induced increase in mMCP-1 was abrogated by boiling (Figure 5.12E).

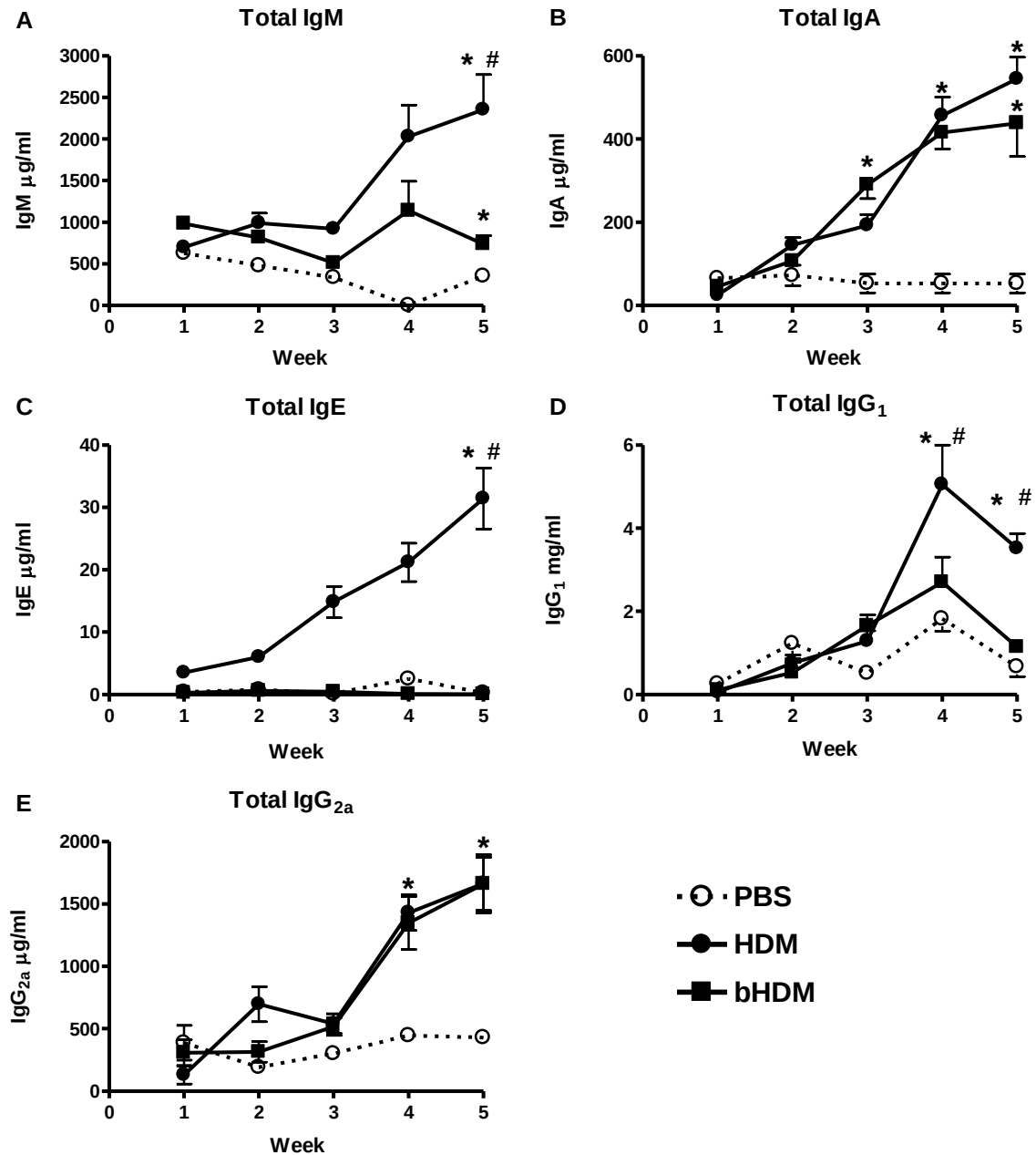


Figure 5.10. Altered immunoglobulin profile in response to bHDM.

Total (A) IgM, (B) IgA, (C) IgE, (D) IgG₁ and (E) IgG_{2a} levels were measured in the serum at weekly time points. All levels were determined by ELISA. (n=5 PBS and n=7 HDM or bHDM at each time point). *, P < 0.05 (Mann-Whitney *U* test) HDM or bHDM compared with PBS. #, P < 0.05 (Mann-Whitney *U* test) bHDM compared with HDM. Data are mean ± SEM. Representative of two experiments.

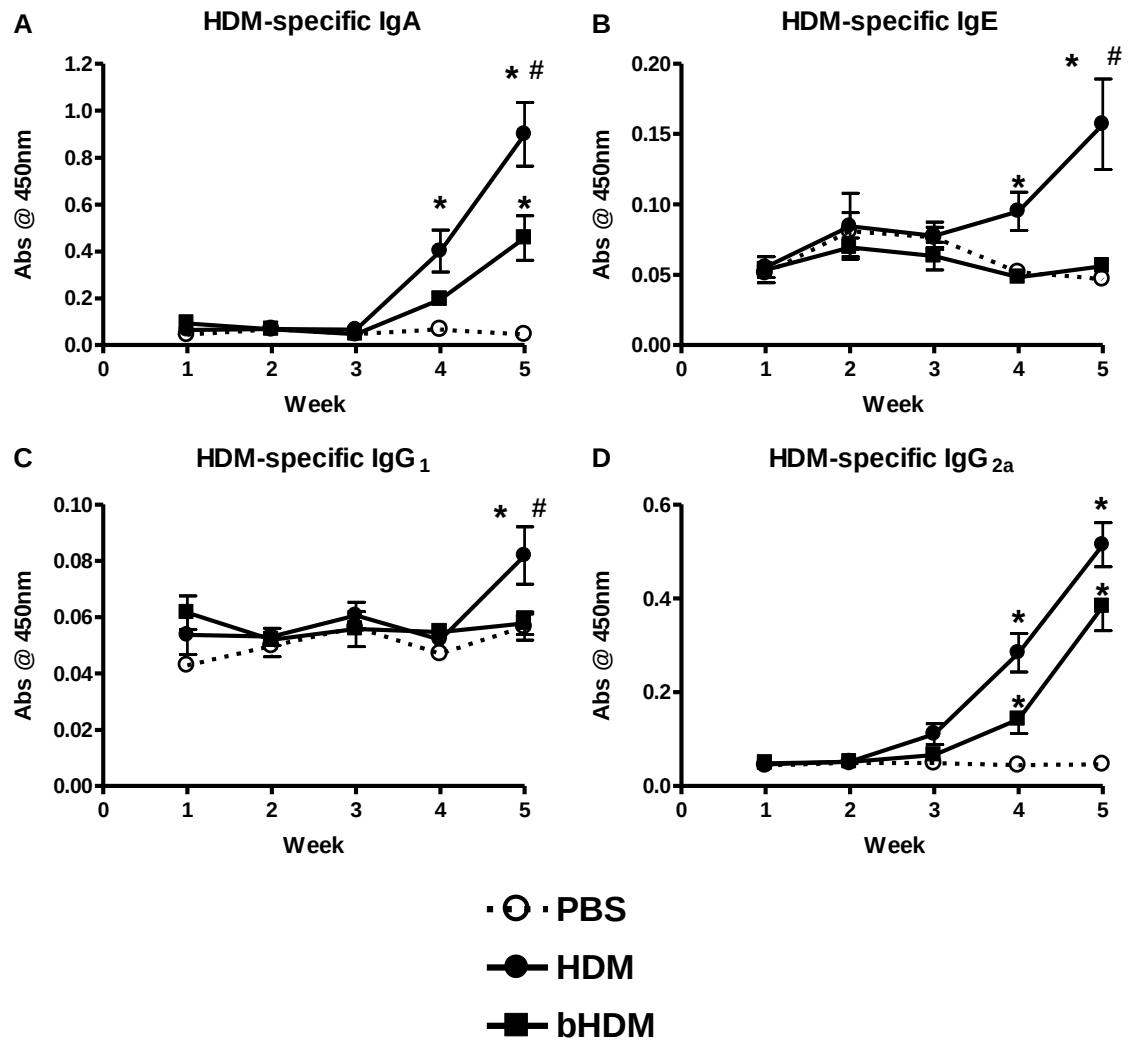


Figure 5.11. Altered HDM-specific immunoglobulin profiles.

HDM-specific (A) IgA, (B) IgE, (C) IgG₁ and (D) IgG_{2a} levels were measured in the serum at weekly time points. All levels were determined by ELISA. (n=5 PBS and n=7 HDM or bHDM at each time point). *, P<0.05 (Mann-Whitney *U* test) HDM or bHDM compared with PBS. #, P<0.05 (Mann-Whitney *U* test) bHDM compared with HDM. Data are mean ± SEM. Representative of two experiments.

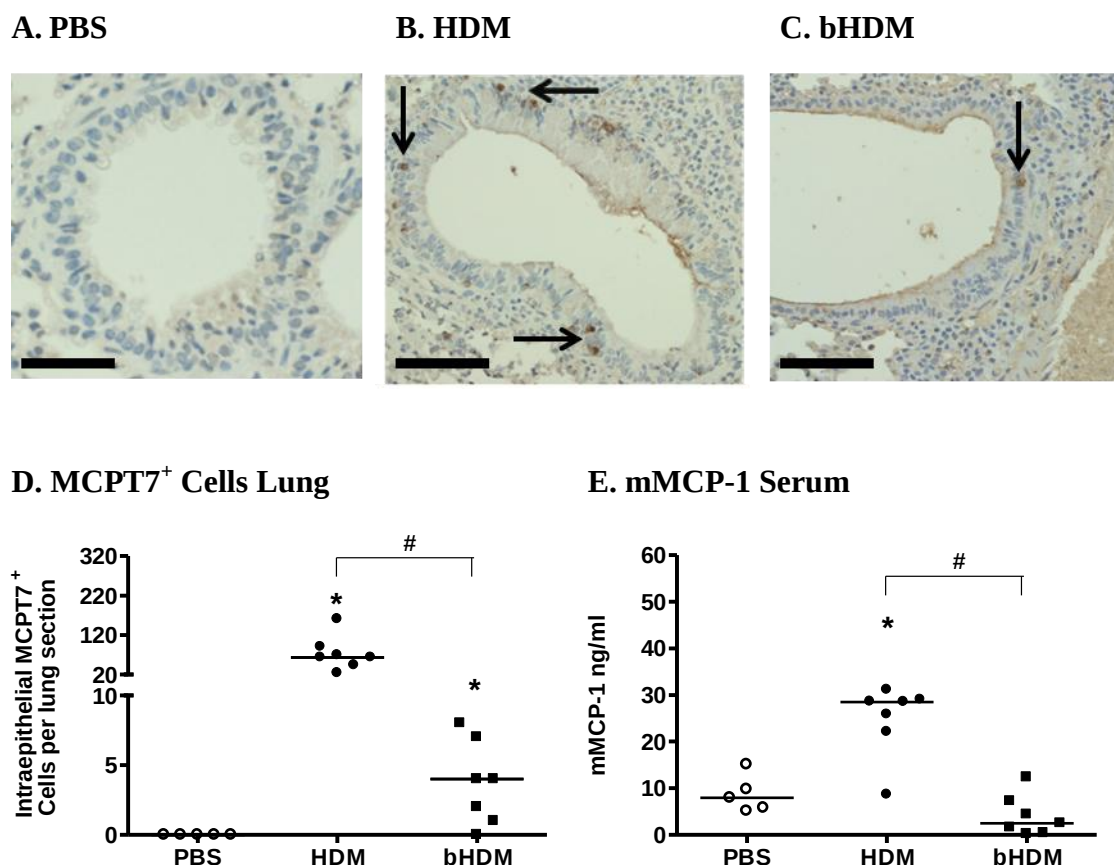


Figure 5.12. Reduction in mast cell numbers and activation in response to bHDM.

Paraffin embedded lung sections from (A) PBS, (B) HDM and (C) bHDM-treated mice were stained with mast cell tryptase 7 (MCPT7) by immunohistochemistry. MCPT7⁺ cells are indicated by the black arrows (original 20X magnification). Scale bar = 50 μ m. (D) The number of MCPT7⁺ cells located in the airway epithelium of PBS, HDM and bHDM-treated mice were quantified per lung section. (E) mMCP-1 was quantified in the serum 24h post final challenge by ELISA. Bars depict the median of the groups. (n=5 PBS and n=7 HDM or bHDM). *, P<0.05 (Mann-Whitney *U* test) HDM or bHDM compared with PBS. #, P<0.05 (Mann-Whitney *U* test) bHDM compared with HDM. Representative of two experiments.

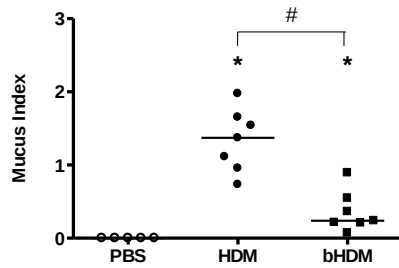
5.4.1.10. Airway remodelling is attenuated in bHDM-treated mice

To assess the impact of boiling of HDM on the development of airway remodelling, the degree of goblet cell hyperplasia and peri-bronchial collagen deposition were assessed.

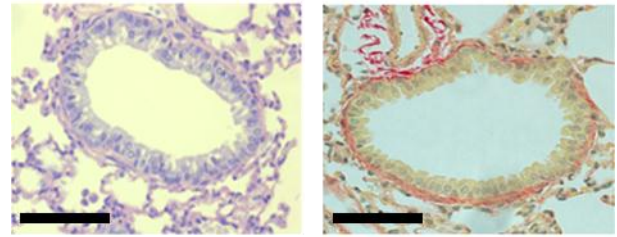
Goblet cell hyperplasia was semi-quantitatively scored on PAS stained lung sections. Both lung sections stained from HDM and bHDM-treated mice stained positive for PAS, however, there was a greater degree of mucus production in the non-boiled HDM-treated group. No positive cells were observed in PBS controls (Figure 5.13A). Representative pictures from PAS stained lung sections are shown (Figure 5.13D – F; left panel).

Total lung collagen was quantified in the lungs from PBS, HDM and bHDM-treated mice using a biochemical Sircol assay. The amount of collagen was significantly greater in HDM-treated mice compared to bHDM-treated mice and PBS controls (Figure 5.13B). Paraffin embedded lung sections from the three groups of mice were stained with sirius red. HDM-treated mice showed a greater mean intensity of peri-bronchial collagen deposition compared to bHDM-treated mice (Figure 5.13C). There was no difference in collagen deposition between PBS and bHDM-treated mice (Figure 5.13B and C). Representative pictures from sirius red stained lung sections are shown (Figure 5.13D – F; right panel). Intense positive red stain is observed around the lung section from HDM-treated mice indicating sub-epithelial collagen deposition, whereas this is not seen in PBS and bHDM-treated mice (Figure 5.13D – F; right panel).

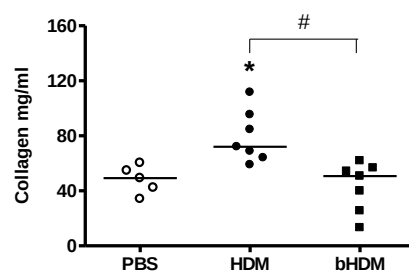
A. Mucus Score



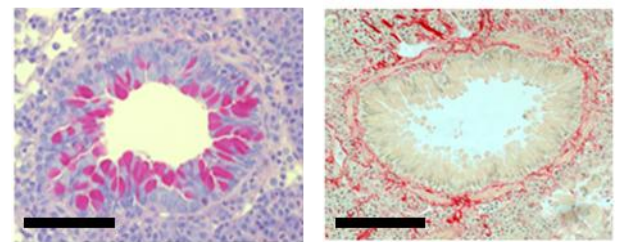
D. PBS



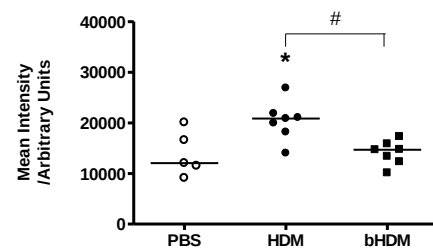
B. Total Lung Collagen



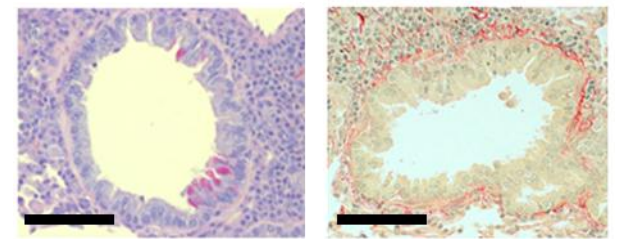
E. HDM



C. Mean Intensity of Peri-bronchial Collagen Deposition



F. bHDM



PAS

Sirius Red

Figure 5.13. Reduction in airway remodelling in bHDM-treated mice.

(A) Semi-quantitative scoring of PAS stained lung sections. (B) Total lung collagen was quantified in the lung tissue 24h post final challenge by a Sircol assay. (C) Quantification of peri-bronchial collagen deposition, expressed as mean intensity. Representative pictures of PAS and sirius red stained lung sections from (D) PBS, (E) HDM and (F) bHDM-treated mice (original 20X magnification). Scale bar = 50 μ m.. (n=5 PBS and n=7 HDM or bHDM). *, $P < 0.05$ (Mann-Whitney U test) HDM or bHDM compared with PBS. #, $P < 0.05$ (Mann-Whitney U test) bHDM compared with HDM. Bars depict the median of the groups. Representative of two experiments.

5.4.1.11. HDM causes the disruption of epithelial tight junctions

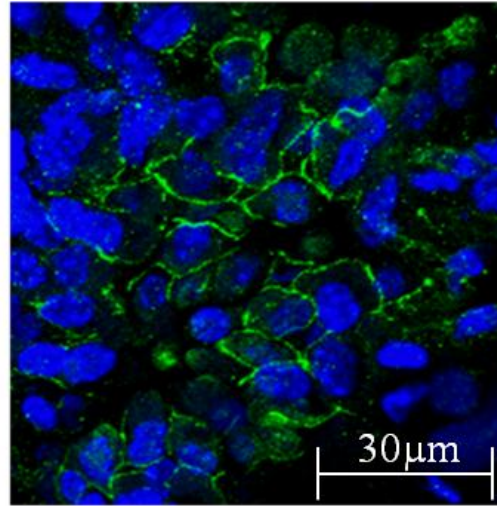
The effect of HDM on the integrity of the tight junctions (TJs) of the airway epithelium was investigated by immunofluorescence. Paraffin embedded lung sections were stained with ZO-1, a key component of TJs (green), and all nuclei stained with DAPI (blue). Sections were sliced through the epithelial cells and viewed as a lateral cross section by confocal microscopy (Figure 5.14A – C). Sections stained from PBS-treated mice showed ZO-1 in the inter-epithelial areas and formed a continuous network around the epithelial cells (Figure 5.14A). In sections from HDM-treated mice, there was interrupted ZO-1 staining suggesting disorganisation of epithelial TJs (Figure 5.14B). In response to bHDM, the ZO-1 was continuous in some areas, but disrupted and disorganised in others (Figure 5.14C), indicating that boiling reduced HDM-mediated TJ disorganisation.

5.4.2. Protocol B: Epithelial permeability in response to inhaled HDM

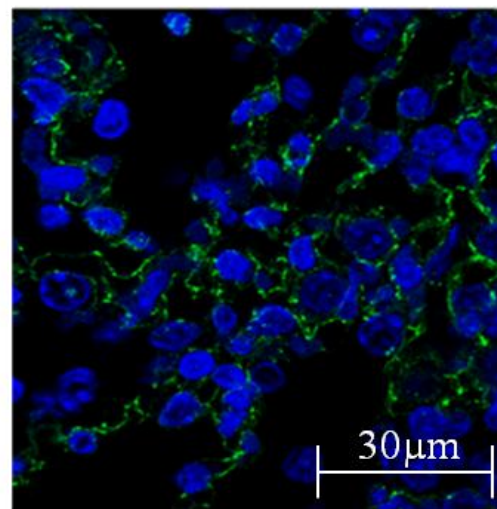
5.4.2.1. HDM exposure increases epithelial permeability

Since HDM was shown to degrade epithelial TJs (Figure 5.14B), it was hypothesised that this could increase epithelial permeability. Thus, to investigate the functional consequences of HDM on airway epithelial permeability *in vivo*, mice were administered either 25 µl PBS, 25 µg HDM or 25 µg bHDM, 5 times a week, for 1 week. Following this HDM exposure, all mice were given FITC-Dextran i.n. FITC-Dextran resides in the airway lumen, as it is of sufficient size not to cross the epithelial barrier (4 kDa). Therefore, the presence of FITC-Dextran in the lung homogenate of HDM-treated mice indicates an increase in epithelial permeability. Increased airway epithelial permeability was observed in HDM-treated mice, whereas this was not observed in mice that had received the boiled allergen (Figure 5.15).

