Immunoregulatory therapies for inflammatory diseases

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Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository**, subject to the provisions of the Copyright Act 1968.

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Statement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers, or carried out in other institutions. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

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To these people and four-legged friends, I dedicate my thesis.

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Synopsis

Respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and influenza affect millions of people worldwide. The lack of effective therapies for these diseases means there is an urgent need to understand the immune mechanisms underpinning these diseases in order to develop more effective therapies.

Asthma is a chronic inflammatory disease of the airways characterised by recurring symptoms of wheezing, coughing and chest tightness. The development and progression of asthma is primarily due to the actions of activated mast cells, eosinophils and type 2 helper (Th2) lymphocytes upon exposure to allergens. These cells release mediators that result in inflammation, oedema and mucus hypersecretion, which are often accompanied by airway hyper-responsiveness (AHR). These features collectively lead to narrowing of the airways and airflow obstruction.

Chronic obstructive pulmonary disease (COPD) is a progressive lung disease characterised by chronic bronchitis, emphysema and airflow limitation, which is not fully reversible. Active smoking of tobacco products remains the most important risk factor for COPD. Recruitment of inflammatory cells and mediators as a result of cigarette smoking leads to oxidative stress and a disruption in the balance between proteases and anti-proteases. Collectively this leads to parenchymal destruction, airway remodelling, narrowing of the airways and ultimately airflow limitation.

Influenza is one of the most important respiratory viral infectious diseases in the world. The virus causes annual epidemics and regular pandemics. Frequent genetic mutation of influenza viruses limits the efficacy of current vaccines and antiviral drugs.

The common theme and aim of my PhD was to investigate the role of novel immune pathways and factors that may underpin the development of asthma, COPD and control the severity of influenza virus infection. The first study demonstrates a novel role

for RelB expression in dendritic cells (DCs) in the development of allergic airway inflammation (AAI). Genetic deletion of RelB led to increased inflammatory cell influx, chemokine and Th2-associated cytokines in the lungs, and airway remodelling that is independent of allergen exposure. Adoptive transfer of RelB-sufficient DCs ameliorated AAI.

The second study investigated the benefits of targeting protein phosphatase 2A (PP2A) and the ubiquitin proteasome system (UPS) using immunomodulatory drugs in allergic airway disease (AAD). Enhancing PP2A activity with 2-amino-4-(4-heptyloyphenol)-2-methylbutanol ($AAL_{(S)}$) is more efficient at suppressing hallmark features of AAD compared to fingolimod (FTY720), while inhibiting proteasome activity with bortezomib (BORT) suppresses certain features of AAD. Our study also demonstrates for the first time that enhancing PP2A and inhibiting proteasome activity at the same time has synergistic effects, and is able to suppress more features of AAD than either $AAL_{(S)}$ or BORT treatment alone.

The third study investigated the novel role of the anti-inflammatory molecule, tristetraprolin (TTP), in an experimental model of cigarette smoke (CS)-induced COPD. Our study identified a novel role for TTP in the pathogenesis of experimental COPD. We demonstrate that active TTP is able to reduce the severity of experimental COPD by suppressing CS-induced pulmonary inflammation, pro-inflammatory cytokine and chemokine expression, airway remodelling and lung function impairment.

The fourth study furthered our investigation into the role of TTP by investigating the importance of active TTP in a mouse model of influenza virus infection. We demonstrate for the first time that active TTP reduced the severity of infection by enhancing protective antiviral responses, decreasing pro-inflammatory cytokine and chemokine production, and suppressing phosphoinositide 3-kinase (PI3K) activity.

Collectively these studies have identified potential novel pathways and molecules that are implicated in asthma, COPD and influenza virus infection. Importantly, these studies have expanded our understanding of disease pathogenesis and demonstrate that therapeutically targeting these pathways and molecules may be novel therapeutic avenues for these respiratory diseases.

Publications arising from this thesis

Publications prepared for submission

These publications form the basis of this thesis:

- **Nair PM**, Starkey MR, Haw TJ, Ruscher R, Maradana MR, Thomas R*, O'Sullivan BJ*, Hansbro PM*. RelB-deficient dendritic cells promote the development of spontaneous allergic airway inflammation. Prepared for submission to *Journal of Immunology*. Presented in chapter 2 of this thesis.
- **Nair PM**, Starkey MR, Haw TJ, Liu G, Horvat JC, Verrills NM, Morris JC, Clark AR, Ammit AJ, Hansbro PM. Enhancing PP2A and inhibiting proteasome activity ameliorates features of allergic airway disease. Prepared for submission to *European Respiratory Journal*. Presented in chapter 3 of this thesis.
- **Nair PM**, Starkey MR, Haw TJ, Liu G, Clark AR, Ammit AJ, Hansbro PM. Enhancing tristetraprolin activity reduces the severity of cigarette smoke-induced experimental chronic obstructive pulmonary disease. Prepared for submission to *American Journal of Respiratory Cell and Molecular Biology*. Presented in chapter 4 of this thesis.
- **Nair PM***, Starkey MR*, Haw TJ, Dua K[1], Deane A[1], Liu G[1], Ammit AJ, Clark AR, Hansbro PM. Enhancing tristetraprolin activity reduces the severity of influenza virus infection in mice. Prepared for submission to *Infection and Immunity*. Presented in chapter 5 of this thesis.

* denotes equal contribution to the manuscript

Other publications

- Liu G, Cooley MA, Jarnicki AG, Hsu AC, **Nair PM**, Haw TJ, Fricker M, Gellatly SL, Kim RY, Inman MD, Tjin G, Wark PA, Walker MM, Horvat JC, Oliver BG, Argraves WS, Knight DA, Burgess JK, Hansbro PM. Fibulin-1 regulates the pathogenesis of tissue remodelling in respiratory diseases. *JCI Insight*. In press 2016 June 16.
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Conference publications

- **Nair PM**, Starkey MR, Haw TJ, Liu G, Clark AR, Ammit AJ, Hansbro PM. Tristetraprolin protects mice against cigarette-smoke induced experimental COPD. Thoracic Society of Australia and New Zealand (TSANZ, 2016).
- **Nair PM**, Starkey MR, Haw TJ, Ruscher R, Maradana MR, Thomas R*, O'Sullivan BJ*, Hansbro PM*. RelB-deficient dendritic cells promote development of spontaneous allergic airway inflammation in mice. European Congress of Immunology, 2015.
- Haw TJ, Starkey MR, **Nair PM**, Hanish I, Nguyen DH, Liu G, Inman M, Kim RY, Collison A, Horvat JC, Foster PS, Yagita H, Mattes J, Hansbro PM. Tumor necrosis factor-related apoptosis-inducing ligand promotes the development of experimental chronic obstructive pulmonary disease. European Congress of Immunology, 2015
- Haw TJ, Starkey MR, **Nair PM**, Hanish I, Nguyen DH, Liu G, Inman M, Kim RY, Collison A, Horvat JC, Foster PS, Yagita H, Mattes J, Hansbro PM. Tumour necrosis factor-related apoptosis inducing ligand promotes the development of experimental chronic obstructive pulmonary disease. American Association of Immunologists (AAI, 2015). J Immunol;194:64.4 2015
- **Nair PM**, Starkey MR, Haw TJ, Ruscher R, Maradana MR, Thomas R*, O'Sullivan BJ*, Hansbro PM*. RelB deficiency promotes allergic airway inflammation in mice. Australasian Society of Immunology (ASI, 2014).
- Liu G, Jarnicki AG, **Nair PM,** Haw TJ, Fricker M, Gellatly S, Kim R, Inman M, Tjin G, Horvat JC, Oliver B, Knight DA, Burgess J, Hansbro PM. Fibulin-1 plays a critical role in the pathogenesis of chronic obstructive pulmonary disease (COPD). Australasian Society of Immunology (ASI, 2014).