A. PBS



B. HDM



C. bHDM

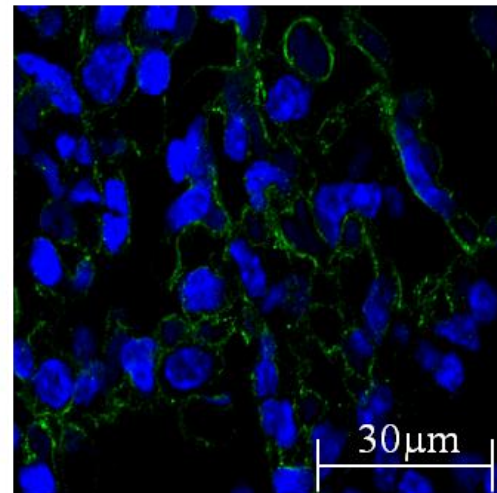


Figure 5.14. Lack of epithelial tight junction organisation in HDM-treated mice. Paraffin embedded lung sections from (A) PBS, (B) HDM and (C) bHDM-treated mice were stained with ZO-1 (green) and the epithelial cell marker DAPI (blue) by immunofluorescence (original 40X magnification). Representative of two experiments.

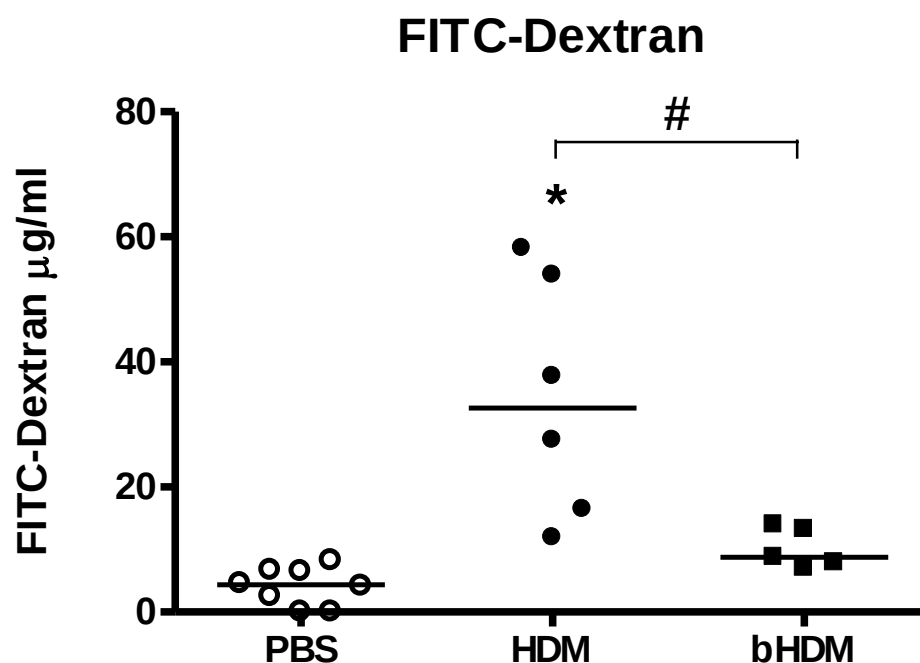


Figure 5.15. Increased epithelial permeability in HDM-treated mice.

FITC-Dextran was quantified in the lung homogenate of PBS, HDM and bHDM-treated mice after 1 week of exposure. FITC-Dextran was quantified by fluorescence read out, as described in the Materials and Methods. (n=8 PBS, n=6 HDM and n=5 bHDM). *, $P < 0.05$ (Mann-Whitney U test) HDM or bHDM compared with PBS. #, $P < 0.05$ (Mann-Whitney U test) bHDM compared with HDM. Bars depict median of the groups.

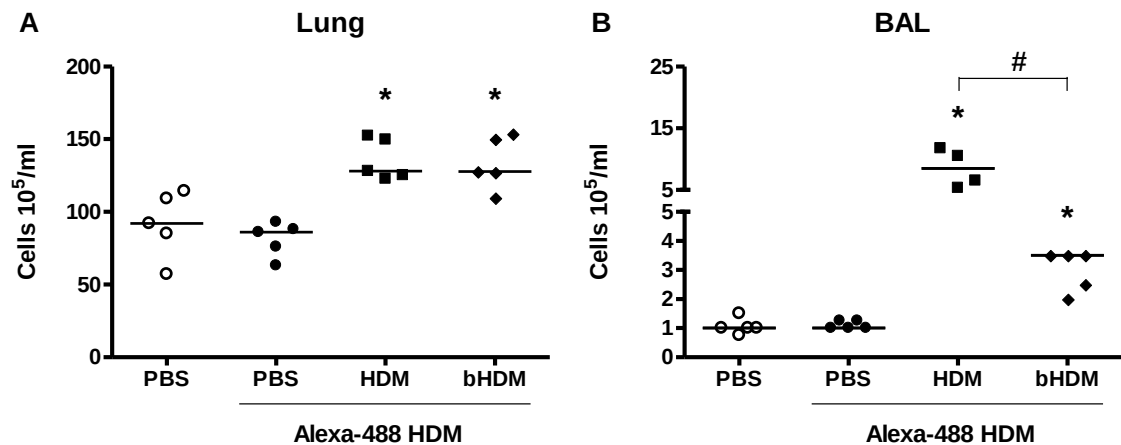
5.4.3. Protocol C: Impact of boiling on the recognition of HDM by APCs

5.4.3.1. Both HDM and bHDM increase total cell numbers in the lung and BAL

In order to determine whether boiling influenced how HDM was recognised by APCs, mice were administered either 25 μ l PBS, 25 μ g HDM or 25 μ g bHDM for 1 week, followed by an i.n. dose of 25 μ g Alexa-488-labelled HDM. A group of PBS-treated mice did not receive Alexa-488-labelled HDM, to test whether there was any intrinsic effect of administering an Alexa-conjugated allergen.

The administration of a single dose of Alexa-488-labelled HDM did not cause an increase in the total number of cells in either the lung or BAL, revealing that there was no inherent effect of administering the Alexa-conjugated allergen alone (Figure 5.16A and B). Following HDM and bHDM pre-treatment, mice showed higher numbers of total cells in the lung (Figure 5.16A). However, the number of total cells recovered from the BAL was greater in HDM exposed mice than bHDM-treated mice (Figure 5.16B).

Microscopic examination of paraffin embedded lung tissue sections revealed distribution of Alexa-488-labelled HDM (green) in the lung parenchyma, with epithelial cell nuclei stained with DAPI (blue) (Figure 5.16C).



C. Distribution of Alexa-488-labelled HDM in the lung parenchyma

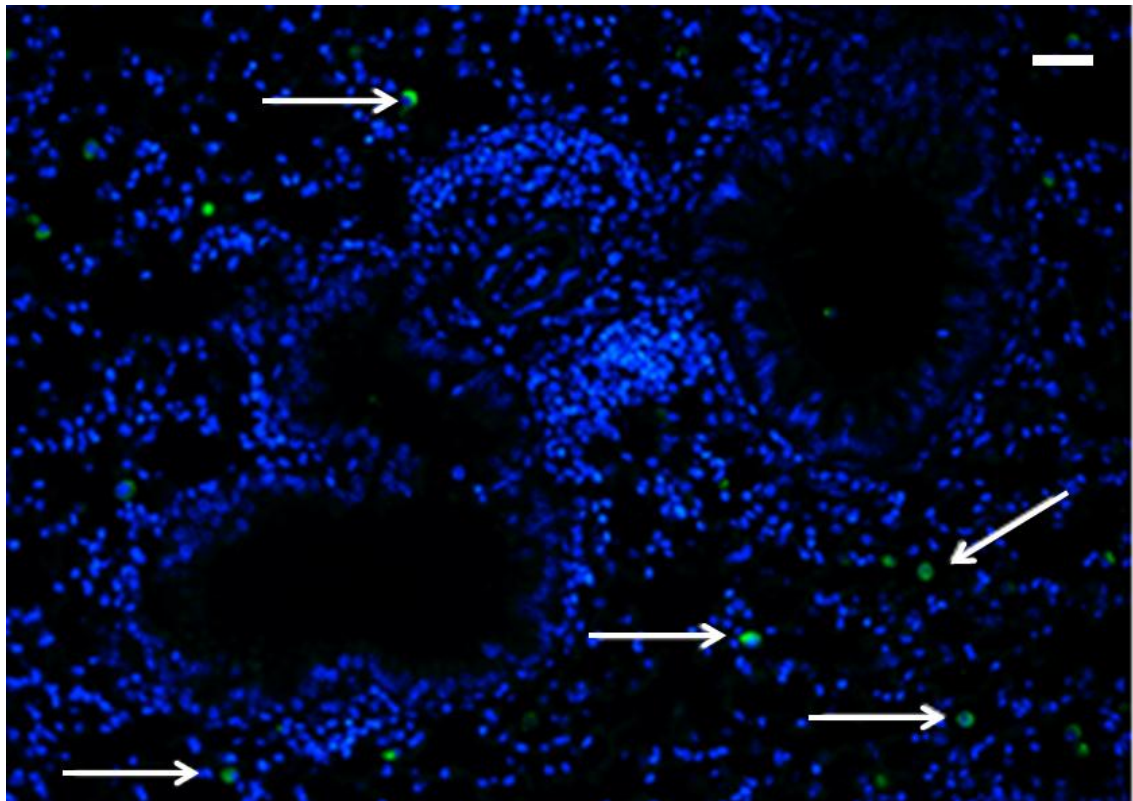


Figure 5.16. Elevated total cells in HDM-treated mice and distribution of Alexa-488-labelled HDM in the lung parenchyma.

Total cells were quantified in the (A) lung and (B) BAL 24h post final challenge. (n=4-5 for each treatment group. Total cell counts were determined using a haemocytometer with white blood cell counting fluid, as described in the Materials and Methods. *, $P < 0.05$ (Mann-Whitney U test) HDM or bHDM compared with PBS-Alexa-488. #, $P < 0.05$ (Mann-Whitney U test) bHDM compared with HDM. Bars depict median of the groups. (C) Paraffin embedded lung section stained with the epithelial cell marker DAPI (blue). The Alexa-488-labelled HDM is green and indicated with the white arrows (original 20X magnification). Scale bar = 50 μm . Representative of two experiments.

5.4.3.2. Inhaled HDM is recognised by APCs in the lung and allergen loaded mDCs migrate to the lymph nodes

mDCs, pDCs and basophils were quantified by flow cytometry in the lung tissue and lymph nodes (LNs) in order to assess which cells localised with the labelled HDM in the lung and which cells then subsequently migrated to the lung draining LNs. Representative flow cytometry plots are shown, with mDCs classed as Gr-1⁻CD11c⁺CD11b⁺MHCII⁺ (Figure 5.17A – B) and pDCs classified as Gr-1^{int}CD11c^{int}CD11b⁻120G8⁺ (Figure 5.18A – B). Basophils could not be detected in either the lung or the LNs in response to HDM administration (plots and data not shown).

Both HDM and bHDM-treated mice showed greater numbers of mDCs in the lung when compared to PBS controls (Figure 5.19A). Boiling did not alter either the number or percentage of mDCs that localised with the Alexa-488 conjugated allergen (Figure 5.19B and C). Neither HDM nor bHDM pre-treatment altered the number of pDCs residing in the lung compared to PBS controls (Figure 5.19D). Far fewer pDCs localised with the labelled antigen as compared to the mDCs and the number of pDCs that localised with Alexa-488-labelled HDM was not altered by boiling (Figure 5.19E and F).

Following allergen challenge, only mDCs were observed in the LNs and the number and percentage of these cells that localised with the Alexa-488-HDM was not altered by boiling of HDM (Figure 5.19G – I).

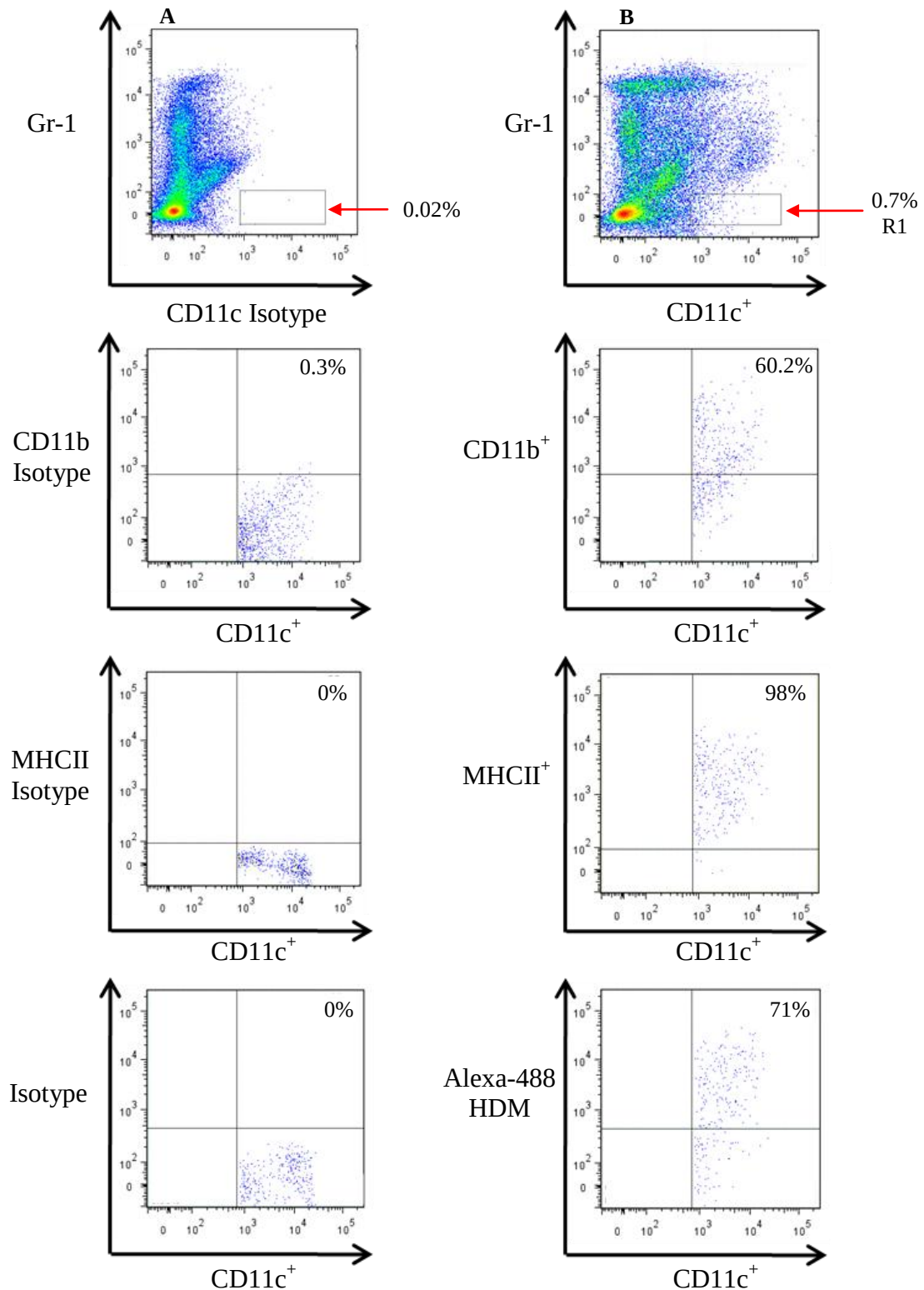


Figure 5.17. Representative plots for mDCs.

After digestion of the lungs, cells were stained for Gr-1, CD11c, CD11b and MHCII expression and gated on live cells. Tissue was stained with either (A) isotype control antibodies or (B) antibodies of interest. From R1 (Gr-1⁻CD11c⁺), mDCs were identified by expression of both CD11b and MHCII and were classed as Gr-1⁻CD11c⁺CD11b⁺MHCII⁺. mDCs that localised with Alexa-488-labelled HDM were quantified by flow cytometry.

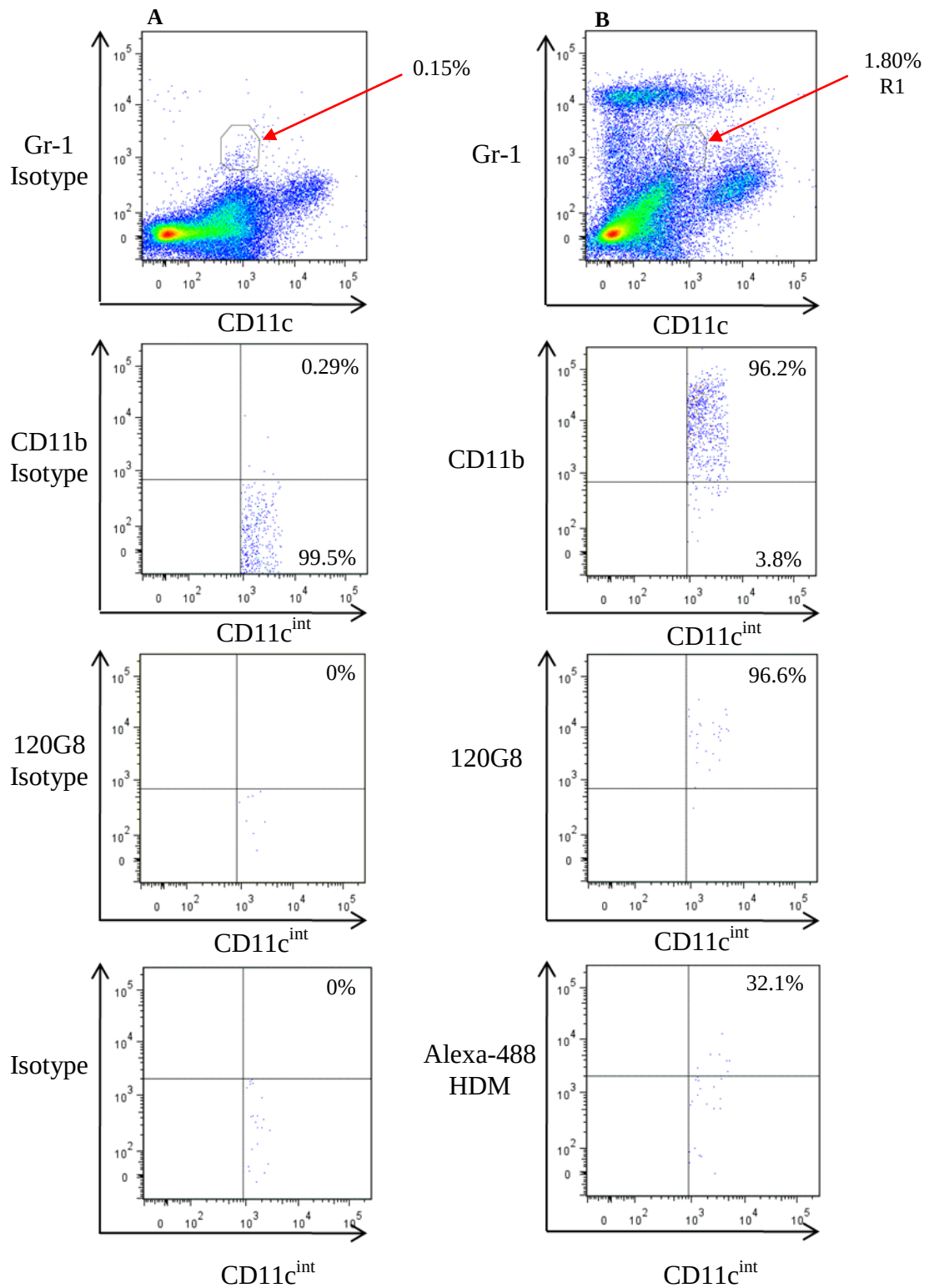
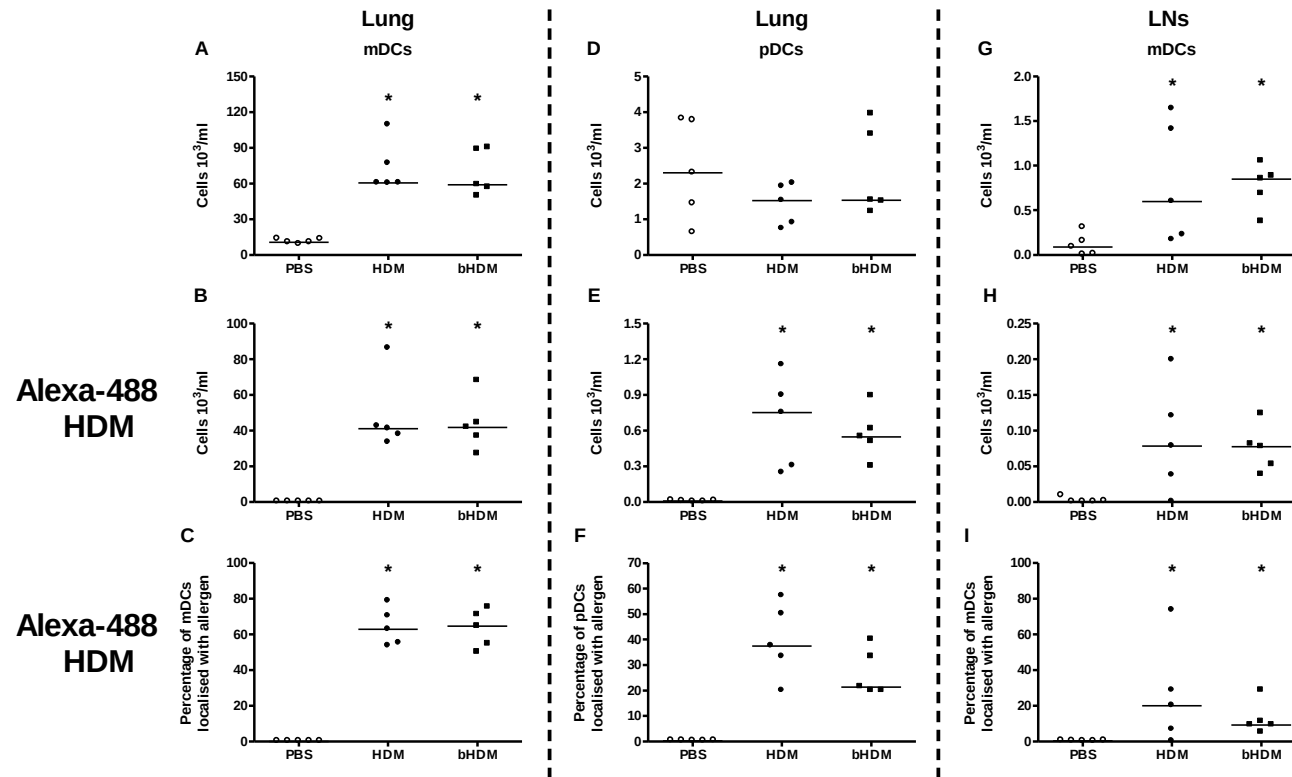


Figure 5.18. Representative plots for pDCs.

After digestion of the lungs, cells were stained for Gr-1, CD11c, CD11b and 120G8 expression and gated on live cells. Tissue was stained with either (A) isotype control antibodies or (B) antibodies of interest. From R1 (Gr-1^{int}CD11c^{int}) pDCs were identified by the absence of expression of CD11b and the presence of 120G8 expression. pDCs were classed as Gr-1^{int}CD11c^{int}CD11b⁻120G8⁺. pDCs that were localised with the Alexa-488-labelled HDM were quantified by flow cytometry.



5.5. Discussion

The experiments in Chapter 5 investigated how boiling influenced the immune response to inhaled HDM *in vivo*. Through these experiments it was observed that boiling reduced 80% of the enzymatic activity within the extract. Continuous administration of the boiled allergen led to a significant reduction in the parameters of pulmonary inflammation, such as the number of total cells and eosinophils recovered from the lung and BAL and pulmonary Th2 cytokine production, compared to mice that received unaltered allergen. Moreover, bHDM-treated mice did not develop AHR and had attenuated features of airway remodelling despite 5 weeks of exposure. Boiling of HDM reduced the impact of HDM on the organisation of the airway epithelium and the production of innate cytokines, since less ZO-1 degradation and abrogated IL-25 (IL-17E) and TSLP production were observed in response to bHDM. Heat treatment of HDM also eliminated the IgE-mast cell and IgG₁ responses *in vivo*, whilst the IgA and IgG_{2a} responses remained intact. However, boiling of HDM did not alter how the inhaled allergen was recognised by APCs residing in the airway, suggesting that boiling does not disrupt how the allergen is initially identified by DCs in the lung.

The results from this Chapter show a striking impact of boiling on the immune response to HDM *in vivo*. The immunogenicity of HDM, although markedly reduced, was not eliminated following boiling since eosinophils and Th2 cells were quantified in the lung and BAL albeit at reduced levels compared to the unaltered allergen. However, a reduction in IgM production in response to bHDM showed that the primary humoral response from B cells, and thus how HDM was recognised as an allergen by B cells, was altered following heat treatment. Further analysis of immunoglobulin class switching from IgM revealed an insight into the mechanisms by which HDM generates Th2 responses, since boiling of HDM did not alter the production of IgA and IgG_{2a}, whereas circulating IgE and IgG₁ levels were ablated following heat treatment. This can be explained by dissection of the precise mechanisms behind IgM class switching to IgE, IgG₁, IgG_{2a} and IgA and how these relate to the various features of the HDM allergen.

T cell-dependent signalling is required for IgE and IgG₁ class switching, whereas non-T cell-dependent and non-HDM-specific signals can mediate IgG_{2a} and IgA class switching. IFN- γ is the only cytokine that promotes IgM class switching to IgG_{2a} (Bossie and Vitetta, 1991) and NK cells have been suggested to play an important role in IgG_{2a} regulation, since activated NK cells secrete large quantities of IFN- γ (Satoskar et al., 1999). NK cells are activated by DCs (Ferlazzo and Munz, 2004), which can recognise antigenic epitopes within the HDM extract that are resistant to boiling, as bHDM was still localised with mDCs in the lung. Thus, systemic activation of innate DCs and NK cells by signals that are resistant to boiling likely explain the IgG_{2a} production. Class switching from IgM to IgA can be induced by either T cell-dependent or T cell-independent mechanisms (Fagarasan and Honjo, 2000). T-cell-independent antigens that activate B cells include LPS, signalling via TLRs (Peng, 2005), and polysaccharides, which activate B cells through the BCR (Mond et al., 1995). Both LPS and the polysaccharide β -glucan are detected in HDM extracts (Douwes et al., 2000) and LPS-induced TNF- α production from macrophages has also been shown to be heat-resistant (Asea et al., 2000; Dybdahl et al., 2002; Gao and Tsan, 2003), suggesting that LPS within the HDM extract is not denatured by boiling. T-cell-independent antigens can also provide additional B-cell-stimulating signals through DCs (Batista et al., 2001; Balazs et al., 2002; Litinskiy et al., 2002; Schneider, 2005; Qi et al., 2006) and epithelial cells (Kett et al., 1986; Hebel et al., 2006; Kato et al., 2006; Xu et al., 2007; He et al., 2007) via ligation of PRRs.

However, in contrast, IgE and IgG₁ class switching from IgM is induced by either IL-4 or IL-13 (Xu and Stavnezer, 1992; Delphin and Stavnezer, 1995). Indeed, there was an absence of IL-4 and IL-13 production in the lung and BAL in response to bHDM. Once class switching occurs, ligation of CD86 on the B cell surface results in increased production of both IgE and IgG₁ (Kasprowicz et al., 2000). In comparison, IFN- γ -driven IgG_{2a} class switching is not subject to up-regulation following signalling through CD86 (Bossie and Vitetta, 1991; Kasprowicz et al., 2000). Together with the serum data this suggests that increased total IgE and IgG₁ levels are associated with heat sensitive epitopes within the HDM extract, whereas IgG_{2a} and IgA levels reflect broader patterns of B cell activation that can be triggered by non-HDM-specific ligands, such as LPS (Lewkowich et al., 2004). Indeed, the absence of IgE production in response to bHDM may be due in part to an absence of CD23

cleavage by *Der p* 1 (Hewitt et al., 1995;Schulz et al., 1995), as *Der p* 1 is inactivated by boiling (Lombardero et al., 1990). Concomitant with an absence of IgE and IL-4, mast cell activation was ablated in response to bHDM. The lack of mast cell activation and absence of IL-13 in response to bHDM likely contribute to the absence of development of AHR in these mice (Wills-Karp, 2004;Yu et al., 2006).

Boiling of HDM reduced allergen-induced inflammatory chemokine production in the lung tissue. Thus, concomitant with decreased eotaxin-1/CCL11, KC/mCXCL1 and TARC/CCL17 production, fewer numbers of eosinophils, neutrophils and Th2 cells were present in the airways. This may occur as a result of boiling preventing *Der p* 1, 3, 5 and 9-induced release of these chemokines from the airway epithelium. The attenuated pulmonary Th2 responses can also be explained by the ablated production of TSLP and IL-25 (IL-17E), as well as decreased IL-33 production in response to bHDM, since all three cytokines promote Th2 responses (Al-Shami et al., 2005;Angkasekwinai et al., 2007;Kondo et al., 2008). However, it is important to recognise that the remaining IL-33 and unaltered CCL20/MIP-3 α production in response to bHDM would still be able to exert a degree of Th2 polarisation and recruitment of immature DCs. Since CCL20/MIP-3 α secretion is unaltered by boiling, this also suggests that the β -glucan structures within the HDM, responsible for CCL20/MIP-3 α production (Nathan et al., 2009), are not denatured by boiling.

Staining of ZO-1, a key component of epithelial TJs, revealed that disruption of epithelial TJ organisation could still occur in response to bHDM and that the protease activity of *Der p* 1, 3, 6 and 9 are not the sole contributors to this TJ disruption. In view of the fact that eosinophils and neutrophils were present in the lung in response to bHDM, it is likely that these cells are responsible for the damage to the epithelial TJs, as both eosinophils and neutrophils can cause tissue damage via the release of stored mediators (Kato et al., 1991;Hogan et al., 2008). The production of eotaxin-1/CCL11, IL-5, IL-33 and KC/mCXCL1, although reduced, was not ablated in bHDM-treated mice, therefore, allowing for the recruitment of a small number of eosinophils and neutrophils to the lung. However, only HDM-induced epithelial TJ disruption resulted in the functional consequence of increased epithelial permeability *in vivo*. This likely contributes to the increased Th2 inflammation in response to HDM, as the allergen would have greater access to cells residing in the airway

mucosa, including DCs and basophils. Not only may this result in more APCs recognising the allergen, but *Der p 1* can induce IL-4 release from both DCs (Hammad et al., 2003) and basophils (Phillips et al., 2003), which may further amplify Th2 and IgE responses.

In view of the fact that boiling HDM dramatically reduced in the *in vivo* response to the allergen, the effects of boiling on the allergenic epitopes within the extract identified by APCs were investigated. Heat treatment of 100°C for 10 minutes, in addition to denaturing enzymes, may also alter the tertiary and quaternary structures of other allergenic proteins. Thus, the reduction in the immune response to HDM with boiling cannot be solely attributed to a lack of protease activity. In agreement with others, mDCs were identified as the primary APC subset which recognised inhaled HDM in the airway (Hammad et al., 2010). Interestingly boiling did not alter either the number of mDCs recruited to the lung, the number of mDCs that localised with the Alexa-conjugated allergen or the number of mDCs present in the LNs. Despite not altering how HDM was identified by mDCs, other aspects of the antigen presentation process may be altered. This could include how HDM allergens are processed and presented to naive T cells in the LNs, and whether boiling influences DC maturation or co-stimulatory molecule expression. Heat treatment of HDM will also prevent *Der p 1*-mediated Th2 cytokine release from DCs, which has been shown *in vitro* (Hammad et al., 2003). Therefore, in order to proceed from the experiments in this Chapter, inflammatory DCs should be stimulated with either HDM or bHDM *ex vivo* and the cytokine production and the degree of DC maturation assessed.

The observations from this Chapter raise further important questions regarding the precise interactions between HDM and the airway epithelium and how immune responses are generated. This includes determining the contribution of PAR2 to the *in vivo* response to HDM, since a vital role has been proposed *in vitro* (Asokanathan et al., 2002a; Winter et al., 2006) and it is unclear whether HDM-induced epithelial TJ disorganisation and increased epithelial permeability occur as a result of PAR2 cleavage. Since ZO-1 is located in the intracellular regions of airway epithelial cells, it is unlikely to be directly cleaved by extracellular HDM proteases. Alterations to ZO-1 are therefore likely caused by either PAR2-mediated signalling cascades or as a

consequence of direct TJ disruption by cleavage of extracellular claudins and occludin by HDM proteases. If indeed PAR2 is required for TJ degradation, this suggests that therapeutic targeting of this receptor would be beneficial to asthmatics in preventing epithelial damage and injury, especially since PAR2 expression has been shown to be elevated on respiratory epithelial cells from asthmatics (Knight et al., 2001).

Another important question that arises from the results in this Chapter, together with the current literature, is whether protease active epitopes within HDM enhance the allergenicity of other allergens. Therefore, in addition to epithelial TJ disruption and inducing pro-inflammatory cytokine and chemokine production, protease active allergens may promote IgE responses to other allergens. *Der p 1* has been shown to enhance the IgE response to OVA (Gough et al., 2001) and collaboration between *Der p 1* and other HDM allergens has also been illustrated. *Der p 1* has been implicated in promoting the activation of *Der p 3* (Dumez et al., 2008) and protease-active *Der p 1* increases *Der p 1*-specific IgE compared to protease-inactive *Der p 1* (Gough et al., 1999). Therefore, it would be important in understanding HDM functionality to investigate the effects of *Der p 1*, 3, 6 and 9 on the sensitisation to *Der p 2* to further elucidate these intricate and complex interactions. For example, *Der p 1*, 3, 6 and 9 cleavage of epithelial TJs could enhance *Der p 2* activation on TLR4 on DCs and *Der p 1* cleavage of DCs by facilitating greater epithelial permeability and access to these cells residing in the airway mucosa. It has also been shown that in response to *Der p 1*, total IgE and *Der p 1*-specific IgE production were reduced in mice treated with inactive *Der p 1* (Gough et al., 2003; Kikuchi et al., 2006). Thus, these findings along with the results obtained here, suggest that protease active *Der p 1* is vital for the induction of IgE responses to *Der p 1* and HDM.

Protease activity is not unique to HDM and the mechanisms regarding HDM sensitisation and requirement of proteases likely translate to other allergens, since other allergens associated with allergy and atopic asthma possess protease activity. Removal of proteases from *Aspergillus fumigatus* (Kheradmand et al., 2002), German cockroach frass (Page et al., 2008), American cockroach *Per a 10* antigen (Sudha et al., 2009), *Epi p 1* antigen from the fungus *Epicoccum purpurascens* (Kukreja et al., 2008) or *Cur 11* antigen from the mold *Curvularia lunata* (Tripathi et al., 2009) has

been shown to decrease airway inflammation and AHR in mice. Co-administration of either *Der p 1* or active proteases from *Aspergillus fumigatus* with OVA resulted in enhanced IgE production (Ghaemmaghami et al., 2001; Kheradmand et al., 2002). However, it is important to recognise that each allergen has a distinct nature and subtle differences may still occur. For example, different allergen-specific memory T cell responses have been observed following exposure to either perennial allergens, such as HDM (*Der p 1*), or seasonal allergens, such as birch pollen (*Bet v 1*), therefore suggesting that memory CD4⁺ responses can vary between protease-active allergens (Wambre et al., 2011). Conversely, it would also be necessary to determine exactly how non-enzymatic allergens, such as the major timothy grass pollen allergen *Phl p 1* (Röschmann et al., 2009), promote immune responses and whether this can be applied to HDM.

From Chapter 5, it can be concluded that boiling substantially reduces the intrinsic protease activity, as well as allergenicity of HDM, and this has a profound effect on reducing the development of disease pathogenesis *in vivo*. Thus, the results obtained support a vital role for proteases in the induction of Th2 responses to HDM and enhance our understanding regarding the mechanisms by which HDM elicits an immune response *in vivo*. It was revealing that even with boiling, the breakdown of NBP-VANA was not completely ablated. The likely causes of this are that heat stable proteases are present within the HDM extract and that these are derived from bacterial contaminants within the HDM preparation. HDM extracts have been found to contain various bacterial species including *Bartonella* (*henselae*, *quintana*, *vinsonii*, and *grahamii*), *Escherichia coli*, α -proteobacteria, *Pseudomonas* species, and *Acinetobacter* species (Valerio et al., 2005). Both *Pseudomonas* (Jackman et al., 1983; Stepaniak and Fox., 1985) and α -proteobacteria (Durham et al., 1987) can produce heat stable proteases which likely explain why boiling does not eliminate NBP-VANA breakdown. In addition to this, there may be as yet other unidentified proteases within the HDM extract that are heat stable and account for the residual protease activity, such as heat stable aspartic proteases (Rao et al., 1998) and MMPs (Bracho and Haard, 1995). It is thus plausible that any heat-resistant proteases within the HDM could contribute to ZO-1 degradation in bHDM-treated mice. Prior to carrying out the experiments in this Chapter, dissecting the specific roles of cysteine and serine proteases in the immune response to HDM had been an avenue of interest.

However, using the available serine and cysteine protease inhibitors, AEBSF and E-64 respectively, only a 40% reduction in the protease activity of HDM was observed. Therefore, this was not pursued *in vivo* as boiling of the allergen had a greater impact on reducing the protease activity of HDM.

Better molecular understanding of the activities of protease allergens on the airway epithelium could lead to the development of new therapeutic strategies against allergic asthma. Indeed, the results from this Chapter suggest that targeting proteases within HDM would be of clinical benefit, since the severity of disease pathogenesis was dramatically reduced with boiling concomitant with an 80% reduction in enzymatic activity. Subcutaneous injection of the serine protease inhibitor nafamostat mesilate during sensitisation to HDM has been shown to attenuate the development of allergic inflammation and AHR (Chen et al., 2006). However, it would be impossible to treat HDM with protease inhibitors prior to human exposure and unsafe to treat humans with protease inhibitors, as endogenous proteases are vital to microbial defence (Reece et al., 2010). Serine proteases make up an important component of the host defence against *Mycobacterium tuberculosis* infection (Reece et al., 2010) and a delicate protease-anti-protease balance is required for lung mucosal homeostasis (Greene and McElvaney, 2009). This therefore leads to the idea that protecting or treating an already injured airway epithelium may be a potential therapeutic avenue, as epithelial injury and damage are routinely seen in asthmatics (Bertorelli et al., 1998; Bucchieri et al., 2002; Shebani et al., 2005; Shahana et al., 2006). Not only has epithelial damage associated with asthma been replicated here *in vivo* following exposure to HDM, potential new treatment strategies can also be tested in this model. Thus through the experiments undertaken in this Chapter it can be concluded that protease activity of the HDM allergen plays a critical role in causing epithelial damage and specifically promoting Th2 responses.

Chapter 6 - The role of TGF- β in HDM-induced allergic airways disease

6.1. Introduction

The transforming growth factor- β (TGF- β) superfamily consists of approximately 30 mammalian cytokines, including three TGF- β isoforms (TGF- β 1, - β 2 and - β 3), activins and bone morphogenetic proteins (BMPs) (Attisano and Wrana, 2002). TGF- β 1 is the most prominent and widely studied family member and is produced by both structural cells, including epithelial cells and fibroblasts, and immune cells, such as eosinophils and mast cells (Sporn and Roberts, 1990).

TGF- β is encoded as a biologically large precursor peptide (pre-pro-TGF- β) (Rifkin et al., 1993). Endopeptidases catalyse the cleavage of these precursor molecules, which then yield mature TGF- β molecules (Dubois et al., 1995). Mature TGF- β is secreted as a heterodimeric complex with latency-associated protein (LAP) (Saharinen et al., 1999) and latent TGF- β is activated *in vivo* via multiple mechanisms that are not fully understood (Khalil, 1999). TGF- β signalling is facilitated through the binding of two specific subfamilies of cell transmembrane receptors, type I (T β R-I) and type II receptors (T β R-II) (Piek et al., 1999). Both receptor types have intrinsic serine/threonine kinase activity and both are essential for transducing TGF- β signals (Piek et al., 1999).