- Haw TJ, Starkey MR, **Nair PM**, Hanish I, Nguyen DH, Liu G, Inman M, Kim RY, Collison A, Horvat JC, Foster PS, Yagita H, Mattes J, Hansbro PM. Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) promotes pulmonary inflammation and emphysema-like alveolar enlargement in experimental COPD. Australasian Society of Immunology (ASI, 2014).
- Starkey MR, Hanish I, Dua K, Hsu A, **Monogar P**, Foster PS, Kinght DA, Horvat JC, Wark PA, Hansbro PM. Interleukin-13 predisposes mice to more severe influenza infection by suppressing interferon responses and activating microRNA-21/PI3K. International Cytokine and Interferon (ICIS) Annual Meeting, 2014.
- Starkey MR, Hanish I, Dua K, Hsu A, **Monogar P**, Foster PS, Kinght DA, Horvat JC, Wark PA, Hansbro PM. Interleukin-13 predisposes mice to more severe influenza infection and exacerbated allergic airways disease. American Thoracic Society, 2014.

* denotes equal contribution to the manuscript

Conference presentations

- Oral presentation at 11th Annual Newcastle Asthma meeting, Newcastle, Australia, October 2015. Title: Role of tristetraprolin in respiratory diseases
- Oral presentation at 4th European Congress of Immunology, Vienna, Austria 2015. Title: RelB-deficient dendritic cells promote development of spontaneous allergic airway inflammation in mice. *(presented by Dr.Malcolm Starkey).*
- Oral presentation at Australian Respiratory Virology meeting, Canberra, Australia 2015. Title: Tristetraprolin is protective against influenza virus infection in mice. *(presented by Dr.Malcolm Starkey).*
- Oral presentation at 10th Annual Newcastle Asthma meeting, Newcastle, Australia, October 2014. Title: Role of RelB in allergic airway inflammation
- Oral presentation at 44th ASI annual meeting, Wollongong, Australia, December 2014. Title: RelB deficiency promotes allergic airway inflammation in mice

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Abbreviations

AAD: Allergic airway disease

$AAL_{(S)}$: 2-amino-4-(4-heptyloyphenol)-2-methylbutanol

AhR: Aryl hydrocarbon receptor

AHR: Airway hyperresponsiveness

APC: Antigen presenting cell

ARE: AU-rich element

ATP: Adenosine triphosphate

AU: Adenosie and uridine

BAL: Bronchoalveolar lavage

BM: Basement membrane

BMAL: Aryl hydrocarbon receptor nuclear translocator-like

BORT: Bortezomib

cDNA: Complementary deoxyribonucleic acid

cRNA: Complementary ribonucleic acid

CARD: Caspase activation and recruitment domain

Cbl-b: Casitas B lineage lymphoma B

CCCH: Cysteine-cysteine-cysteine-histidine

CCL: Chemokine (C-C motif) ligand

CD: Cluster of differentiation

CLOCK: Clock circadian regulator

COPD: Chronic Obstructive Pulmonary Disease

COX-2: Cyclooxygenase-2

CS: Cigarette smoke

CXC: Chemokine (C-X-C motif) ligand

DCs: Dendritic cells

DNA: Deoxyribonucleic acid

dpi: Days post infection

ELISA: Enzyme-linked immunosorbent assay

ERK: Extracellular signal-regulated kinase

FcεRI: High affinity IgE receptor

FACS: Fluourescence activated cell sorting

FEV_1: Forced expiratory volume in 1 second

FVC: Forced vital capacity

FTY720: Fingolimod

GM-CSF: Granulocyte macrophage colony-stimulating factor

GOLD: Global Initiative for Chronic Obstructive Pulmonary Disease

H&E: Haematoxylin and eosin

HA: Haemagglutinin

HDM: House dust mite

HPRT: Hypoxanthine-guanine phosphoribosyltransferase

IκB: Inhibitor of κB

ICAM-1: Intracellular adhesion molecule 1

IDO: Indoleamine 2,3-dioxygenase

IFN: Interferon

Ig: Immunoglobulin

IKK: IκB kinase

IKK-i: IκB kinase-i

IL: Interleukin

ILC2: Type 2 innate lymphoid cells

i.n: Intranasal

i.p: Intraperitoneal

IPS-1: IFN-β promoter stimulator 1

LCMT1: Leucine carboxyl methyltransferase 1

IRF7: IFN regulatory factor 7

LPS: Lipopolysaccharide

LTβR: Lymphotoxin beta receptor

MACS: Magnetic-activated cell sorting

MAPK: Mitogen-activated protein kinase

mDCs: Myeloid dendritic cells

MG-132: carbobenzoxy-L-leucyl-L-leucinal

MHC: Major histocompatibility complex

MID1: Midline 1

miRNA: Micro RNA

MK2: MAPK-activated protein kinase 2

mRNA: Messenger ribonucleic acid

MMP: Matrix metalloproteinase

MSC: Mucus secreting cells

Muc5AC: Mucin 5AC

NA: Neuraminidase

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NIK: NF- κB inducing kinase

NK: Natural killer

NLRP3: NOD-like receptor family pyrin domain containing 3

NS: Non structural

Nup475: Nuclear protein 475

OVA: Ovalbumin

PA: Polymerase acidic

PAMP: Pathogen-associated molecular pattern

PAS: Periodic acid-Shiff

PB: Polymerase basic

pBEC: Primary bronchial epithelial cell

PBMC: Peripheral blood mononuclear cell

pfu: Plaque forming units

PKC: Protein kinase C

PI3K: Phosphoinositide 3-kinase

PIP_2: Phosphatidylinositol 4,5-bisphosphate

PIP_3: Phosphatidylinositol (3,4,5)-trisphosphate

PPP: Phosphoprotein phosphatase

PP2A: Protein phosphatase 2A

PRR: Pattern recognition receptor

PTP: Protein tyrosine phosphatase

qPCR: Quantitative polymerase chain reaction

RIG-I: Retinoic acid-inducible gene I

RNA: Ribonucleic acid

RNP: Ribonucleoprotein

ROS: Reactive oxidant species

s.e.m: Standard error of the mean

siRNA: Small interfering RNA

SIPR1: Sphingosine-1-phosphate receptor

SNP: Single nucleotide polymorphism

STAT6: Signal transducer and activator of transcription-6

TANK: TRAF family member-associated NF-κB activator

TBK1: TANK binding kinase-1

TGF-β: Transforming growth factor beta

Th: T helper lymphocyte

TLR: Toll-like receptor

TNF: Tumour necrosis factor

TRAF: TNF receptor associated factor

TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand

Tregs: Regulatory T cells

TSLP: Thymic stromal lymphopoietin

TTP: Tristetraprolin

TIS11: TPA-induced sequence 11

UCHL-1: Ubiquitin carboxyl-terminal hydrolase L1

US: United States

VCP: Valosin-containing protein

WT: Wild-type

ZFP36: Zinc finger protein 36

γδ T cell: Gamma delta T cell

CHAPTER 1:

Introduction

In this chapter, I review the literature on the epidemiology, pathogenesis and immune responses associated with asthma, chronic obstructive pulmonary disease (COPD) and influenza virus infection. I also provide important background information on the key immune pathways and factors such as RelB, protein phosphatase 2A (PP2A), the ubiquitin proteasome system (UPS) and tristetraprolin (TTP) that are explored in this thesis. The rationale and aims of the project are described at the end of this chapter.

1.1 Asthma

1.1.1 Epidemiology of asthma

Asthma is a chronic inflammatory disease of the airways that has doubled in prevalence over the past three decades, although this increase has now plateaued. Global estimates of asthma prevalence suggests that 300 million people have asthma worldwide and an additional 100 million people are predicted to be diagnosed with this disease by 2025 [1]. Approximately 8% of the population in the United Stated (US) were diagnosed with asthma in 2009 with prevalence higher in children than in adults, and around 9 people dying from asthma each day in the US [2]. In Australia, approximately 10% of the population were diagnosed with asthma in 2004-2005 with higher prevalence in females than males, and 0.29% of all deaths in 2009 were reported to be due to asthma [1, 3]. Asthma is the main cause of school absenteeism among children and accounts for approximately 10 million hospital outpatient visits each year in the US [2, 4, 5]. In addition to morbidity, the economic cost of asthma is considerable. In Australia, asthma expenditure was $655 million with the greatest proportion attributed to prescription pharmaceuticals in 2008/2009 [6].

Current treatments for asthma include the use of corticosteroids and long-acting beta agonists that are generally effective in suppressing the symptoms of asthma. While they alleviate the disease symptoms, they do not treat the underlying cause of asthma and about 50% of asthmatics do not respond adequately to corticosteroid treatment [7, 8]. The importance of asthma and steroid resistance in subsets of patients means that there is an urgent need to develop more effective therapeutic strategies that targets the underlying mechanisms of pathogenesis.

1.1.2 Pathophysiology of asthma

Asthma is characterised by recurring symptoms of wheezing, coughing and chest tightness [9, 10]. Environmental factors, such as exposure to air pollutants and allergens are triggers of asthmatic episodes, termed exacerbations [11]. Familial clustering of asthma suggests the possibility of genetic predisposition. Multiple genes have been identified that have been linked to increased susceptibility to asthma including *ADAM33*, *PHF11*, *DPP10* and *TIM-1* [12-14]. The 'hygiene hypothesis' proposes that the lack of exposure to certain infections in early life increases the susceptibility to allergic disorders [15]. Improved hygiene, smaller family sizes and increased antibiotic use and vaccination leads to reduced childhood exposure to infectious agents in Western societies and these are proposed to contribute to the increased prevalence of asthma as well as other allergic disorders [16, 17].

The development and progression of mild to moderate allergic asthma is primarily considered to be due to the actions of activated mast cells, eosinophils and type 2 helper (Th2) lymphocytes upon exposure to allergens. The release of mediators by these cells results in bronchoconstriction, inflammation, oedema, structural changes such as hypertrophy and hyperplasia of smooth muscle and mucus hypersecretion [9, 10]. These changes are often accompanied by airway hyperresponsiveness (AHR), which is an increased susceptibility of the airways to constrictor agonists such as histamine and methacholine (**Figure 1.1**) [18, 19]. The degree of individual responses to challenge is thought to correlate with the clinical severity of allergic asthma. Several mechanisms have been postulated to influence AHR including inflammation and structural changes. While airway inflammation has been associated with the development of AHR, these features may also be independent of each other [18-20]. The continuous process of tissue damage and repair leads to airway remodelling with permanent changes in the airways that include

sub-epithelial fibrosis, thickening of sub-basement membrane and mucus gland hyperplasia and hypersecretion [21, 22]. These structural changes are often associated with progressive loss of lung function and are not fully reversible by current therapies [23, 24]. Collectively, these symptoms may result in hospitalisation and in severe cases death due to asphyxiation [25, 26].

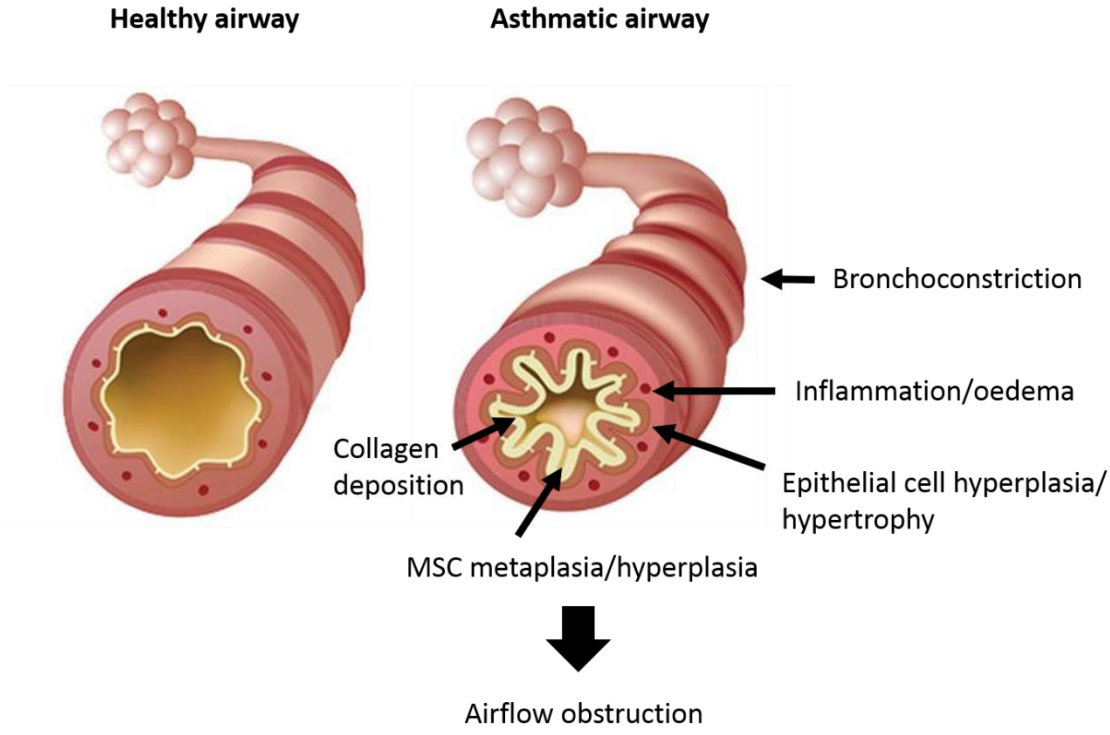


Figure 1.1: Pathophysiology of asthma

Schematic representation of the hallmark features of asthma in an asthmatic airway. Exposure to allergens leads to activation of inflammatory cells, which release mediators resulting in inflammation/oedema, bronchoconstriction and mucus hypersecretion. Chronic inflammation results in airway remodelling with epithelial cell hyperplasia/hypertrophy, mucus secreting cell metaplasia/hyperplasia and collagen deposition around the airways. These features collectively result in airflow obstruction. Figure incorporates images adapted from NIH Medline Plus [27]. *MSC: mucus secreting cells*