TGF- β induces its cellular responses through the formation of receptor complexes involving interaction between T β R-I and T β R-II dimers (Yamashita et al., 1994). The activated T β R-I directly phosphorylates specific intracellular target proteins called Smads, that transduce extracellular signals from TGF- β to the nucleus where they activate downstream TGF- β gene transcription (Heldin et al., 1997). There are three families of Smads: receptor-activated (or R-) Smads (Smads 1, 2, 3, 5 and 8), common-partner (or Co-) Smads (Smads 4a and 4b) and inhibitory (or I-) Smads (Smads 6 and 7) (Abdollah et al., 1997). Smads 2 and 3 are activated by TGF- β (Piek et al., 1999). Co-Smads interacts with R-Smads to participate in signalling (Shi et al., 1997) and I-Smads block the activation of R-Smads and Co-Smads (Itoh et al., 2001). A summary of the TGF- β signalling pathway is shown in Figure 6.1 (Pinzani and Marra, 2001).

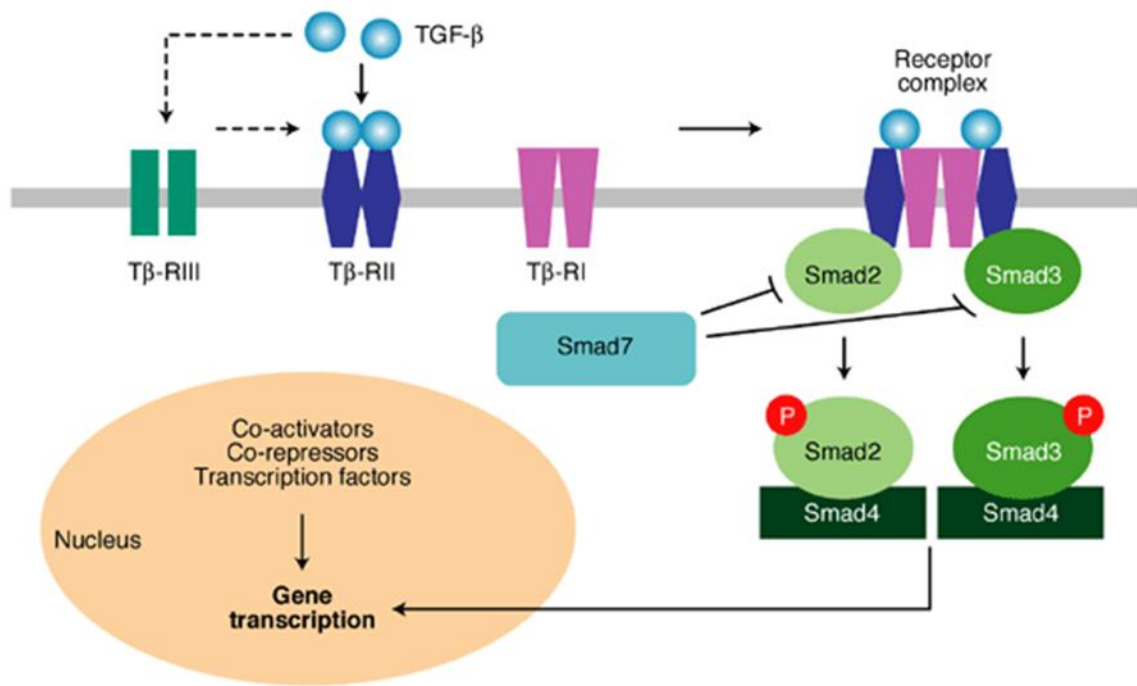


Figure 6.1. The TGF- β signalling pathway.

TGF- β binds to the receptor T β R-II and this binding can be enhanced by the presence of T β R-III. After binding to TGF- β , T β R-II recruits and phosphorylates T β R-I, leading to activation of Smad2 and Smad3 by phosphorylation (P). This process is inhibited by Smad7. Activated Smad2 and Smad3 form heterodimers with Smad4 and translocate to the nucleus. Together with co-activators, co-repressors and other transcription factors, the Smad complex regulates gene expression (Pinzani and Marra, 2001).

TGF- β has been strongly implicated in the development of airway remodelling in asthma. Clinical studies have shown increased TGF- β expression in lung biopsies (Ohno et al., 1996; Minshall et al., 1997; Magnan et al., 1997; Vignola et al., 1997) and the BAL of asthmatics (Redington et al., 1997). TGF- β is essential for the transformation of fibroblasts into myofibroblasts, which are major producers of collagen, and gene transfer of TGF- β 1 in mouse lungs promotes lung fibrosis with ECM protein deposition (Sime et al., 1997; Kolb et al., 2002). In addition to asthmatic studies, TGF- β expression has also been shown to be increased in murine models of allergic airway disease, induced by fungal extracts (Blease et al., 2002) and OVA respectively (Kumar et al., 2004; McMillan and Lloyd, 2004; Kelly et al., 2005). Indeed, TGF- β levels were higher in the lung tissue of HDM-treated mice than PBS controls in Chapter 3 (Figure 3.7E). TGF- β has been directly implicated in the

development of lung structural changes as the blockade of TGF- β reduced OVA-induced airway remodelling (McMillan et al., 2005;Alcorn et al., 2007). TGF- β signalling through Smad 2/3 also mediates fibroblast α -smooth muscle actin expression, which is a marker of myofibroblast differentiation, and Smad 3 KO mice demonstrate reduced OVA-induced airway remodelling (Kobayashi et al., 2006;Le et al., 2007). However, concurrent with the work in this Chapter, in a HDM model of allergic airways disease, TGF- β was observed to regulate airway inflammation but not remodelling. Blockade of TGF- β worsened HDM-induced AHR and BAL eosinophilia and Smad 3 KO mice developed airway remodelling to the same extent as HDM-exposed wt mice (Fattouh et al., 2008).

TGF- β plays a critical role in maintaining lung immune homeostasis and actively suppressing immune responses. One of the major mechanisms by which TGF- β exerts immunosuppressive actions is through the tight control of T cell development, tolerance and differentiation. Several studies have shown that transgenic mice with T cells that are unresponsive specifically to TGF- β develop autoimmune diseases, indicating that TGF- β signalling is essential for T cell homeostasis (Gorelik and Flavell, 2000;Marie et al., 2006;Li et al., 2006a). In the absence of TGF- β signalling specifically in T cells, T cells undergo hyperproliferation, activation, and effector T cell differentiation that results in the infiltration of leukocytes into multiple vital organs (Marie et al., 2006;Li et al., 2006a). A vital role for TGF- β -mediated immune suppression is through the induction of regulatory T cells (Tregs) (O'Garra et al., 2004). TGF- β has been shown to induce FoxP3 transcription (Chen et al., 2003), which is the key transcription factor for naturally occurring Tregs (Marie et al., 2005;Josefowicz and Rudensky, 2009). Deficiency of FoxP3 in mice results in T cell-dependent inflammatory phenotype similar in severity to that of T cell-specific TGF- β receptor II-deficient mice (Brunkow et al., 2001;Fontenot et al., 2003;Marie et al., 2005). Tregs are also a major source of TGF- β in the immune system. The latent form of TGF- β exists as a dimeric pro-protein, known as latency-associated peptide (LAP) and Tregs express LAP on their membrane surface at high levels (Chen et al., 2008). CD4⁺CD25⁺LAP⁺ Tregs are more potent in their regulatory activity than CD4⁺CD25⁺LAP⁻ T cells (Nakamura et al., 2004;Chen et al., 2008).

The precise mechanism by which TGF- β blockade worsens HDM-induced airway inflammation and AHR remains to be elucidated. In view of the fact that TGF- β has a central role in controlling T cell homeostasis and maintaining lung immune homeostasis, a potential mechanism by which the blockade of TGF- β may worsen HDM-induced AHR and BAL eosinophilia is through reduced Treg-mediated immunosuppression.

6.2. Hypothesis and Aims

6.2.1. Hypothesis

Therapeutic blockade of TGF- β will worsen HDM-induced airway inflammation and AHR by reducing the numbers of Tregs in the lung.

6.2.2. Aims

The aims of Chapter 6 are to determine how therapeutic neutralisation of TGF- β impacts on the development of HDM-induced airway remodelling and determine how the blockade of TGF- β leads to exaggerated AHR and airway inflammation.

6.3. Experimental Plan

6.3.1. Protocol A: Induction of HDM-induced allergic airways disease and therapeutic blockade of TGF- β

Female BALB/c mice were administered either 15 μ l PBS or 15 μ g (in 15 μ l) HDM i.n. 3 times a week for 5 weeks. A therapeutic regimen of anti-TGF- β Ab was instigated at the beginning of week 3 and mice were given 100 μ l of either 0.5 mg/Kg or 5 mg/Kg i.p. before each airway challenge. Control mice received an isotype (IgG₁) Ab. Mice were culled either at 4h post-final challenge (for analysis of pulmonary inflammatory cells, IgE levels, mucus production and cytokine production) or at 24h (for lung function analysis).

6.3.2. Protocol B: Induction of HDM-induced allergic airways disease and prophylactic blockade of TGF- β

Female BALB/c mice were administered either 15 μ l PBS or 15 μ g (in 15 μ l) HDM i.n. 3 times a week for 3 weeks. A prophylactic regimen of anti-TGF- β Ab was instigated prior to the first i.n. dose and mice were given 100 μ l of either 0.5 mg/Kg or 5 mg/Kg i.p. before each airway challenge. Control mice received an isotype (IgG₁) Ab. Mice were culled 24h post-final challenge.

The doses of anti-TGF- β were chosen as these have been used previously *in vivo*. McMillan and colleagues used a 0.5 mg/Kg dose to show that blockade of TGF- β reduced airway remodelling in OVA exposed mice (McMillan et al., 2005), and Fattouh and colleagues used doses equivalent to 0.5 mg/Kg and 5 mg/Kg in the HDM studies (Fattouh et al., 2008). The blocking Ab neutralises all mouse isoforms of TGF- β (McMillan et al., 2005).

6.4. Results

6.4.1.1. Therapeutic Blockade of TGF- β : Impact of anti-TGF- β on pulmonary TGF- β expression

The levels of TGF- β were analysed in the lung tissue following HDM exposure and respective Ab treatment. There was a reduction, but a non-significant decrease, in the levels of TGF- β in the lung following the administration of the 0.5 mg/Kg dose. However, HDM-induced increase in TGF- β was completely abolished by the administration of the 5 mg/Kg dose (Figure 6.2).

6.4.1.2. Blockade of TGF- β exacerbates HDM-induced AHR

Increased airway resistance in response to inhaled HDM was not decreased by either dose of anti-TGF- β . Interestingly, at the highest dose of neutralising antibody, airway resistance was exacerbated (Figure 6.3A - D). Similarly, this was also observed with the airway compliance. All HDM-treated mice showed decreased airway compliance as compared to the respective control groups. HDM exposed mice, treated with 5 mg/Kg anti-TGF- β , showed decreased airway compliance as compared to Ig-treated HDM exposed mice (Figure 6.3E - H).

6.4.1.3. Neutralisation of TGF- β aggravates BAL inflammation

The administration of anti-TGF- β did not alter the total number of cells or eosinophils present in the lung in response to HDM (Figure 6.4A and B). Both Ig and anti-TGF- β -treated HDM exposed mice also showed greater numbers of total cells and eosinophils in the BAL as compared to the respective PBS control groups. At both doses of TGF- β neutralising Ab, the numbers of eosinophils in the BAL were increased (Figure 6.4C and D).

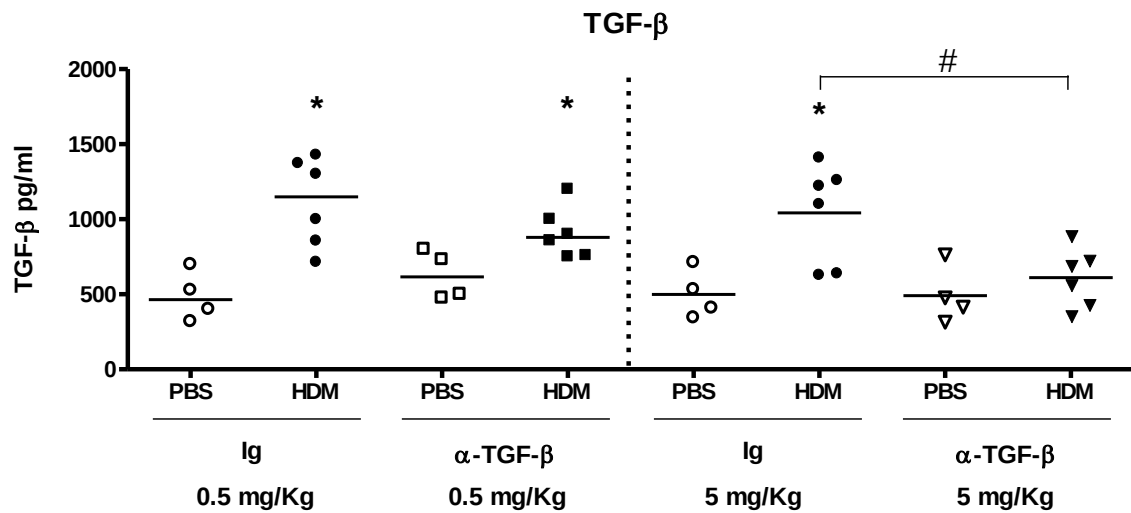


Figure 6.2. Impact of anti-TGF- β on active pulmonary TGF- β expression.

TGF- β was measured in the lung 4h after the final challenge. TGF- β was quantified by ELISA. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β and n=6 HDM and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. #, $P < 0.05$ (Mann-Whitney U test) HDM- α -TGF- β compared with HDM-Ig. Bars depict the median of the groups. Representative of two experiments.

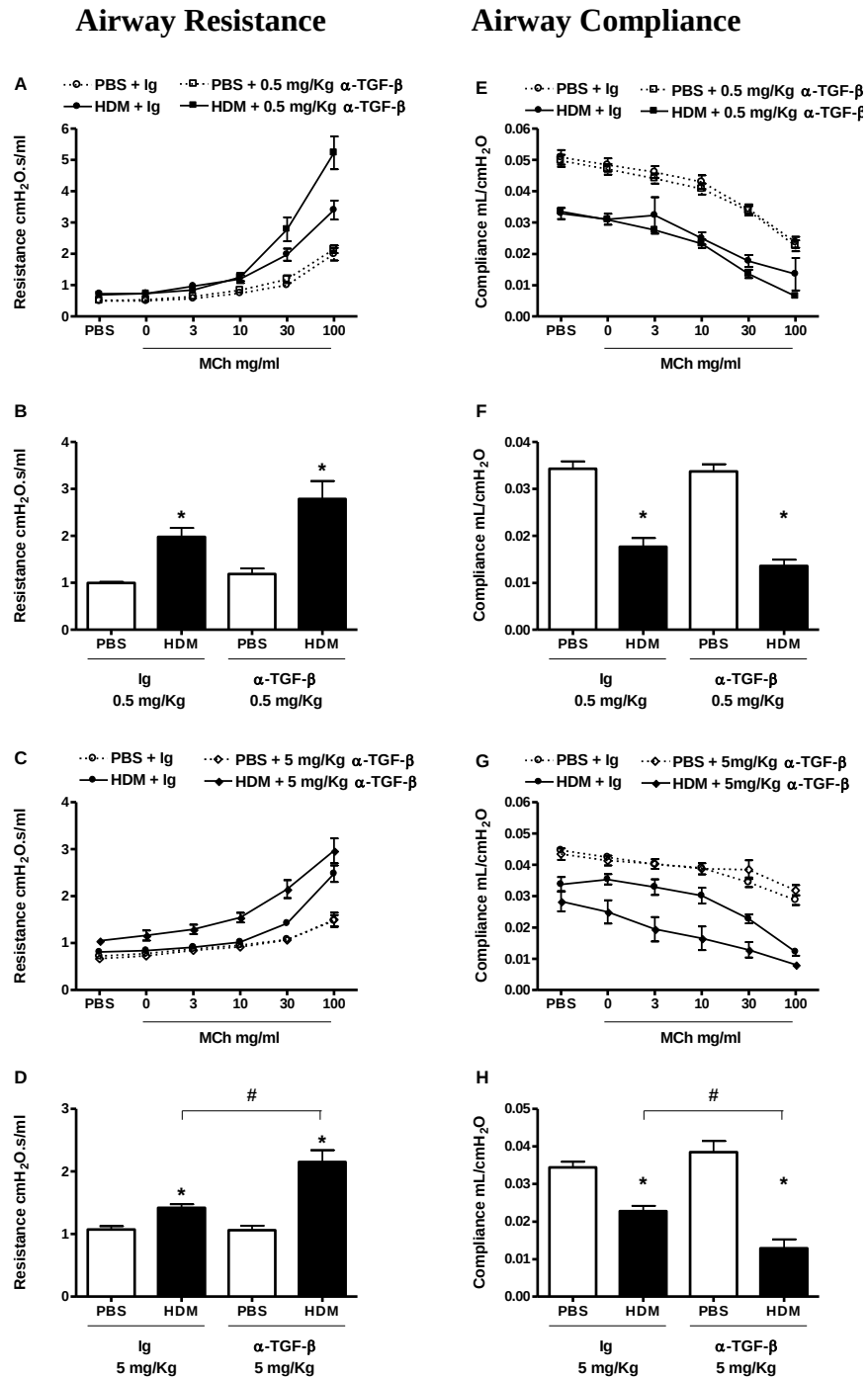


Figure 6.3. Therapeutic blockade of TGF- β worsens HDM-induced AHR.

(A) Airway resistance of PBS and HDM-treated mice at 24h post final challenge treated with either Ig or 0.5 mg/Kg anti-TGF- β . (B) Airway resistance is expressed as mean $\pm$ SEM for the 30mg/ml dose of methacholine. (C) Airway resistance of PBS and HDM-treated mice at 24h post final challenge treated with either Ig or 5 mg/Kg anti-TGF- β . (D) Airway resistance is expressed as mean $\pm$ SEM for the 30mg/ml dose of methacholine. (E) Airway compliance of PBS and HDM-treated mice at 24h post final challenge treated with either Ig or 0.5 mg/Kg anti-TGF- β . (F) Airway compliance is expressed as mean $\pm$ SEM for the 30mg/ml dose of methacholine. (G) Airway compliance of PBS and HDM-treated mice at 24h post final challenge treated with either Ig or 5 mg/Kg anti-TGF- β . (H) Airway compliance is expressed as mean $\pm$ SEM for the 30mg/ml dose of methacholine. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β and n=6 HDM and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β). *, P<0.05; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. #, P<0.05 (Mann-Whitney U test) HDM-anti-TGF- β compared with HDM-Ig. Data are expressed as mean $\pm$ SEM. Representative of two experiments.

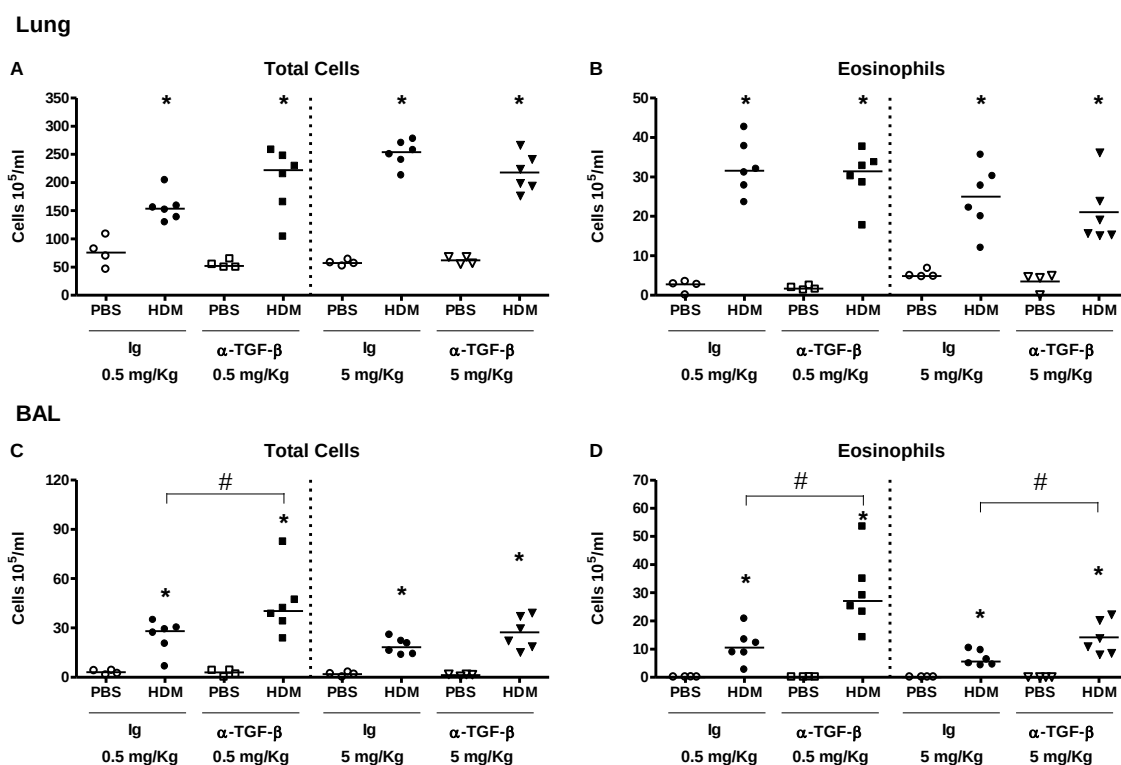


Figure 6.4. Therapeutic neutralisation of TGF- β elevates HDM-induced BAL eosinophilia.