1.1.3 Immunopathogenesis of asthma

Asthma is a heterogeneous and complex disorder of the conducting airways characterised by aberrant immune responses to innocuous stimuli such as air pollution, respiratory infections, food products and inhaled allergens such as grass pollen, house dust mite (HDM) and cockroach antigens [11, 28]. The asthmatic response to different stimuli can be divided into early and late phase responses. The early phase response involves the activation of mast cells and release of mediators, which then leads to the initiation of the late phase response, characterised by the activation and infiltration of Th2 cells and eosinophils. Recently, the importance of epithelial cells and type 2 innate lymphoid cells (ILC2), has been implicated in the initiation and progression of asthma.

1.1.3.1 Early phase response

The early or immediate phase response occurs within minutes of allergen exposure and causes the activation of mast cells, which occurs through the cross linking of immunoglobulin E (IgE) to the high affinity IgE (FcεRI) receptors on the surface of these cells [29]. Antigen presenting cells (APCs) such as dendritic cells (DCs) that line the airway epithelium and submucosa play a crucial role in initiating the synthesis of IgE and the subsequent allergic response. Inhaled allergens are taken up and processed by DCs. These cells then migrate to the lung draining mediastinal lymph nodes where they present antigens to naïve T cells through the interaction between major histocompatibility complex (MHC) class II molecules on their surface and T cell receptors [30]. In the presence of the Th2 cytokine interleukin (IL)-4 and proper co-stimulation through the interaction of cluster of differentiation (CD)28 with CD80 or CD86 on DCs, naïve T cells differentiate into Th2 cells, which secrete cytokines such as IL-4, IL-5, IL-9, IL-13 and granulocyte macrophage colony–stimulating factor (GM-CSF) [10, 31]. These cytokines play a role in modulating the allergic responses mainly by ensuring Th2 cell survival (IL-4 *via*

a positive feedback loop), basophil recruitment (GM-CSF) as well as mast cell (IL-9, IL-13) and eosinophil (IL-5, GM-CSF) differentiation, maturation and survival [10, 31-33]. The presence of IL-4 and IL-13 promotes B cell switching from IgG to IgE antibody production that activates mast cells to release mediators such as histamine, prostaglandins and leukotrienes. The release of these mediators promote the induction of cellular and vascular leakage, bronchoconstriction and recruitment of inflammatory cells. (**Figure 1.2**) [30, 34]. Mast cells can also release cytokines such as IL-4 and IL-13, which are important in the late phase response [30].

1.1.3.2 Late phase response

The more persistent late phase response that typically occurs 4-6 hours after allergen exposure involves the activation and infiltration of leukocytes, consisting mainly of eosinophils, and Th2 cells into the sites of allergic reactions [10, 30]. Upon activation, eosinophils degranulate and release toxic granular proteins such as eosinophilic cationic protein, reactive oxygen species (ROS), lipid mediators, cytokines and chemokines. These promote inflammation, airway epithelial damage and increase vascular permeability [35, 36]. Th2 cells release the cardinal Th2-associated cytokines, IL-4, IL-5 and IL-13. IL-4 plays a role in epithelial hyperplasia [37, 38], B class switching and Th2 cell differentiation [30, 39, 40]. IL-5 is involved in eosinophil development, activation and recruitment [41], while IL-13 initiates Th2 responses, mucus production and AHR [10, 42-44]. IL-13 also induces the production of chemokines such as eotaxin that together with IL-5 recruits eosinophils into the lung. Chemokines such as chemokine (C-C motif) ligand (CCL) 2, CCL3, CCL4, CCL5, CCL17, chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10 and CXCL13 have all been implicated in allergic inflammation and found to be elevated in asthmatic patients [45]. These chemokines control the migration and activation

of the different leukocyte populations, such as eosinophils and lymphocytes during the asthmatic response [45].