(A) Total cells and (B) eosinophils in the lung and (C) total cells and (D) eosinophils in the BAL 4h after the final challenge. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β and n=6 HDM and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β). Total cell counts were determined using a haemocytometer with white blood cell counting fluid and eosinophils were quantified from Wright-Giemsa stained cytopins as described in the Materials and Methods. *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. #, $P < 0.05$ (Mann-Whitney U test) HDM-anti-TGF- β compared with HDM-Ig. Bars depict the median of the groups. Representative of two experiments.

6.4.1.4. Blockade of TGF- β increases Th2 cell populations

All HDM-treated mice showed higher numbers of Th2 (CD4⁺ T1ST2⁺) cells in the lung tissue and BAL compared to PBS controls. However, greater numbers of these cells were observed in the lung and BAL of 0.5 mg/Kg anti-TGF- β -treated HDM exposed mice than Ig-treated HDM exposed mice (Figure 6.5A and B).

6.4.1.5. Neutralisation of TGF- β reduces regulatory T cell populations

Representative flow cytometry plots are shown from lung tissue that was either unstained or had received either FoxP3 or IL-10 isotype control Abs. Representative dot plots are also shown of stained lung tissue samples from PBS and HDM-treated mice (Figure 6.6A - D and 6.7A - D). All HDM-treated mice had higher numbers of CD4⁺CD25⁺FoxP3⁺ and CD4⁺IL-10⁺ cells in the lung tissue than the respective PBS control groups. However, 5 mg/Kg anti-TGF- β -treated HDM exposed mice showed lower numbers of CD4⁺CD25⁺FoxP3⁺ and CD4⁺IL-10⁺ cells than Ig-treated HDM exposed controls (Figure 6.8A and B).

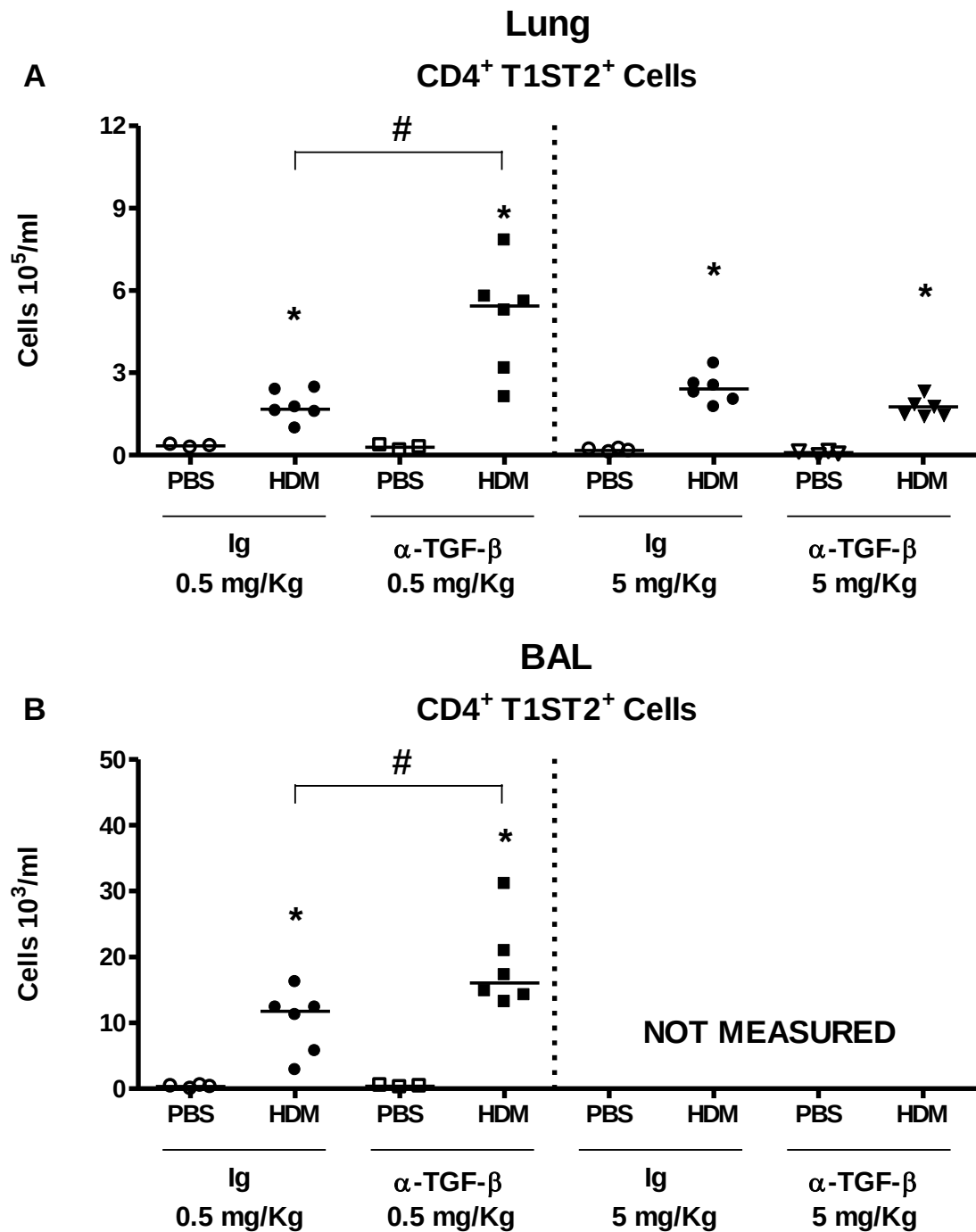


Figure 6.5. Blockade of TGF- β increases Th2 cell numbers.

CD4⁺T1ST2⁺ T Cells recovered from the lung (A) and BAL (B) 4h after the final challenge (n=3-4 PBS Ig, n=6 HDM Ig, n=3-4 PBS and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β and n=6 HDM and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β). Cells were quantified by flow cytometry. *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. #, $P < 0.05$ (Mann-Whitney U test) HDM-anti-TGF- β compared with HDM-Ig. Bars depict the median of the groups. Representative of two experiments.

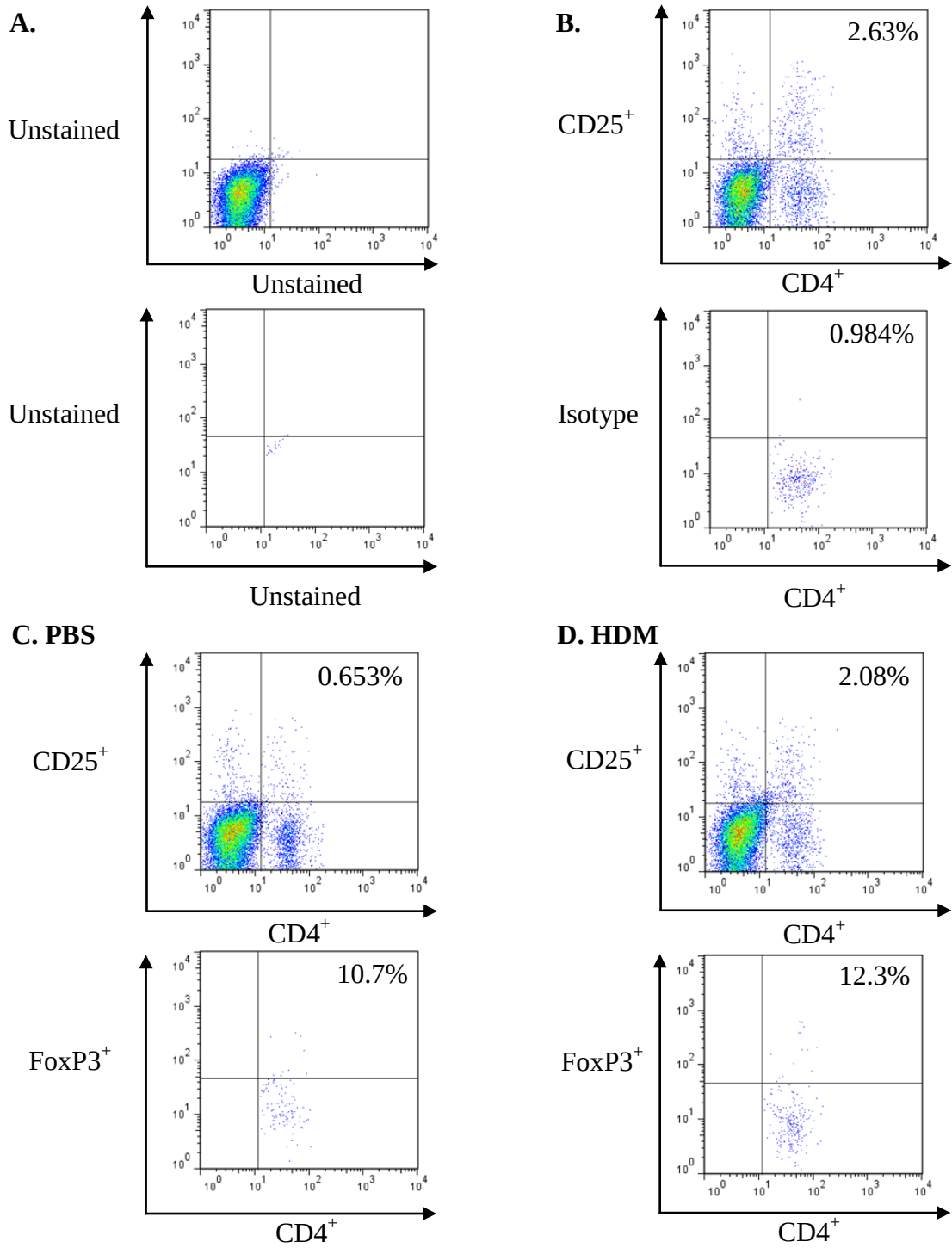


Figure 6.6. Flow cytometry plots for staining of CD4⁺CD25⁺FoxP3⁺ regulatory T cells.

Representative dot plots from (A) unstained cells, (B) CD4⁺CD25⁺FoxP3 isotype stained cells, (C) CD4⁺CD25⁺FoxP3⁺ cells from PBS-treated mice and (D) CD4⁺CD25⁺FoxP3⁺ cells from HDM-treated mice.

Top panel of B - D: Percentage of CD4⁺ cells expressing CD25⁺.

Bottom panel of B - D: Percentage of CD4⁺CD25⁺ cells expressing FoxP3⁺.

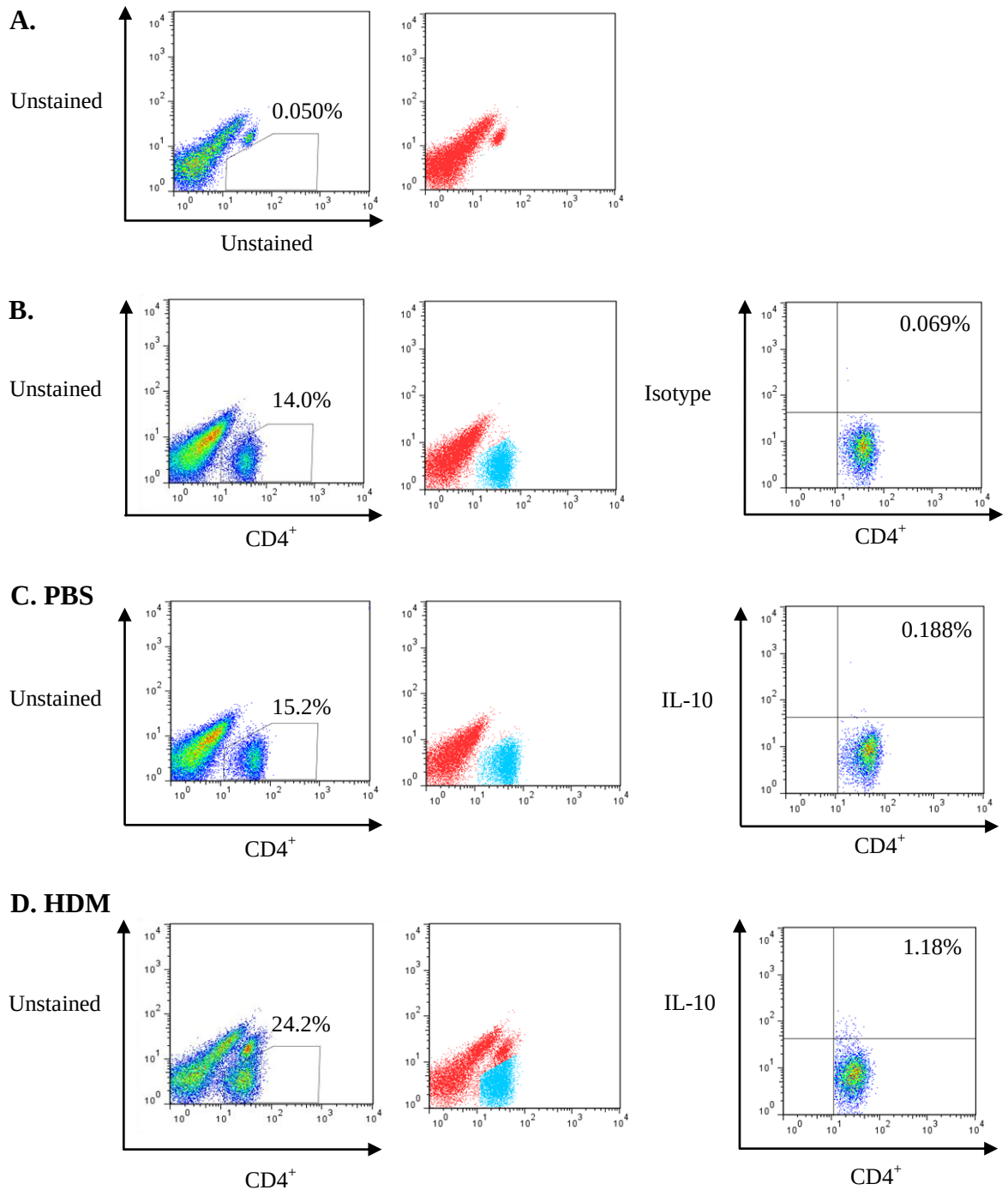


Figure 6.7. Flow cytometry plots for staining of CD4⁺IL-10⁺ cells.

Representative dot plots from (A) unstained cells, (B) CD4⁺IL-10 isotype stained cells, (C) CD4⁺IL-10⁺ cells from PBS-treated mice and (D) CD4⁺IL-10⁺ cells from HDM-treated mice.

Left panel A - D: Percentage of CD4⁺ lymphocytes from total lymphocytes.

Middle panel A - D: CD4⁺ lymphocytes in blue and CD4⁻ lymphocytes in red.

Right panel B - D: Percentage of CD4⁺ cells expressing IL-10.

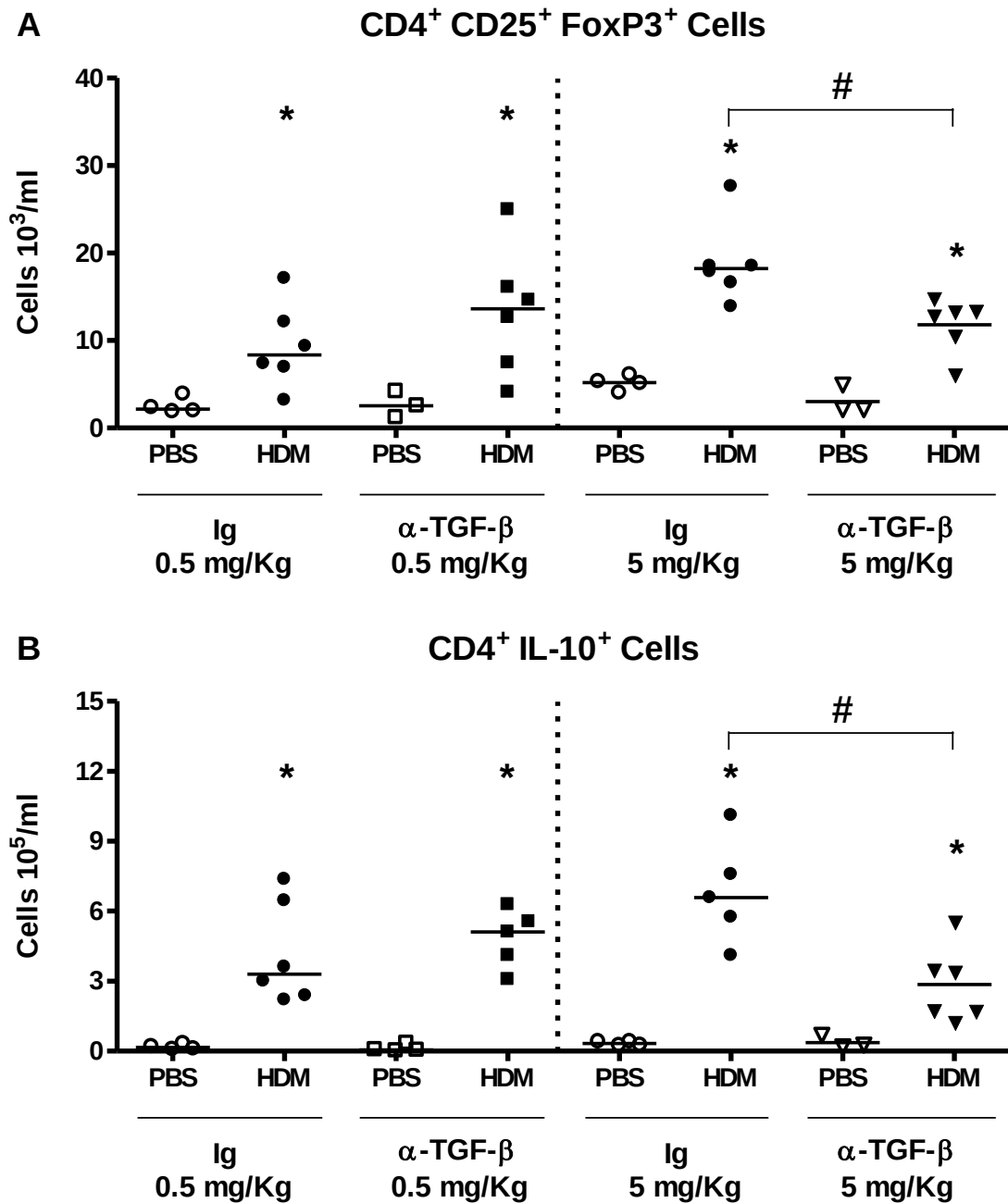


Figure 6.8. Blockade of TGF- β reduces regulatory T cell numbers in the lung.

(A) $CD4^+ CD25^+ FoxP3^+$ and (B) $CD4^+ IL-10^+$ T Cells recovered from the lung 4h after the final challenge (n=4 PBS Ig, n=5-6 HDM Ig, n=3-4 PBS and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β and n=5-6 HDM and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β). Cells were quantified by flow cytometry. *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. #, $P < 0.05$ (Mann-Whitney U test) HDM-anti-TGF- β compared with HDM-Ig. Bars depict the median of the groups. Representative of two experiments.

6.4.1.6. Neutralisation of TGF- β results in elevated IL-13 and IL-33 levels

The signature Th2 cytokines IL-4, IL-5 and IL-13, as well as IL-17, were quantified in the BAL and lung to investigate whether the effects of blocking TGF- β on T cell populations influenced the pulmonary production of these cytokines. IL-4 and IL-5 production was not altered by either dose of anti-TGF- β (Figure 6.9A – D). IL-13 levels were elevated in the BAL and lung of all HDM-treated mice as compared to PBS controls. Greater IL-13 production was observed in 5 mg/Kg anti-TGF- β -treated HDM exposed mice compared to Ig-HDM-treated mice (Figure 6.9E - F). IL-17 was also elevated in the BAL of all HDM-treated groups compared to the respective PBS control groups. The levels of IL-17 in the BAL were lower in 5 mg/Kg anti-TGF- β -treated HDM exposed mice than Ig-treated HDM exposed mice (Figure 6.9G). Both doses of anti-TGF- β further enhanced HDM-induced production of IL-33 (Figure 6.9H).

6.4.1.7. Neutralisation of TGF- β does not alter mast cell activation

Mast cell activity was determined by quantification of mMCP-1 in the serum. The administration of either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β did not alter HDM-induced production of mMCP-1 in the serum, suggesting that mast cell activity is not influenced by blocking TGF- β (Figure 6.10A).

6.4.1.8. Blockade of TGF- β does not elevate IgE production

To investigate whether the humoral response to HDM, was influenced by neutralisation of TGF- β , total and HDM-IgE were quantified in the serum. All HDM-treated mice showed higher IgE levels in the serum than the respective control groups and there was no effect of anti-TGF- β on the IgE response to HDM (Figure 6.10B and C).

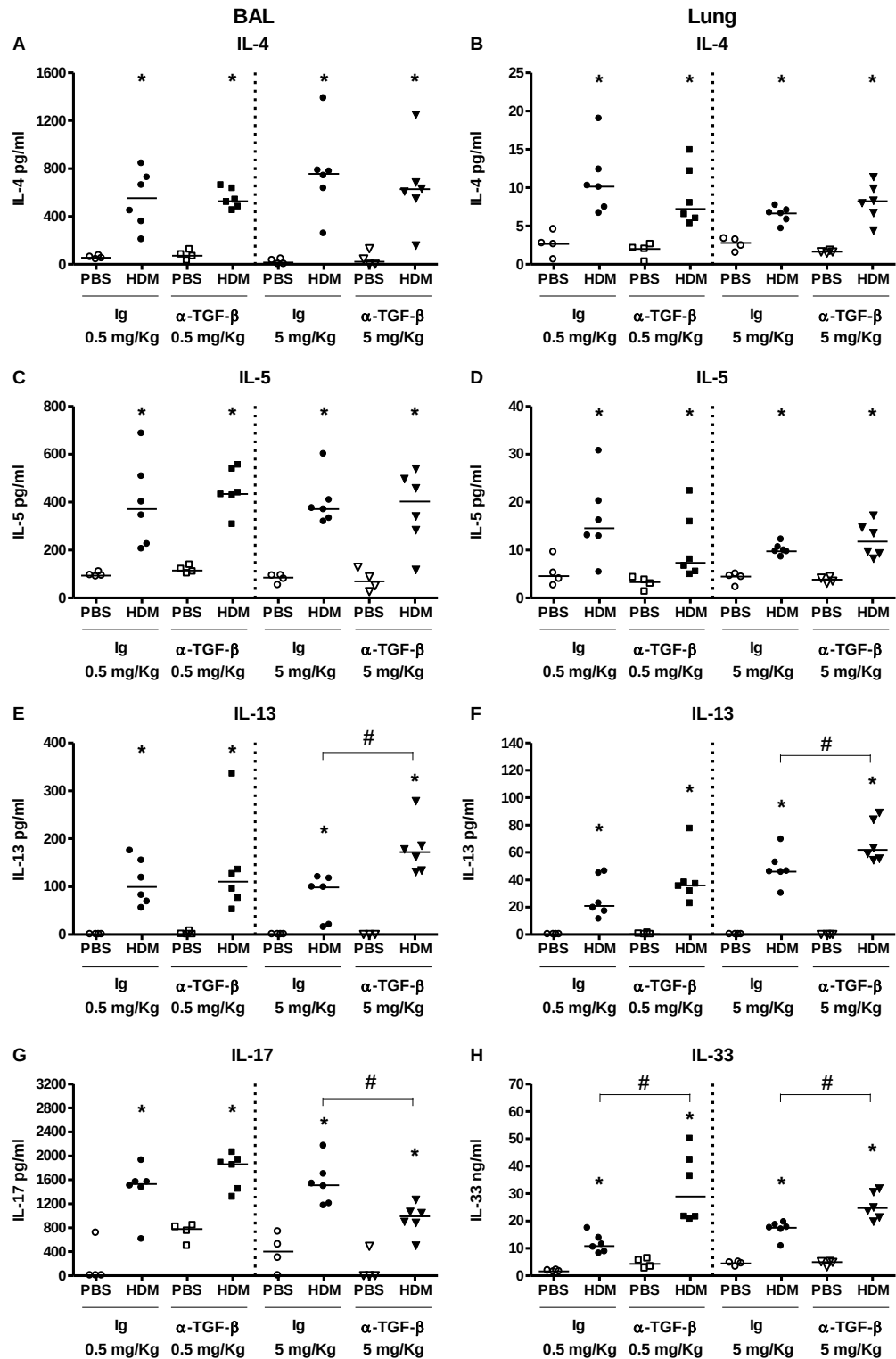


Figure 6.9. Blockade of TGF- β increases IL-13 production in the BAL and lung.

IL-4, IL-5 and IL-13 measured in the BAL (A, C and E) and lung (B, D and F), IL-17 measured in the BAL (G) and IL-33 measured in the lung (H) 4h after the final challenge. IL-4, IL-5 and IL-17 were quantified by MSD and IL-13 and IL-33 by ELISA. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β and n=6 HDM and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. #, $P < 0.05$ (Mann-Whitney U test) HDM-anti-TGF- β compared with HDM-Ig. Bars depict the median of the groups. Representative of two experiments.

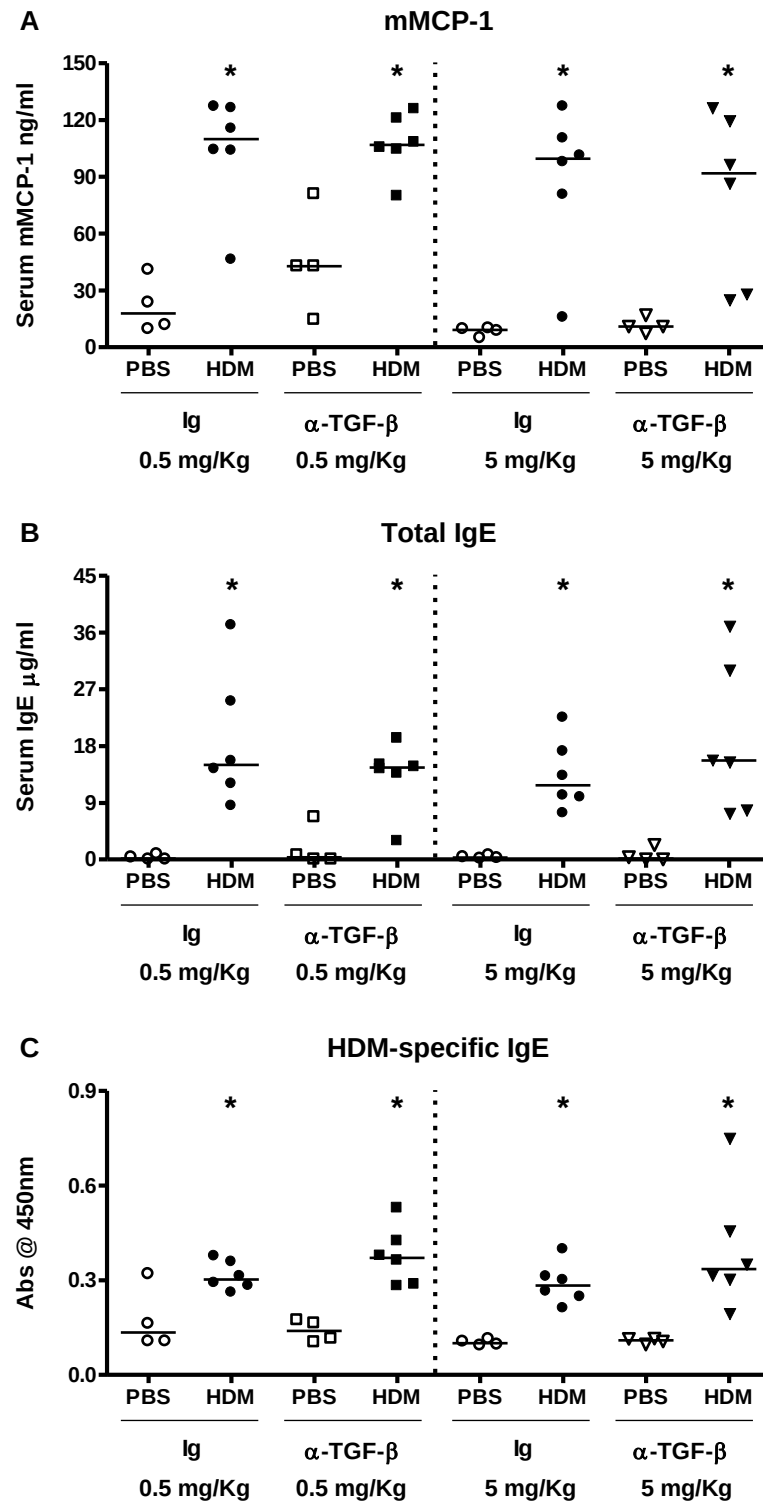


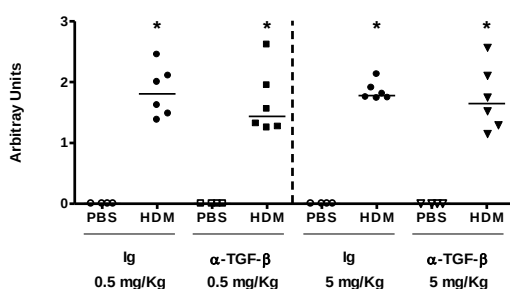
Figure 6.10. Blockade of TGF- β does not alter mast cell activity or IgE production.

(A) mMCP-1, (B) total IgE and (C) HDM-specific IgE measured in the serum 4h after the final challenge. All mediators were quantified by ELISA. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β and n=6 HDM and either 0.5 mg/Kg or 5 mg/Kg anti-TGF- β). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. Bars depict the median of the groups. Representative of two experiments.

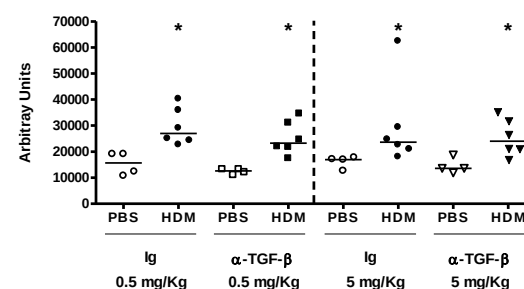
6.4.1.9. Blockade of TGF- β does not influence HDM-induced mucus production or collagen deposition

To investigate whether *in vivo* blockade of TGF- β influenced HDM-induced airway remodelling, the degree of goblet cell hyperplasia and peri-bronchial collagen deposition were then assessed. HDM exposed mice treated with either Ig, 0.5 mg/Kg anti-TGF- β or 5 mg/Kg anti-TGF- β developed goblet cell hyperplasia and showed peri-bronchial collagen deposition. There was no effect of the blockade of TGF- β on the degree of goblet cell hyperplasia or collagen deposition (Figure 6.11A and B). Representative PAS and sirius red stained lung sections from the respective groups of mice are shown (Figure 6.11C – H).

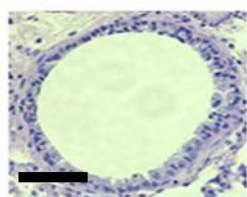
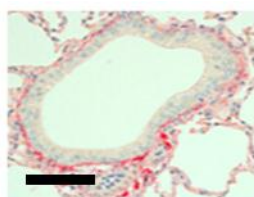
A. Mucus Score



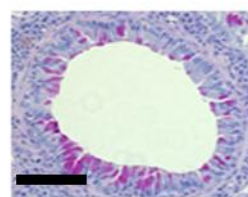
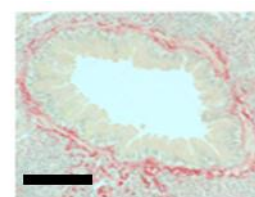
B. Mean Intensity of Peri-bronchial Collagen Deposition



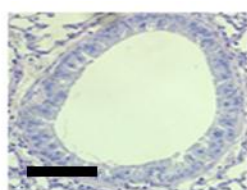
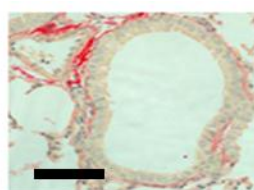
C. PBS + Ig



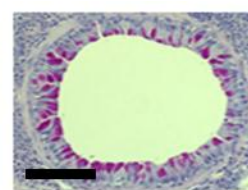
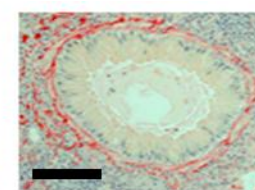
F. HDM + Ig



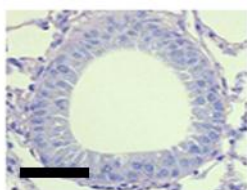
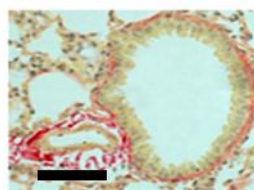
D. PBS + 0.5 mg/Kg α-TGF-β



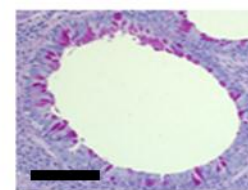
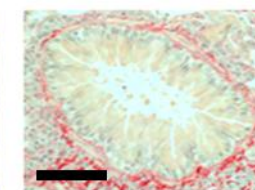
G. HDM + 0.5 mg/Kg α-TGF-β



E. PBS + 5 mg/Kg α-TGF-β



H. HDM + 5mg/Kg α-TGF-β



PAS

Sirius Red

PAS

Sirius Red

Figure 6.11. Development of goblet cell hyperplasia and peri-bronchial collagen deposition following TGF-β neutralisation.

(A) Semi-quantitative scoring of PAS stained lung sections. (B) Quantification of peri-bronchial collagen deposition, expressed as mean intensity. Bars depict the median of the groups. *, $P < 0.05$; (Mann-Whitney U test) HDM compared with PBS. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and either 0.5 mg/Kg or 5 mg/Kg anti-TGF-β and n=6 HDM and either 0.5 mg/Kg or 5 mg/Kg anti-TGF-β). Representative pictures of PAS and sirius red stained lung sections from (C) Ig PBS, (D) 0.5 mg/Kg anti-TGF-β-PBS, (E) 5 mg/Kg anti-TGF-β-PBS, (F) Ig HDM, (G) 0.5 mg/Kg anti-TGF-β-HDM and (H) 5 mg/Kg anti-TGF-β-HDM-treated mice (original 20X magnification). Scale bar = 50 μ m. Representative of two experiments.

6.4.2. Protocol B: Prophylactic blockade of TGF- β

6.4.2.1. Prophylactic treatment with anti-TGF- β does not alter HDM-induced AHR or airway inflammation

When the anti-TGF- β Ab was administered concurrently with HDM exposure for three weeks, no alterations were observed in the parameters of either AHR or airway inflammation. The 0.5 mg/Kg dose of anti-TGF- β Ab did not alter HDM-induced AHR (Figure 6.12A - D), eosinophilia or pulmonary IL-4, IL-5 and IL-13 production (Figure 6.13A – H) and the 5 mg/Kg dose of Ab did not alter pulmonary eosinophilia or Th2 cytokine levels (6.14A – F).

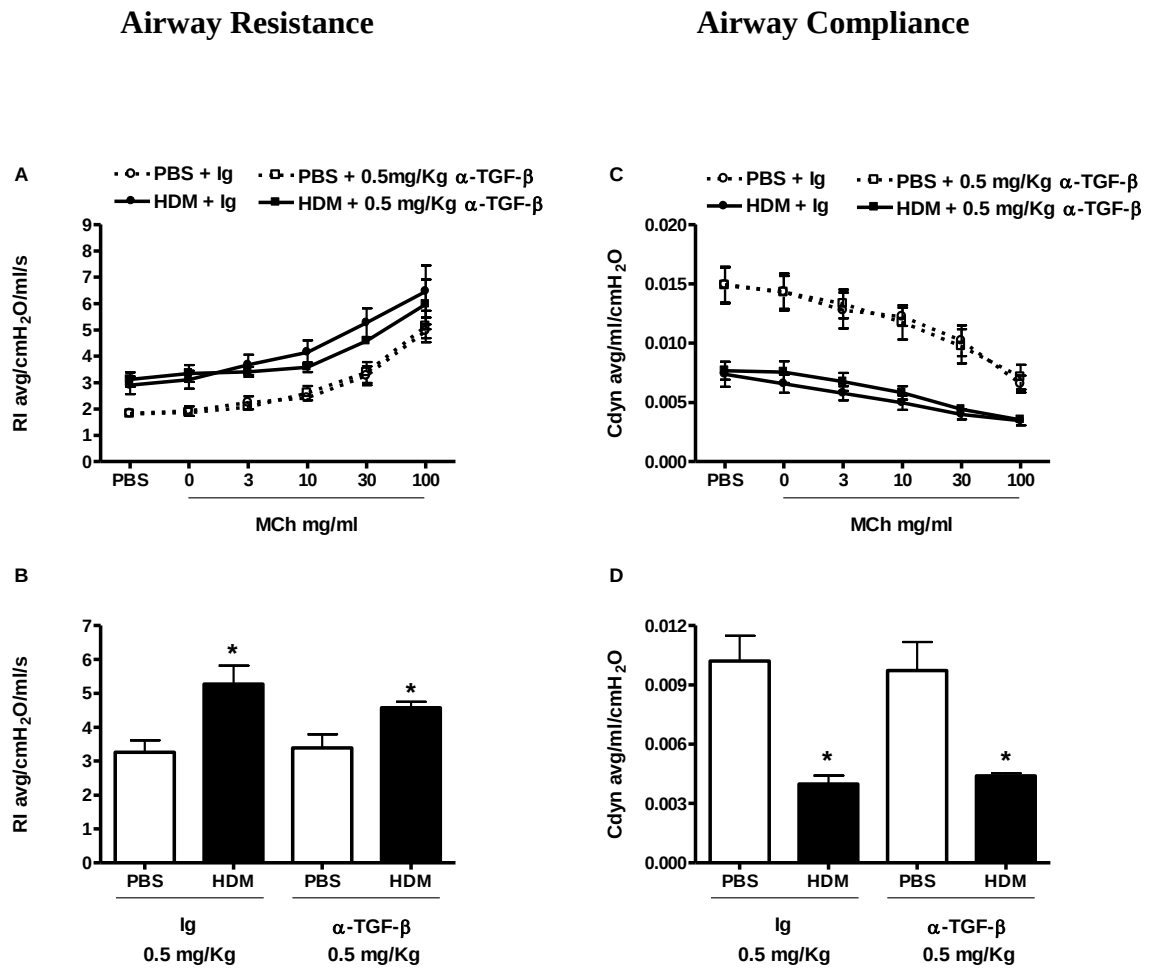


Figure 6.12. Prophylactic TGF- β neutralisation does not alter HDM-induced AHR.

(A) Airway resistance of PBS and HDM-treated mice at 24h post final challenge, treated with either Ig or 0.5 mg/Kg anti-TGF- β . (B) Airway resistance is expressed as mean RI $\pm$ SEM for the 30mg/ml dose of methacholine. (C) Airway compliance of PBS and HDM-treated mice measured 24h post final challenge, treated with either Ig or 0.5 mg/Kg anti-TGF- β . (D) Airway compliance is expressed as mean Cdyn $\pm$ SEM for the 30mg/ml dose of methacholine. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and 0.5 mg/Kg anti-TGF- β and n=6 HDM and 0.5 mg/Kg anti-TGF- β). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. Data are expressed as mean $\pm$ SEM. One experiment.