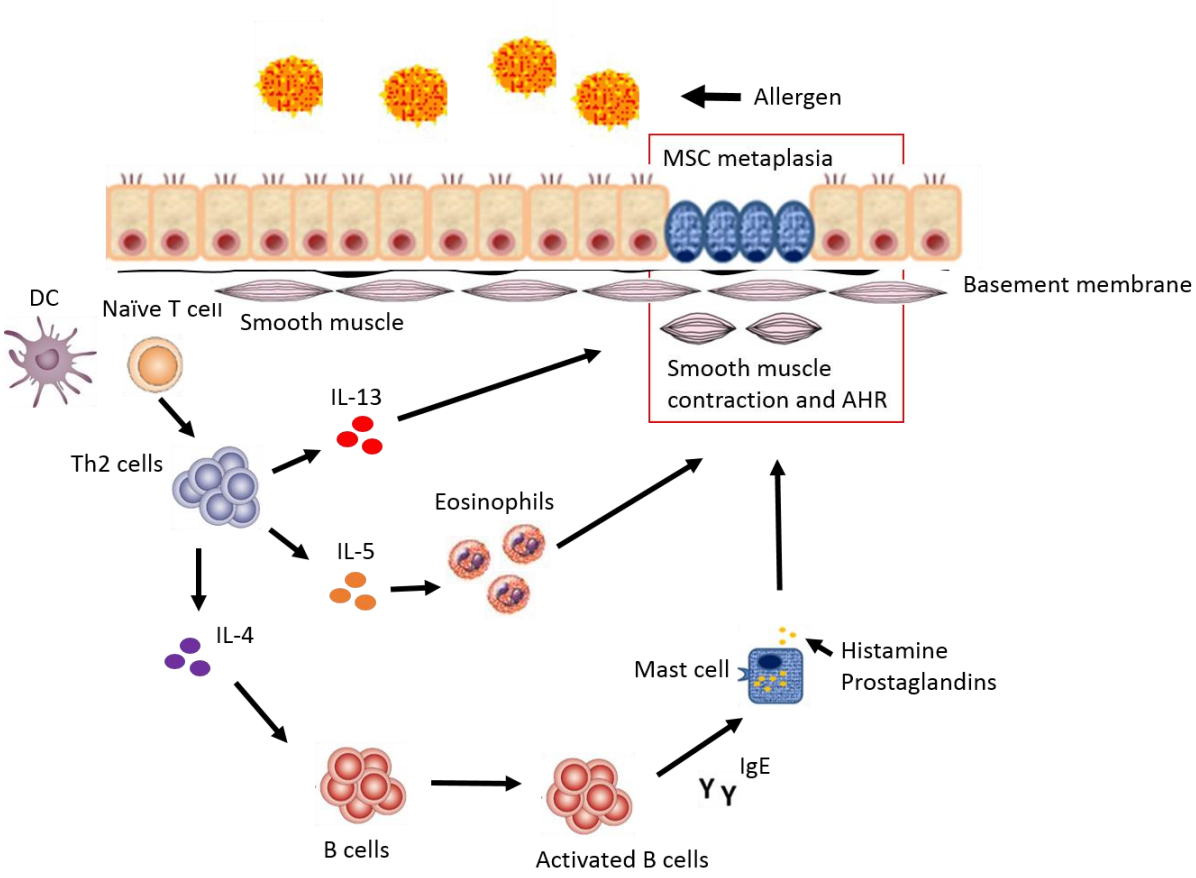


Figure 1.2: Immune responses in allergic asthma

Within minutes of allergen exposure, dendritic cells capture and present processed antigens to T cells. T cells induce the release of IgE from B cells, which binds to high affinity FcεRI on mast cells resulting in the release of histamine and prostaglandin mediators. A late phase response results in the infiltration of Th2 cells and eosinophils. Collectively, these events promote mucus secreting cell metaplasia and airway hyperresponsiveness. *AHR: airway hyper-responsiveness, MSC: mucus secreting cells, Ig: immunoglobulin, IL: interleukin, DC: dendritic cell*

1.1.4 Involvement of other cell types

Airway epithelial cells represent the first line of defence against allergens and microorganisms. They act as a link between innate and adaptive immune responses by detecting stimuli and secreting danger signals following the activation of pattern recognition receptors (PRR) [46]. Airway epithelial cells release cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) upon exposure to allergens [47]. These cytokines activate and expand ILC2s to produce IL-4 and IL-13 [48]. IL-25 plays a role in the development of allergic inflammation by promoting AHR, eosinophilia and mucus hypersecretion in mice [49, 50]. IL-33 leads to the production of Th2 cytokines and serum IgE and IgA. TSLP primes the differentiation of T cells into Th2 cells, through the costimulatory action of OX40 ligand expressed on DCs, triggering allergic inflammation [51, 52]. Impairment of the barrier function of the airway epithelium has been found in asthma. [53]. Continuous damage to the epithelium and incomplete repair processes lead to the secretion of growth factors, such as transforming growth factor beta (TGF-β) that subsequently promotes airway remodelling [54].

ILC2s have recently been identified as mediators of type 2 immune responses in the lung. ILC2s react to epithelium-derived cytokines IL-25, IL-33 and TSLP and produce Th2 cytokines (IL-4, IL-5 and IL-13) that eventually leads to the generation of eosinophils, mucus hypersecretion and AHR [48]. Local or systemic administration of IL-25 or IL-33 is able to induce the proliferation of ILC2s [55-57]. Mouse models of asthma have been shown to have increased ILC2s in the lung and bronchoalveolar lavage (BAL) fluid, and these represent a significant proportion of IL-5 and IL-13 producing cells in the lung [48, 56]. ILC2 have been found in nasal polyps of patients with chronic rhinosinusitis and lesional skin from patients with atopic dermatitis [58, 59]. These studies highlight that

ILC2s may be vital for the induction of allergic airway inflammation (AAI) and play a significant role in the pathogenesis of asthma.

While Th2 cells are the main cell type involved in the pathogenesis of mild to moderate allergic asthma, other cell types have also been implicated in this and other forms of the disease. Th1 cells that produce interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) have been associated with severe asthma and exacerbations [60, 61]. These and Th17 cells have been associated with neutrophilic inflammation and steroid-resistant asthma [62]. Cytokines produced by Th17 cells such as IL-17, IL-21 and IL-22 play important roles in neutrophil recruitment, airway remodelling and amplification of inflammatory responses [31, 63]. Another subset of T cells, natural killer (NK) T cells have also been implicated in asthma and are thought to be involved in the induction of AHR [64]. High levels of NKT cells have been detected in the sinus mucosa and sputum of asthmatics, suggesting their involvement in asthma [65, 66].

The heterogeneity and the complexity of the immune responses involved in the pathogenesis of asthma poses a major barrier to identifying effective therapeutic targets for this disease. Increasing our understanding of the mechanisms underlying the development and progression of the disease as well as investigating the different immunological processes involved in the pathogenesis of the disease could pave the way in finding effective preventative and therapeutic strategies for asthma.

1.1.5 Animal models of asthma

Animal models of asthma typically involve the induction of allergic airway disease (AAD) with allergens such as ovalbumin (OVA) or HDM extract, which is a clinically relevant allergen [67]. Acute OVA-induced AAD involves systemic sensitisation followed by airway challenges while acute HDM-induced AAD involves local HDM sensitisation followed by airway challenges [68-70]. These models induce the cardinal

features of AAD, similar to those observed in human asthma, including airway inflammation with increased numbers of eosinophils, elevated circulating levels of IgE, Th2 cytokine responses (IL-4, IL-5, IL-13), mucus hypersecretion and AHR [68-70]. Chronic models involving repeated exposure to low levels of allergens for prolonged periods (up to 12 weeks) are employed to investigate airway remodelling, another important feature of human asthma [71].

1.2 COPD

1.2.1 Epidemiology of COPD

COPD is a heterogeneous disease characterised by chronic airway inflammation, airway remodelling, emphysema and airflow limitation. It is currently the third leading cause of mortality and morbidity worldwide and affects approximately 65 million people [72]. COPD accounted for almost 5% of deaths or 3 million people globally in 2005 [73]. In Australia, COPD is one of the leading causes of death and hospitalisation and accounted for 4.2% of all deaths in the year 2003 [74]. COPD has also been listed as an associated cause of death and is often linked to other comorbidities such as cardiovascular disease, diabetes and recurrent respiratory tract infections [74]. COPD also causes significant economic burden. The health expenditure for COPD in Australia in 2008/2009 was estimated to be $929 million with the greatest proportion of expenditure attributed to hospital admissions [75]. There is currently no cure for COPD. Current mainstay therapies include glucocorticoids, long acting muscarinic antagonists and anti-cholinergic agents. These are only generally effective in managing the symptoms of COPD and do not treat the underlying cause of the disease [76]. Lifestyle changes such as smoking cessation help improve symptoms but do not halt the progression of the disease. Lung volume reduction surgery and lung transplantation may be an option in severe cases. However, these procedures are rare and not common due to the high-risk of the procedures and lack of organ donors [74, 77]. Hence, there is an urgent need to further understand the immunological mechanisms underlying COPD to facilitate the development of more effective therapies.