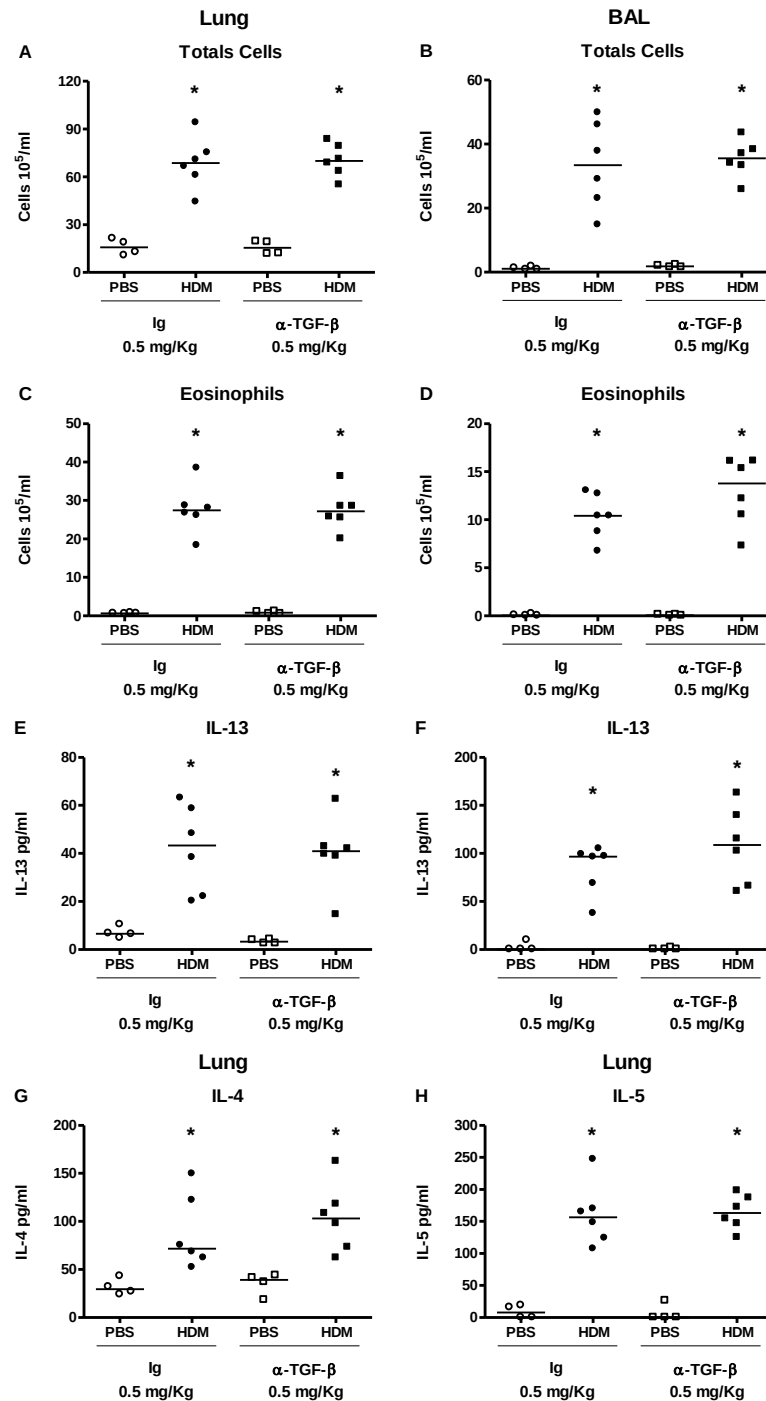


Figure 6.13. Preventative TGF- β blockade does not alter HDM-induced airway inflammation.

Total cells, eosinophils and IL-13 in the lung (A, C and E) and BAL (B, D and F) and (G) IL-4 and (H) IL-5 in the lung 24h post final challenge. Total cell counts were determined using a haemocytometer with white blood cell counting fluid and eosinophils were quantified from Wright-Giemsa stained cytopins, as described in the Materials and Methods. IL-4, IL-5 and IL-13 were quantified by ELISA. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and 0.5 mg/Kg anti-TGF- β and n=6 HDM and 0.5 mg/Kg anti-TGF- β). *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. Bars depict the median of the groups. One experiment.

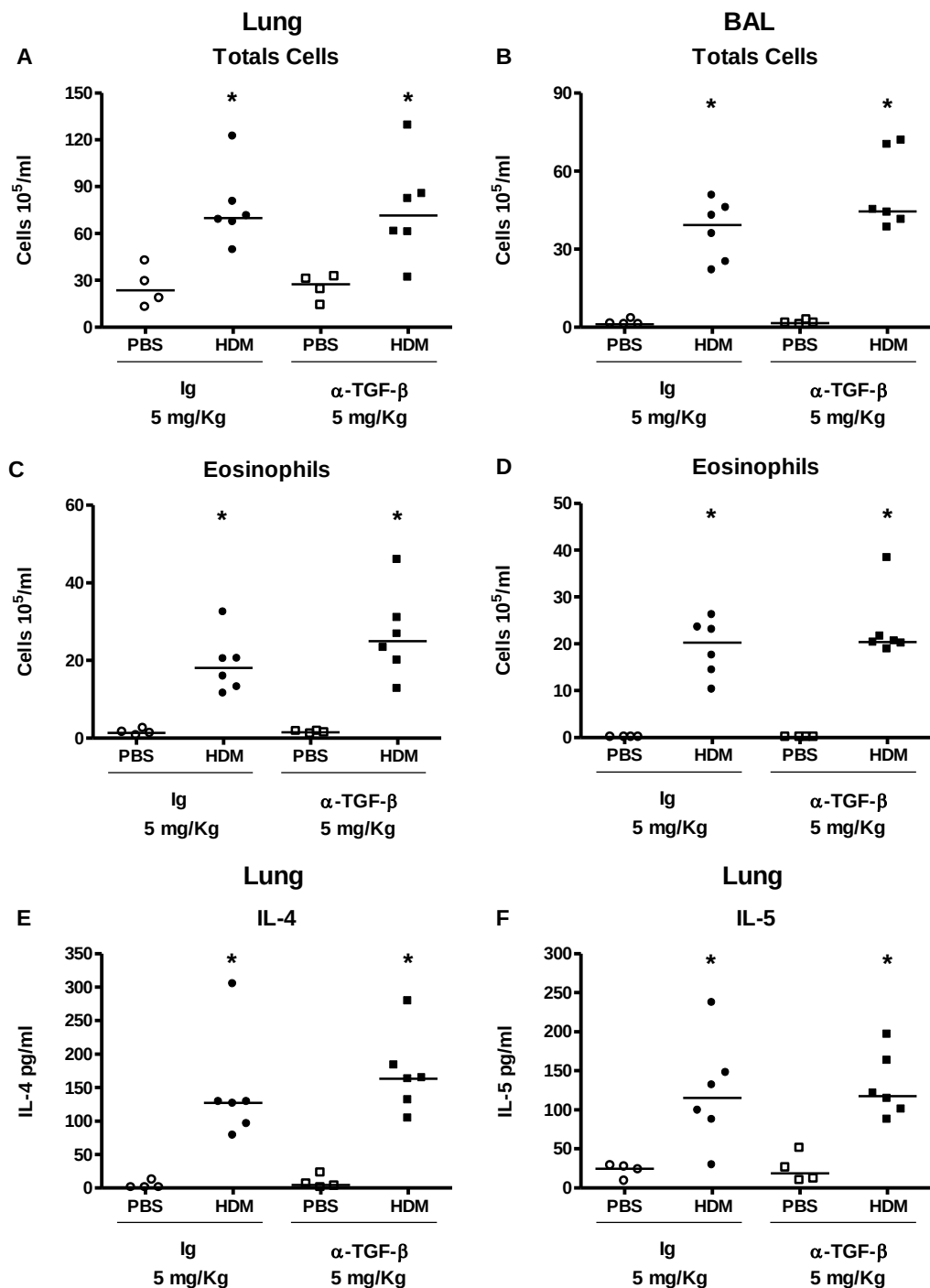


Figure 6.14. Prophylactic TGF- β neutralisation does not alter HDM-induced airway inflammation.

Total cells and eosinophils in the lung (A and C) and BAL (B and D) and (E) IL-4 and (F) IL-5 quantified in the lung 24h post final challenge. (n=4 PBS Ig, n=6 HDM Ig, n=4 PBS and 5 mg/Kg anti-TGF- β and n=6 HDM and 5 mg/Kg anti-TGF- β). Total cell counts were determined using a haemocytometer with white blood cell counting fluid and eosinophils were quantified from Wright-Giemsa stained cytopins, as described in the Materials and Methods. IL-4 and IL-5 were quantified by ELISA. *, $P < 0.05$; (Mann-Whitney U test) HDM compared with corresponding PBS-treated control group. Bars depict the median of the groups. One experiment.

6.5. Discussion

Following therapeutic blockade of TGF- β in a HDM model of allergic airways disease there was no impact on peri-bronchial collagen deposition or mucus production, which are key features of airway remodelling. However, neutralisation of TGF- β led to worsened AHR and BAL eosinophilia, together with elevated levels of IL-13 and IL-33 in the lung. Conversely, the numbers of CD4⁺IL-10⁺ and CD4⁺CD25⁺FoxP3⁺ Tregs were decreased in the lung following the blockade of TGF- β *in vivo*. Thus, these results show a vital role for TGF- β in regulating HDM-induced airway inflammation and AHR, while airway remodelling progressed independently of TGF- β .

The elevated HDM-induced AHR following blockade of TGF- β can be attributed to the decreased numbers of Tregs and elevated IL-13 and IL-33 levels in the lung. Naturally occurring CD4⁺CD25⁺ Tregs have been shown to suppress OVA-induced AHR in mice, via a TGF- β -dependent mechanism (Joetham et al., 2007). The anti-TGF- β Ab administered would therefore prevent the inhibitory effects of these cells on AHR here in the current study. CD4⁺CD25⁺FoxP3⁺ cells have also been shown to induce Th2 cell apoptosis and increase tolerance to OVA, which contribute to the resolution of AHR (Strickland et al., 2006; Finotto et al., 2007). Adoptive transfer of antigen-specific CD4⁺CD25⁺ Tregs suppressed OVA-induced AHR (Kearley et al., 2005) via IL-10 production from bystander CD4⁺ cells (Kearley et al., 2008). Depletion of CD4⁺CD25⁺ cells has also been shown to elevate HDM-induced AHR (Lewkowich et al., 2005). Consequently, following anti-TGF- β Ab administration, there would be a loss of suppression of AHR with a reduction in the number of the CD4⁺CD25⁺FoxP3⁺ and CD4⁺IL-10⁺ cells in the lung. The reduction in the number of CD4⁺CD25⁺FoxP3⁺ cells in anti-TGF- β -treated HDM exposed mice would occur as a result of blocking CD4⁺CD25⁺FoxP3⁺ differentiation from naive CD4⁺ cells, as TGF- β is required for this process (Chen et al., 2003; Fontenot et al., 2003; Ramsdell, 2003). However, CD4⁺CD25⁺FoxP3⁺ cells are not completely absent from the lungs of anti-TGF- β -treated HDM exposed mice because the therapeutic protocol was only instigated after 2 weeks of HDM exposure, thereby allowing for the differentiation of these cells to occur prior to Ab administration.

IL-13 is central to the development of AHR (Wills-Karp et al., 1998;Wills-Karp, 1999;Wills-Karp, 2004) and IL-33 also promotes AHR (Kondo et al., 2008). Therefore, elevations in the levels of these mediators in the lung are also likely to contribute to the exaggerated AHR when TGF- β was neutralised. However, exactly how the blockade of TGF- β results in heightened levels of these mediators in the lung remains to be determined. Decreased numbers of CD4⁺CD25⁺FoxP3⁺ cells in the lung are the likely cause since these cells can suppress both OVA (Joetham et al., 2007) and *Der p* 1-induced IL-13 production (Leech et al., 2007). Indeed, depletion of CD4⁺CD25⁺ cells also elevated HDM-induced Th2 cytokine production by increasing the ability of DCs to present HDM (Lewkowich et al., 2005). In addition to blocking TGF- β -mediated FoxP3 differentiation, TGF- β neutralisation would also inhibit CD4⁺CD25⁺FoxP3⁺-TGF- β -mediated suppression of Th2 responses. A possible mechanism by which IL-33 is elevated following TGF- β blockade in HDM exposed mice is that there is a loss of regulation of IL-33 production. Mice lacking TGF- β responsiveness in epithelial cells or T cells develop severe intestinal inflammation (Beck et al., 2003;Fahlen et al., 2005) and TGF- β limits IL-33 production from alveolar macrophages (Rani et al., 2011). Therefore, a loss of TGF- β -mediated regulation of IL-33 from alveolar macrophages may explain the increased mediator production in the lung.

The heightened BAL eosinophilia, following blockade of TGF- β , can be attributed to the decreased regulatory T cell populations and elevated IL-33 levels. Transfer of IL-10 to the lungs and adoptive transfer of IL-10-transfected T cells block eosinophilic inflammation in the airways of mice (Stampfli et al., 1999;Oh et al., 2002) and CD4⁺CD25⁺ Tregs also suppress OVA-induced eosinophilia (Kearley et al., 2005;Joetham et al., 2007;Kearley et al., 2008). This is not specific to OVA as CD4⁺CD25⁺FoxP3⁺ regulatory cells attenuate *Der p* 1-induced eosinophilia in the lung (Leech et al., 2007). However, mast cell activity was not altered by the blockade of TGF- β , despite CD4⁺CD25⁺FoxP3⁺ Tregs having been shown to suppress mast cell degranulation, via interference with OX40-OX40L interactions (Gri et al., 2008). The increased IL-33 levels may also contribute to the heightened eosinophilia, since IL-33 has been shown to exacerbate eosinophil-mediated inflammation (Cherry et al., 2008;Pecaric-Petkovic et al., 2009;Chow et al., 2010;Stolarski et al., 2010). Indeed, with both doses of blocking Ab, elevated eosinophilia was observed in the BAL

together with heightened IL-33 production. However, the increased eosinophilia here was not observed as a consequence of increased IL-5 levels, as the blockade of TGF- β did not enhance pulmonary IL-5 production. This conflicts with Fattouh and colleagues, who observed elevated IL-5 levels in cultured splenocytes (Fattouh et al., 2008) and may reflect the limitations of using the *ex vivo* restimulations.

The findings reported in this Chapter are important in furthering our understanding of the mechanisms regulating both airway inflammation and the development of airway remodelling following HDM exposure. However, it is important to consider that the experimental system in which a mediator with a wide range of functions, such as TGF- β , is investigated likely influences certain observations. Previously, TGF- β neutralisation was shown to prevent the progression of airway remodelling in OVA challenged mice (McMillan et al., 2005), whereas this does not occur when HDM is used as the sensitising allergen. This is not completely unforeseen due to differences between the OVA and HDM models, whereby Th2 immune responses to OVA require peripheral sensitisation with alum, whereas with HDM sensitisation occurs in the airway mucosa. Thus, OVA and HDM are likely to possess distinct immunological profiles even though the same outcomes of airway inflammation, AHR and airway remodelling occur.

The complexity of understanding the role of TGF- β has also been illustrated when subtle differences in observations have occurred within both OVA and HDM models of allergic airways disease. Using different approaches to investigate the role of TGF- β , several studies have illustrated a role for TGF- β in regulating OVA-induced allergic airways disease. Nakao and colleagues showed in mice expressing Smad7 (an intracellular antagonist of TGF- β signalling) selectively in mature T cells developed enhanced AHR and pulmonary eosinophilia and IL-5 production in response to OVA compared to *wt* controls (Nakao et al., 2000). Schramm and colleagues observed in mice possessing impaired TGF- β signalling in T cells, via the expression of a dominant-negative TGF- β type II receptor, that these mutant mice showed increased AHR, pulmonary eosinophilia and IL-13 production in response to OVA (Schramm et al., 2003). Scherf and colleagues using mice heterozygous for the deletion of the TGF- β 1 gene showed elevated eosinophilia, goblet cell hyperplasia and pulmonary IL-4, IL-5 and IL-13 levels in response to OVA (Scherf et al., 2005). In contrast to

the Nakao and Schramm studies, Scherf and colleagues did not observe heightened AHR in response to OVA, indicating that 30% of the normal TGF- β levels can regulate OVA-induced AHR (Scherf et al., 2005). TGF- β neutralisation with a blocking Ab has also been shown to increase AHR in an acute OVA model with increased production of IL-4 and IL-13, however, these authors reported no increased airway eosinophilia (Alcorn et al., 2007). Indeed, the complexity of elucidating a systemic role for TGF- β was also underscored in this Chapter, whereby anti-TGF- β Ab administered concurrently with HDM sensitisation had no impact on the development of airway inflammation and AHR, whereas there was a clear loss of regulation of AHR when the Ab was administered therapeutically. Certainly, the role of TGF- β is likely to be influenced by the cytokine environment in the lung at the time of Ab administration, as following two weeks of HDM exposure Th2 cytokines and eosinophils are present in the lung (Gregory et al., 2009), whereas this is not the case in the preventative blocking experiment.

The results from the therapeutic intervention study accompany the fundamental roles for TGF- β and Tregs in maintaining lung immune homeostasis. However, despite the reduction in regulatory T cell numbers and elevation of both IL-13 and IL-33 production, the systemic neutralisation of TGF- β may also influence other key immunosuppressive mechanisms in the lung which may contribute to loss of regulation of AHR and eosinophilia. For example, mouse and human airway epithelial cells secrete TGF- β that inhibits DC maturation and subsequent T cell activation (Bilyk and Holt., 1993;Bleck et al., 2006;Mayer et al., 2008;Wang et al., 2009). Epithelial production of IL-10 and TGF- β also maintain the expression of the CD200 receptor (CD200R) on macrophages, which is important in lung homeostasis and for the resolution of myeloid cell activity (Snelgrove et al., 2008). Further to this, alveolar macrophages are poor APCs (Steinmuller et al., 2000;Janssen et al., 2008), and actively inhibit T cell responses in part via the secretion of TGF- β (Roth and Golub, 1993;Chelen et al., 1995;Balbo et al., 2001). Therefore, the therapeutic systemic blockade of TGF- β may increase T cell activation and DC activity, while reducing regulatory CD200R expression, which could also contribute to the worsened HDM-induced airway inflammation. For those reasons, to proceed forward from the experiments in Chapter 6, investigating the specific role of TGF- β signalling in a single cell type would further enhance our understanding of the role of TGF- β at a

specific cellular level in HDM-induced allergic airways disease. To date, cell-specific blockade of TGF- β signalling has been carried out in OVA-based models and should be pursued with HDM, as the observations may not directly translate between systems. Indeed, TGF- β secreted by T cells was shown to suppress immune responses to high doses of OVA (Haneda et al., 1999) and Th cells engineered to express latent TGF- β abolished OVA-induced AHR and airway inflammation (Hansen et al., 2000). Transgenic mice with selective expression of Smad 7 in mature T cells to block TGF- β signalling in T cells showed enhanced OVA-induced airway inflammation and AHR (Nakao et al., 2000). The Nakao study therefore accompanies a crucial role of TGF- β plays in maintaining T cell tolerance and homeostasis (Li et al., 2007). However, TGF- β is also secreted from epithelial cells and the impact on OVA-induced airway inflammation has also been assessed. In contrast to when TGF- β signalling in T cells was inhibited, blockade of TGF- β signalling in airway epithelial cells attenuated OVA-induced Th2 cytokine production and slowed the development of airway remodelling (Luo et al., 2010). These opposing roles of TGF- β , comparing T cell to epithelial signalling, further underscore the importance of TGF- β in modulating local inflammatory responses. However, epithelial blockade of TGF- β signalling when HDM is the sensitising allergen should be carried out as a pro-inflammatory and pro-remodelling role for epithelial TGF- β signalling with OVA, may not translate to HDM.

The data obtained in this Chapter also reveal an important insight into HDM-induced airway remodelling and the relationship with AHR. Despite the neutralisation of TGF- β there was no impact on features of airway remodelling, while AHR was elevated, suggesting that the degree of structural changes in the lung do not directly correlate with the severity of AHR. Indeed, eosinophils, which have been shown by some research groups to be the major source of TGF- β in asthmatics (Minshall et al., 1997; Vignola et al., 1997; Flood-Page et al., 2003), have also been shown to be dispensable for the development of HDM-induced airway remodelling *in vivo* (Fattouh et al., 2011). However, an important consideration with the interpretation of results from the experiments in this Chapter is that the other TGF- β family members contribute to airway remodelling and the continued presence of these mediators may explain why TGF- β neutralisation had no impact on airway structural changes. One such family member that has been implicated as a key factor in the development of

airway remodelling is activin-A. Adenoviral-induced over-expression of Smad 2 in airway epithelial cells resulted in enhanced HDM-induced airway remodelling and AHR, together with elevated production of activin-A and IL-25 (IL-17E). Neutralisation of activin-A *in vivo* attenuated airway remodelling and AHR together with a decrease in IL-25 (IL-17E) production (Gregory et al., 2010).