1.2.2 Pathophysiology of COPD

COPD is a complex disease comprising of airway inflammation that leads to chronic bronchitis, airway structural changes such as parenchymal destruction, loss of elastic recoil and airway remodelling, and most importantly airflow limitation, which is

largely irreversible and causes the hallmark features of breathing difficulties [78]. Active smoking of tobacco products remains the most significant risk factor for COPD. Not all smokers develop COPD, indicating the possibility that genetics may play a role in its development [79]. Host factors such as α1-antitrypsin deficiency predisposes an individual to COPD due to the imbalance protease/antiproteases that are crucial in protecting the lung from damage [80, 81].Other risk factors associated with the aetiology of COPD include occupational exposure (e.g. coal miners), exposure to outdoor pollution and poor indoor air quality due to the use of biomass fuel and cooking oil along with poor ventilation [82-84].

Airway inflammation in the lungs as a result of exposure to cigarette smoke (CS) or other inhaled irritants leads to the influx of a wide range of inflammatory cells and mediators including macrophages, neutrophils and $CD8^+$ T cells. These cells trigger a series of inflammatory cascades that result in oxidative stress and the disruption in the balance between proteases and anti-proteases [85-87]. Consequently, there is a breakdown in the connective tissue in the lung parenchyma and destruction of the tissue leading to the development of emphysema [88-90]. Inflammation also leads to mucocilliary dysfunction with accumulation of excess mucus in the airway [91]. Narrowing of the airways and the development of scar tissue due to damage to the airways over time leads to airway remodelling [92, 93]. All these effects eventually contribute to airflow limitation and breathing difficulties (**Figure 1.3**). The severity of airflow limitation in COPD patients is classified according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) standard based on FEV_1 (forced expiratory volume in 1 second) and FEV_1/FVC (forced expiratory volume in 1 second/forced vital capacity) parameters. Patients with FEV_1/FVC ratio lower than 0.7 and FEV_1 more than 80% predicted are classified as GOLD 1 or "mild" in terms of severity. Lower FEV_1 value indicates worse airflow

limitation and this value declines as COPD progresses. GOLD 2 indicating "moderate" severity is classified in patients with FEV_1 predicted between 50-79%, GOLD 3 with FEV_1 predicted between 30-49% indicating "severe" and GOLD 4 with FEV_1 predicted lower than 30% representing "very severe" COPD (**Table 1.1**) [79].

Table 1.1: GOLD classification of severity of airflow limitation in COPD

GOLD Classification	**Criteria ($FEV_1/FVC<0.70$)**	**Severity**
GOLD 1	$FEV_1 \geq 80\%$ predicted	Mild
GOLD 2	$50\% \leq FEV_1 < 80\%$ predicted	Moderate
GOLD 3	$30\% \leq FEV_1 < 50\%$ predicted	Severe
GOLD 4	$FEV_1 < 30\%$ predicted	Very severe

GOLD: Global Initiative for Chronic Obstructive Lung Disease, FEV_1: Forced expiratory volume in 1 second, FVC: Forced vital capacity

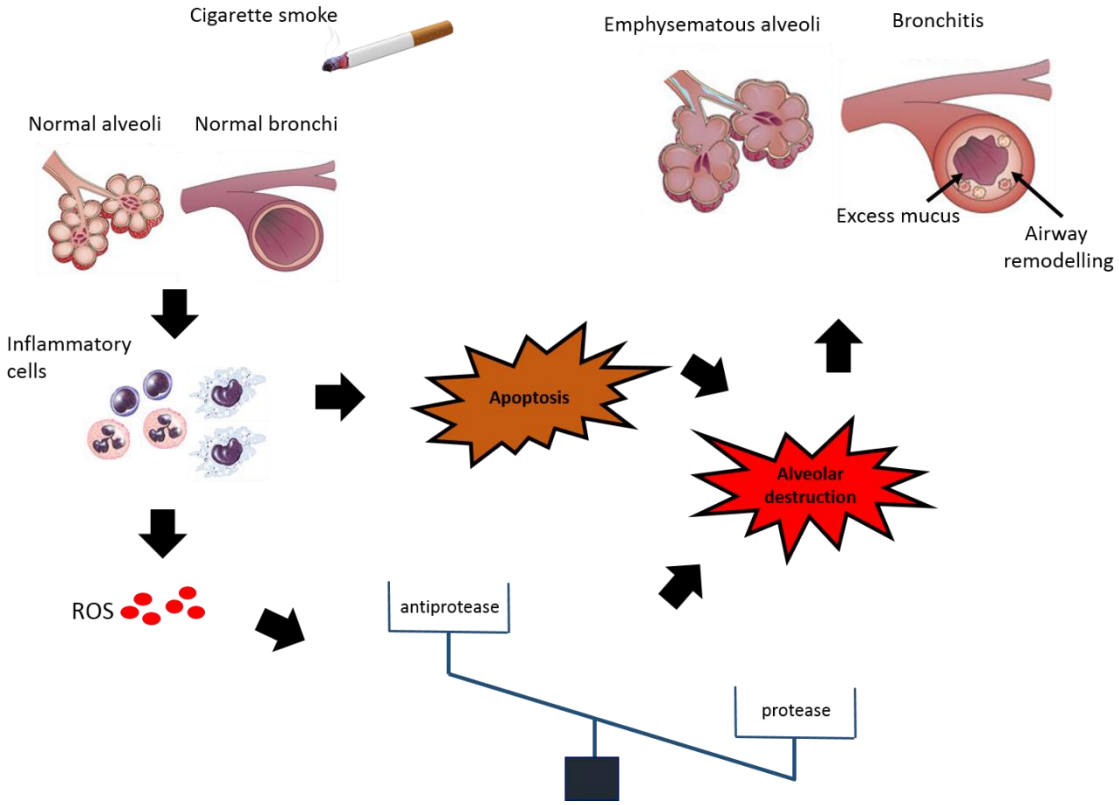


Figure 1.3: Pathogenesis of COPD

Exposure to cigarette smoke and other irritants results in the influx of inflammatory cells that secrete inflammatory mediators and reactive oxygen species. These lead to apoptosis of target cells and protease-antiprotease imbalance that results in loss of parenchymal elastic recoil and subsequently alveolar destruction. Collectively, these events lead to emphysematous destruction in the lung, bronchitis, excess mucus and airway remodelling that ultimately results in lung function decline. *ROS: reactive oxygen species*

1.2.3 Immunopathogenesis of COPD

COPD is a disease with complex pathology involving multiple intricate immunological mechanisms. The airway epithelium represents the first line of defence against exposure to irritants such as CS. Exposure of these cells to CS reduces their barrier function and the epithelial integrity of the epithelium [94, 95]. The activation of epithelial cells by CS causes the release of inflammatory mediators such as IL-6, IL-8, IL-1β and GM-CSF [96]. Importantly, it can also induce the production of TGF-β that contributes to the development of fibrosis[97].