The findings from Chapter 6 address the role of TGF- β in the context of Th2 driven inflammation in the lung *in vivo* following exposure to the environmental allergen HDM. Here, TGF- β has been shown to contribute to the regulation of HDM-induced Th2-driven airway inflammation and it is thus likely that TGF- β plays a dual role in asthma by regulating both the inflammatory and injury repair responses. TGF- β may dampen and oppose the inflammatory response elicited upon HDM exposure while also initiating tissue repair cascades in order to protect the lung from inflammation and tissue damage. Overall the data from this Chapter suggests that targeting TGF- β with a blocking Ab in HDM-induced allergic airways disease is not a viable therapeutic avenue for the treatment of asthma, as there was no improvement in airway remodelling and there was a loss of regulation of the inflammatory response. The loss of regulation of HDM-induced airway inflammation following systemic neutralisation of TGF- β is supportive of the vital role TGF- β and Tregs play in suppressing allergen-driven Th2 inflammation in the lung.

Chapter 7 - General Discussion

The main findings from this thesis were that the TLR-TRIF signalling pathway and the protease activity of HDM played prominent roles in the immune response to the inhaled allergen and were vital for disease pathogenesis. In addition to this, TGF- β was shown to regulate HDM-driven airway inflammation and AHR, whereby systemic *in vivo* blockade resulted in elevated IL-13 and IL-33 levels, concomitant with a decrease in regulatory T cell numbers.

7.1. Murine models and relation to man

In the last several years murine models of allergic airways disease have moved from OVA sensitisation and challenge models, to those which use environmental allergens, such as HDM, that negate the requirement for adjuvant and provide a highly physiological system to study asthma pathology. HDM was selected for this thesis since this allergen is ubiquitous in the environment and exposure is almost unavoidable. Asthmatics are also not subjected to seasonal variations associated with other allergens, such as grass and pollens, and worldwide sensitisation to HDM is the strongest risk factor for the development of asthma (Sears et al., 2003). The establishment and manipulation of a HDM murine model of allergic airways disease, has allowed insights into underlying disease mechanisms, as well as allowing dissection of the roles of particular features of the allergen in disease pathogenesis. Furthermore, refinement and characterisation of this model, as well as improved understanding of the complex immune response to HDM, will allow for the development of novel therapeutic strategies to treat asthma.

The use of HDM *in vivo* will build on knowledge from OVA-based systems which may be misleading with regard to essential contributions from innate immune pathways and therefore has implications for understanding asthma pathogenesis. In addition to HDM, a diverse repertoire of other common allergens including ragweed, pollen, animal dander and cockroach are also associated with allergic asthma. The observations in this thesis regarding the roles of TLR signalling, proteases and TGF- β in the immune response to HDM likely translate to these other allergens since all the afore mentioned allergens possess intrinsic protease activity and upon inhalation first come into contact with the lung at the airway epithelium. However, it must be stressed that although these allergens in mice may produce similar characteristics of

asthma, such as AHR, airway inflammation and airway remodelling, the distinct nature of these allergens needs to be recognised.

Other research groups have also investigated HDM-induced allergic airways disease using different dosing protocols and time points of analysis which may influence the relative importance of different signalling pathways (Phipps et al., 2009; Hammad et al., 2009). However, these differences could reflect variations that might occur in humans and, rather than being contradictory, may provide key insights into subpopulations of people with asthma (Moore et al., 2010). For the purpose of this thesis, a continuous HDM exposure protocol was followed as this most closely replicates natural human exposure to environmental HDM and, rather than isolated *Der p* proteins, a complete HDM extract was utilised in these studies. However, future research should also continue to use purified natural or recombinant mite allergens in addition to whole mite extracts. Different allergens within HDM extracts may stimulate distinct aspects of the innate immune system and possess unique mechanisms of sensitisation, which may be difficult to delineate from whole HDM extracts. Recombinant *Der p* 2, and not a whole HDM extract, was utilised *in vitro* and *in vivo* by Trompette and colleagues to show that this allergen acted as an MD-2 homologue (Trompette et al., 2009) and thus to define mechanisms of sensitisation for *Der p* 5 and 7 that are currently unknown, single recombinant allergens should be used for these investigations.

7.2. HDM immune response: remaining questions

HDM allergy is strongly associated with asthma (Sears et al., 2003) and this is likely because HDM possesses multiple antigenic epitopes that can promote the immune response. Unpublished data from our laboratory suggests that the allergenicity of HDM is determined by a delicate balance between several components of the allergen. A separate batch of HDM obtained from a different source from the Greer HDM (used in this thesis), was observed to induce a comparatively low immune response *in vivo* compared to the Greer HDM, despite the non-Greer HDM possessing vastly greater protease activity. Upon further analysis of this allergen, the non-Greer HDM contained approximately a 100-fold less *Der p* 2 content than the Greer HDM. This can be expected to contribute to the functional *in vivo* immune

response as *Der p 2* has the highest rate of skin test positivity in HDM allergic patients (Kidon et al., 2011). It is therefore plausible that proteolytic activity contributes to the potency of *Der p 2*, since an increased protease activity alone does not correlate with an elevated immune response.

The observations from the non-Greer HDM studies suggest that the allergenic potency of HDM is determined by a delicate balance of several different allergenic epitopes and that no single feature of the HDM allergen dominates in the development of the immune response. This is further illustrated when LPS alone is administered to mice, whereby LPS administration does not recapitulate the full asthma phenotype and the inflammatory response differs markedly to that of HDM (Szarka et al., 1997). One possible scenario is that the protease activity of *Der p 1*, 3, 5 and 9 enhances the allergenicity of non-protease allergens, such as *Der p 2*, by facilitating easier access to TLR-expressing DCs residing in the airway mucosa. When *Der p 2* was administered *in vivo* by Trompette and colleagues and Ye and colleagues, *Der p 2* was shown to elicit the features of allergic airways disease when accompanied by an exogenous adjuvant (Trompette et al., 2009; Ye et al., 2011). Thus it may be that *Der p 2*, within a whole extract, is able to promote an immune response *in vivo* because of the adjuvant properties of the proteases *Der p 1*, 3, 6 and 9 (Chapman et al., 2007). In view of the finding that *Der p 1* can promote the activation of *Der p 3* (Dumez et al., 2008), the potency of other HDM allergens, such as *Der p 5* which is a lipid binding protein (Mueller et al., 2010b), may also be augmented by protease activity. Therefore, the synergistic effects of biologically active enzyme allergens on Th2 responses and inflammation may explain why HDM is the predominant allergen associated with atopic asthma. However, other important asthma-associated allergens are not proteolytic enzymes and it is therefore difficult to identify a unifying theory of allergenicity based on biological function. Clearly, both enzymatic and non-enzymatic allergens can mediate Th2 responses, inflammation, and asthma and there is also the further complication as to whether augmentation of these responses to mite allergens is influenced by asthma susceptibility genes or gene-environment interactions.

Several important questions remain concerning the precise contributions of PRRs and pathways, or combinations of receptor pathways that mediate Th2-driven immune

responses to HDM. Concurrent with the work described in this thesis several important PRRs and associated signalling molecules have been shown to be important in driving the immune response to inhaled HDM *in vivo*, most notably TLR4 (Hammad et al., 2009; Trompette et al., 2009), MyD88 (Phipps et al., 2009) and Dectin-2 (Barrett et al., 2011). Important roles for the MR (Deslee et al., 2002; Royer et al., 2010) and DC-SIGN (Hsu et al., 2010) present on DCs have also been shown *in vitro* in promoting the immune response to HDM allergens. In the future, studies should focus on the contribution of other PRRs to HDM immune responses including nucleotide-binding oligomerisation domain protein (NOD)-like receptors (NLRs). This is necessary because NLR polymorphisms have been associated with asthma (Hysi et al., 2005) and *Der p* 1 has recently been shown to trigger the assembly of NLRP3 inflammasome in keratinocytes (Dai et al., 2011). Although not investigated in this thesis, there are many structures within HDM, such as *Der p* 5 and 7 that are lipid-binding proteins (Mueller et al., 2010a; Mueller et al., 2010b). The receptor signalling pathways lipid binding proteins engage require identification because in addition to TLR activation, lipids can stimulate the activation of innate lymphocyte populations (Brutkiewicz, 2006). Carbohydrate structures, such as β -glucans are present in HDM (Douwes et al., 2000; Fahlbusch et al., 2003) and are also strong Th2 stimulators (Nathan et al., 2009). The precise carbohydrate moieties associated with allergens and the array of PRRs and signalling pathways they activate warrant further investigation. Moreover, studies are also required to determine the molecular and cellular mechanisms by which allergen engagement of PRRs, such as TLR4, preferentially drive Th2 inflammatory responses as opposed to either Th1, Th17 or tolerogenic responses, and whether these can be manipulated. It would also be necessary to determine if allergic diseases are associated with functional variants in allergen-activated PRRs or signalling pathways and which of these pathways is the most attractive therapeutic target.

A concept that has recently emerged is receptor cooperativity, which has been shown between TLRs and PAR2 *in vitro* and *in vivo*, in the context of orthomyxovirus and paramyxovirus infections (Nhu et al., 2009). Activation of PAR2 leads to intracellular signals that intersect with TLR/IL-1R signalling pathways (Fyfe et al., 2005; Ostrowska et al., 2007; Trinchieri and Sher, 2007; Uehara et al., 2008) and PAR2 activation has been shown to synergistically enhance LPS-induced IL-8 production in

HEK293 cells (Rallabhandi et al., 2008). PAR2 activation has also been shown to increase TLR3-driven expression of IL-8, whereas RANTES/CCL5 was suppressed in mucosal epithelial cell lines (Nhu et al., 2009). Both TLRs and PARs have been implicated in the development of HDM-induced allergic airways disease and in view of the fact that HDM possess both TLR and PAR ligands, it is plausible that this receptor interplay occurs in response to HDM *in vivo*. To test this hypothesis further, the effect of HDM on this receptor interplay should be investigated firstly *in vitro* in airway epithelial cell lines and then investigated in TLR4-PAR2 double KO mice. This *in vivo* experiment may also shed further light on how a dependence on a single receptor or pathway, such as TLR4, may be overridden in continuous uninterrupted HDM dosing protocols, as proteinase-rich microenvironments may influence the composition of infiltrating leukocytes. Moreover, receptor cooperativity may not be restricted to PARs and TLRs, as HDM can trigger CLRs, PAR2 and TLRs and these receptors share similar signalling pathways and molecules, specifically MAPK and NF- κ B (Jacquet, 2011).

HDM and associated proteases have not only been linked with the development of atopic asthma, but also to atopic dermatitis (Lee et al., 2010). Atopic dermatitis is an inflammatory skin disease which is commonly associated with atopic asthma and is characterised by genetic barrier defects and allergic inflammation (Lee et al., 2010). The mechanisms by which HDM exerts its effects on the airway epithelium likely translate to the skin in atopic dermatitis as HDM is also linked with the development of this disorder (Sanda and Yasue, 1991;Tupker et al., 1996;Tan et al., 1996;Ricci et al., 1999) and atopic dermatitis is characterised by epithelial barrier dysfunction (Cork et al., 2009). In view of this, *Der p* 1 has also been shown to be a danger signal via activation of the inflammasome on keratinocytes (Dai et al., 2011) and this may occur with airway epithelial cells in the lung.

To date, modelling asthma in mice has largely focused on immune-mediated mechanisms and phenotypic changes to mesenchymal cells and the products that they secrete. However, there is an opportunity to use these models to study the impact of structural and functional changes to the airway epithelium and translate these results back to human disease, as epithelial injury is frequently seen in asthmatics (Hackett and Knight., 2007;Bhure et al., 2009). In view of the finding that uric acid has been

shown to play a key role in HDM-induced airway inflammation (Kool et al., 2011), this DAMP may be released because of protease-induced epithelial damage and injury. It is also important to recognise that epithelial injury may occur as a consequence of chronic HDM exposure as opposed to acute exposure, since epithelial barrier function remained intact following a single dose of HDM *in vivo* (Turi et al., 2011) but not after multiple doses (Chapter 5). Since airway epithelial injury is associated with asthma, the development of therapies targeting epithelial injury and repair are an attractive proposition. The HDM model described in this thesis could be used for such a purpose, since impaired epithelial barrier function and epithelial TJ disorganisation was observed following chronic HDM exposure. Restoration of full barrier function has been seen when EGF and keratinocyte growth factor were applied to a damaged asthmatic epithelium *in vitro* and *in vivo* (Berlanga et al., 2002; Basuroy et al., 2006; Tillie-Leblond et al., 2007). Clinical trials are also currently progressing to investigate the effect of surfactant as a potential epithelial barrier treatment for chronic asthma, by preventing environmental allergens from damaging the airway wall (Babu et al., 2003).

7.3. Asthma Therapies: Where next?

One of the key aims of this thesis was to relate properties of the HDM allergen to the pathological features of the disease and a vital role of the intrinsic protease activity of the HDM extract to the development of the immune response was determined. Also from this thesis, as well as from the current literature, inhibitors of the innate pro-allergic cytokines IL-25 (IL-17E), IL-33 and TSLP, or the respective receptors for these cytokines, may offer new therapeutic options for the treatment of atopic asthma. These cytokines were routinely elevated in HDM exposed mice, and boiling of HDM, to inactivate proteases dramatically reduced the production of these cytokines, together with reduced airway inflammation and remodelling. In addition to this, a newly identified innate cell population that promotes the development of Th2 cell-dependent inflammation at mucosal sites in the gut may also provide a new therapeutic target. These new cell populations were termed either natural helper cells (NHCs) (Price et al., 2010), multi-potent progenitor type 2 (MPP^{type2}) cells (Saenz et al., 2010), nuocytes (Neill et al., 2010) or innate type 2 helper (Ih2) cells (Moro et al., 2010) and are required for the type-2 response to the helminthic parasite

Nippostrongylus brasiliensis (Neill et al., 2010). Both IL-25 (IL-17E) and IL-33 can induce the production of IL-4, IL-5 and IL-13 (Neill et al., 2010) from these cells and this has raised important questions about the mechanisms by which cells of the innate immune system promote adaptive Th2 responses. In addition to being sources of Th2 cytokines, NHCs, MPP^{type2}, nuocytes and Th2 cells may act as APCs. Therefore, it is important to determine whether these cells have a role in HDM-induced disease and whether this can be directly translated to humans to establish novel therapeutic targets.

Genomic studies have revealed the association between many genes and the development of asthma, such as orosomucoid 1-like 3 (*ORMDL3*) (Moffatt et al., 2007; Galanter et al., 2008) and IL-33 (Moffatt et al., 2010). Mice provide an invaluable tool in further dissecting a role of these genes in allergic airways disease because there are tools available for genetic manipulation in mice and key asthmatic parameters in mice, such as AHR, are well established. Mice also have the advantage over other rodents in that mice share a similar chromosomal arrangement with humans, therefore allowing for a more accurate translation back to the human scenario (Seldin, 2001). In addition to this, gene expression can also be carried out in a tissue or cell specific manner and this is especially important with asthma susceptibility genes that are located in the airway epithelium. The HDM model of allergic airways disease described in this thesis could be used to investigate the effects of manipulating the expression levels of asthma susceptibility genes on the development of disease. Besides genetic factors that may predispose individuals to atopic diseases, considerations should also be made to the environment, age, sex and exposure to viruses and bacteria, as these factors may influence epithelial-allergen interactions.

Indeed, studies have been carried out to investigate the complex interactions behind the development of asthma. Utilising a murine model of HDM-induced allergic airways disease, acute infection with influenza A was observed to promote allergen responsiveness in mice at an age approximately equivalent to the first 2 years of life in humans (Al-Garawi et al., 2011). The study from Al-Garawi and colleagues accompanies the findings that co-exposure to air pollution, LPS, or bacterial and viral TLR ligands significantly enhance inflammatory responses to either OVA or

cockroach extracts (Hamada et al, 2000;Kulhankova et al., 2009;Phipps et al., 2009). However, conversely to respiratory viral infections, a lack of early exposure to microbial antigens is believed to contribute to the increasing incidences of asthma in developed countries because microbial antigens may contribute to the maturation of the pulmonary immune system (Blaser and Falkow, 2009). Arnold and colleagues have recently reported that early infection of mice with the gastric pathogen *Helicobacter pylori* increased the number of Tregs in the airways that prevented the development of both OVA and HDM-induced allergic airways disease (Arnold et al., 2011). Dietary patterns are also believed to participate in the development of asthma, whereby a lack of vitamin D in the diet is likely to contribute to the high prevalence of vitamin D deficiency (54%) and insufficiency (86%) associated with asthma in inner-city African-American children (Freishtat et al, 2010). Decreased respiratory tract infections in children receiving vitamin D supplementations have also been reported (Majak et al., 2011).

A high proportion of asthmatics respond therapeutically to inhaled corticosteroids and long-acting β_2 -adrenoceptor agonists. However, a significant number of asthmatics respond inadequately to the current mainstay of asthma therapy. Substantial efforts have since been made to develop new asthma therapies targeting inflammatory cytokines associated with asthma, such as IL-5. However, it is clear that anti-IL-5 therapy was only efficient in suppressing eosinophilia in the mild asthmatics and not severe asthmatics (Leckie et al., 2000;Flood-Page et al., 2007;Haldar et al., 2009;Nair et al., 2009). This has led onto the development of more patient-specific therapies, especially since the different subtypes of asthma are believed to have distinct underlying pathophysiological mechanisms (Moore et al., 2010). One such example of new therapeutic effort has been in the development of allergen-specific immunotherapy that was designed to treat the underlying cause of the allergic disorder and is aimed at promoting immunological tolerance (Larche et al., 2006;Focke et al., 2010;Frew, 2010). Allergen-specific immunotherapy has been shown to provide long term clinical benefits, including long-term disease remission, prevention of new atopic sensitisations and a reduction in disease progression from rhinitis to asthma (Keles et al., 2011). HDM-directed immunotherapy has been shown to improve lung function in HDM-allergic asthmatics (Abramson et al., 2010) and decrease HDM-specific IgE levels (Blumberga et al., 2011). Certainly, allergen-

specific immunotherapy holds great promise for HDM-allergic patients. However, careful considerations should be made in the design of immunotherapy as patients within the ‘HDM-allergic’ category may be more sensitive to the minor allergens, such as *Der p* 10, as opposed to the major allergens *Der p* 1 and 2 (Resch et al., 2011).

7.4. Concluding Remarks

There is an urgent need for more tailored and specific therapies for the treatment of asthma. This is so because it has emerged that asthma encompasses a range of different disease phenotypes, each with a distinct underlying cause and therefore a single treatment may not be sufficient to adequately treat all asthmatics. Certainly, one of the major obstacles in improving treatments for asthma is an incomplete understanding of the underlying disease mechanisms. The characterisation and development of murine models of allergic airways disease utilising environmental allergens, such as HDM, represent a significant step forward in a bid to delineate the mechanisms of allergic sensitisation and to identify new therapeutic targets for atopic asthma. There are also the added advantages that the mechanisms of allergic sensitisation to HDM translate to other allergens associated with atopic asthma, since all inhaled allergens interact with the airway epithelium in the lung, and that these observations also relate to atopic dermatitis and rhinitis because HDM is associated with the development of these conditions.

The mechanisms underlying the development of airway remodelling remain to be elucidated and this is important for chronic asthma sufferers. The focus for the immediate future should be on the role of structural components of the airway, in particular the airway epithelium, on the development of airway remodelling. The characterisation of a HDM model of allergic airways disease will allow for the mechanisms of airway remodelling to be further investigated by building on knowledge from previous OVA-based models and allow for the development of novel therapeutic targets, thus opening up a new era in drug discovery.

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Publications relating to this thesis

Inhaled house dust mite induces pulmonary Th2 cytokine production

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Background: Inhaled house dust mite results in Th2 type pathology in unsensitised mice, in conjunction with airway hyperreactivity and airway remodelling. However the pulmonary cytokine and chemokine profile has not been reported. **Methods:** We have performed a time course analysis of the characteristic molecular mediators and cellular influx in the BAL and lung in order to define the pulmonary inflammatory response to inhaled HDM extract. Mice were exposed five times a week to soluble HDM extract for three weeks. Lung function was measured in groups of mice at intervals following the final HDM challenge. Recruitment of inflammatory cells and inflammatory mediator production was then assessed in BAL and lungs of individual mice. **Results:** We found that Th2 cytokines were significantly increased in BAL and lung after HDM challenge from as early as 2 hours post final challenge. The levels of cytokines and chemokines correlated with the influx of eosinophils and Th2 cells to the different compartments of the lung. However, the production of key cytokines such as IL-4, IL-5 and IL-13 preceded the decrease in airways resistance. **Conclusion:** Inhaled HDM challenge induces a typical Th2 inflammatory mediator profile in the BAL and lung. These data are important for studies determining the efficacy of novel treatment strategies for allergic airways disease.

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