Upon exposure to CS or other irritants, there is an influx of inflammatory cells such as neutrophils and macrophages into the lungs. This has been shown in many studies of smokers and COPD patients [98-100]. As one of the key players in the development of COPD, neutrophils secrete proteinases such as neutrophil elastase, proteinase 3, cathespin G, matrix metalloproteinase (MMP)-8 and MMP-9 that contribute to alveolar destruction [96]. Further evidence of the role of these cells is that the number of neutrophils in induced sputum and biopsy specimens has been associated with disease severity and lung function decline [98, 101]. Activation of macrophages by CS induces the secretion of inflammatory mediators such as TNF-α, IL-8, ROS and proteases such as MMP-2, 9 and 12, cathespin K, L and S. Increased macrophage numbers has also been associated with the severity of COPD [102].

The exact role of DCs in disease development remains to be fully explored. Increased numbers of DCs have been observed in the airways of smokers [103]. Increased expression of CD80 and CD86 has also been reported in DCs from COPD patients [104]. It has been postulated that DCs take up antigenic materials from apoptotic or necrotic cells in the lung and present them to $CD8^+$ T cells, which are the predominant T cell subset in COPD lungs [105, 106].

The release of perforins and granzymes by $CD8^+$ T cells leads to the apoptosis and necrosis of target cells that contributes to parenchymal destruction [107]. Interestingly, $CD8^+$ T cells have been reported to be the source of IL-4 and IL-13 that contributes to mucus hypersecretion, a characteristic feature of chronic bronchitis [108]. Heightened inflammatory responses in COPD also leads to an imbalance in protease-antiprotease activity. This imbalance results in the breakdown of elastin, a major component of elastic fibres. The breakdown of elastin leads to the loss of parenchymal tissue and elastic recoil, resulting in emphysematous changes in COPD [96]. Inactivation of α1-antitrypsin by oxidants in CS has also been suggested to alter the balance of protease/antiprotease activity [109]. Although reduced activity of α1-antitrypsin in the BAL fluid of smokers has been previously reported [110], research on the function of α1-antitrypsin has not been fully elucidated. Increased numbers of neutrophils and macrophages in COPD result in increased elastase burden as a result of the release of proteases from these cells [96]. Increased elastase burden and proteolytic activity subsequently lead to emphysematous destruction of alveoli and consequently lung function decline [96].

Collectively, these studies highlight the ability of irritants, such as CS, to activate the airway epithelium and inflammatory cells to initiate an inflammatory response that consequently leads to remodelling, parenchymal destruction and lung function decline. COPD is a disease with complex pathology involving multiple intricate immunological mechanisms. A better understanding of the processes involved in disease development and progression would facilitate the discovery of more effective therapeutic strategies.

1.2.4 Animal models of COPD

As CS is the major risk factor for COPD in humans, it is crucial that animal models replicate the pathological effects of CS exposure. Many experimental models of CS exposure have been developed over the years. Acute exposure (nose-only or whole

body) are important in investigating the early CS exposure effects. However, these models do no induce airway remodelling, emphysema or lung function changes, which are important features of human COPD [111, 112]. Whole body exposures also do not reflect the typical mode of pathological exposure in humans. Chronic exposure of CS for 6 months are able to induce these key features, however the long duration of these models means that it can be very costly and labour intensive [112]. Very few studies assess impaired lung function a critical feature of human COPD. We have recently developed a novel short-term mouse model of nose-only CS-induced COPD that recapitulates key features of human COPD including chronic bronchitis, airway remodelling, emphysema and lung function impairment after 8 weeks of CS exposure [113]. Mice are exposed to 12 cigarettes twice per day for up to 12 weeks, with exposures equivalent to a pack-a-day smoker [112]. The development of key features of COPD in a short timeframe enables us to investigate and better understand the underlying mechanisms of COPD.

1.3 Influenza

1.3.1 Epidemiology of influenza

Influenza, which is a major cause of mortality and morbidity, is one of the most important respiratory viral diseases in the world. It was the cause of three major pandemics in the 20th century, namely the 1918 Spanish Flu, 1957 Asian flu and 1968 Hong Kong flu [114]. The 1918 pandemic was the most severe pandemic that resulted in an estimated death toll of between 50-100 million people [115]. It leads to establishment of this virus as the cause of seasonal influenza. Seasonal influenza poses a significant public health problem resulting in annual outbreaks, which peak during winter [116]. This has been thought to be due to increased viral transmission due to population crowding, indoor co-habitation and increased humidity [117, 118]. Influenza affects 5-10% adults and 20-30% children and causes approximately 250,000 to 500,000 deaths each year globally [119]. This leads to substantial economic burden due to loss of productivity, medical and indirect costs of preventative measures such as vaccination programs. In the US, the burden of influenza is estimated to be at $71-167 billion per year [120].

While there are currently vaccines available that are largely effective in preventing influenza, constant genetic changes in the virus means that vaccines must be reformulated each year. Treatments with antiviral drugs such as oseltamivir and zanamivir may reduce severe complications and death, but the development of resistance by some influenza viruses limits their efficacy and there is an urgent need for new treatments. The substantial mortality and morbidity rate, economic burden and issues with current treatments highlight the importance of developing new therapeutic strategies for influenza.

1.3.2 Influenza virus structure, genome and replication

Influenza viruses are ribonucleic acid (RNA) viruses of the Orthomyxoviridae family, which consists of three different types of viruses, Influenza A, B and C [121]. These viruses are distinguished based on the antigenic differences between their matrix and nucleoproteins. Influenza A viruses are the major human pathogens as a result of their ability to cause seasonal and pandemic outbreaks [122].

Influenza A viruses are enveloped single stranded RNA viruses that are approximately 80-120 nm in diameter [123]. The viral genome consists of eight single stranded RNA strands of negative polarity packaged within a viral envelope that contain the surface glycoprotein, haemagglutinin (HA) and neuraminidase (NA). The HA protein is an influenza virus antigenic site and binds to the sialic acid (N-acetylneuraminic acid) residues on host cells and participates in viral entry into target cells [124]. NA cleaves sialic acid from the HA molecule and releases newly formed virions from infected cells [125, 126]. NA is often the primary target for antiviral drugs. Genetic changes that occur in the genes encoding HA and NA result in the ability of the virus to evade the host immune system. "Antigenic drift" occurs when genomic mutations occur in antibody binding sites, resulting in a new strain of virus particle that are no longer recognised by the host immune system [127]. Another potentially more dangerous phenomenon known as "antigenic shift" occurs when genome segments from two or more viruses re-assort during viral replication [127]. This genetic re-assortment results in a completely new strain of influenza virus that has not been previously encountered by the host immune system, leaving the population vulnerable as they have little or no protection against the new strain. This may consequently lead to pandemics such as the 2009 H1N1 pandemic. Besides HA and NA, the viral genome also encodes for other proteins; the RNA polymerase subunits, polymerase basic (PB)1, PB2, PB1-F2 and polymerase acidic (PA) component, which are

responsible for replication and transcription [128], nucleoprotein that encapsulates the negative strand RNA [129], the matrix proteins M1 and M2 that play a role in protein conductance that is crucial for viral replication and the non-structural (NS) proteins (NS1 and NS2) that are involved in the export of virus ribonucleoprotein (RNP) from the nucleus [130-132].

There are several events that are involved in influenza viral replication. This process is thought to begin with viral entry through receptor-mediated endocytosis that occurs through the binding of viral HA to sialic acid receptors on the surface of infected cells [133]. In humans, this is initiated at epithelial surfaces lining the upper and lower respiratory tract. The acidic environment within the endosome triggers a conformational change of the HA protein, resulting in the release of viral RNP complex into the cytoplasm that is subsequently translocated into the nucleus [134]. In the nucleus, the viral RNPs transcribe the negative-sense viral RNAs into two forms of positive-sense RNA; viral messenger RNA (mRNA) and complementary RNA (cRNA). Through the utilisation of 5'-methylated cap structures belonging to host cells, viral mRNA are processed and exported from the nucleus into the cytoplasm to be translated into viral proteins [135]. In contrast, viral cRNA do not undergo this capping process and remain in the nucleus. They are instead utilised as templates for the synthesis of more viral negative sense RNAs. Synthesis of the envelope proteins HA, NA and M2 begin in the cytosol and are transported to the cell surface by the Golgi apparatus. During this phase, several modifications occur such as cleavage of HA by host proteases [134]. The newly formed virus remains on the host cell surface, until the host sialic acid residues are cleaved by NA, releasing the new virion from the cell surface (**Figure 1.4**) [136].

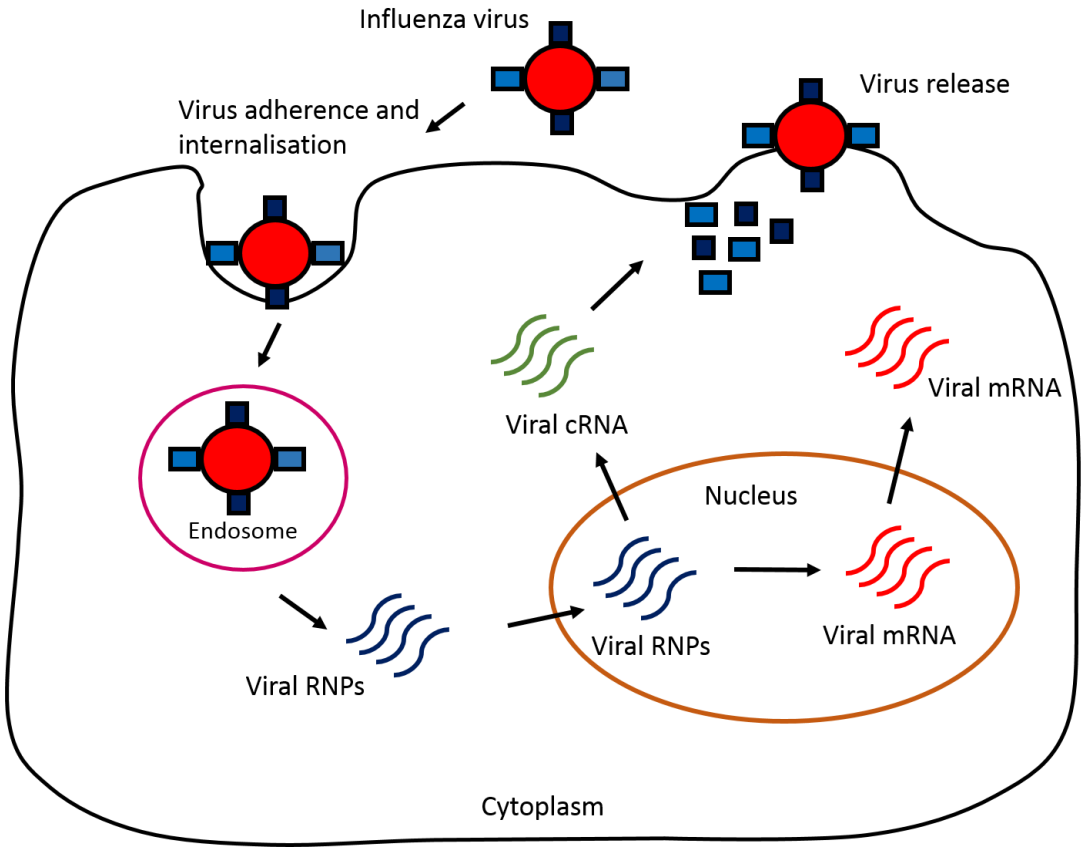


Figure 1.4: Influenza virus replication cycle in host epithelial cells

Influenza viruses adhere to epithelial surfaces lining the respiratory tract and are internalised into host cells. The low pH environment in the subsequently formed endosomes triggers a conformational change of the protein, resulting in the release of viral ribonucleoprotein (RNP) into the cytoplasm, which then translocates into the nucleus. The synthesis of messenger RNA (mRNA) and complementary RNA (cRNA) occurs in the nucleus. Viral mRNA are exported into the cytoplasm and transcribed into viral protein. Viral cRNA remain in the nucleus and are used as templates for the synthesis of new viral negative sense RNAs. Viral components are then transported to the cell surface and new virions are released from the cell surface. *RNP: ribonucoleoprotein, mRNA: messenger ribonucleic acid, cRNA: complementary ribonucleic acid*

Influenza viruses utilise the phosphoinositide-3-kinase (PI3K)/Akt pathway for entry and replication. An initial weak and transient activation of the PI3K signalling pathway occurs during the early phase of infection (15-30 minutes post infection), leading to viral endocytosis and subsequent release of viral genomes into infected cells [137]. A more sustained activation of the pathway occurs during the intermediate to later stages of infection (4-6 hours post infection) that is mediated by the viral NS1 [137]. NS1 binds to the p85β regulatory subunit, which leads to the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and ultimately the phosphorylation of Akt [137, 138]. These events in turn promote viral replication. Inhibition of PI3K by wortmannin and the inhibition of Akt by NH(2)-AVTDHPDRLWAWEKF-COOH suppressed viral entry and replication in infected A549 cells, by inhibiting the association of phosphatidylinositol with Akt and by decreasing the levels of phosphorylated NS1 [137, 139, 140]. Specific inhibition of the PI3K-p110α subunit with PI-103 repressed viral entry and enhanced anti-viral responses in both healthy and COPD primary bronchial epithelial cells (pBECs) infected with influenza viruses H1N1, H3N2 or H11N9 [141].

1.3.3 Pathophysiology of influenza

The pathogenicity and virulence of influenza viruses is determined by host factors such as the presence of specific target receptors on host cells and the ability of the host immune system to control viral replication. Viral factors such as the ability to bind to host cells and to evade the host immune system by evolution of antigenic variation also determine the pathogenicity and virulence of influenza viruses. In humans, the virus can be transmitted by direct contact with infected individuals, by aerosols and droplets or by contact with fomites. Upon exposure, the virus enters the host through the respiratory tract where the columnar epithelial cells of the lower respiratory tract is the initial target

of the virus. HA is thought to bind to sialic acid residues on the surface of host cell. The virus is then endocytosed and RNA components are transported into the nucleus for replication, transcription and translation. Viral replication typically occurs within hours after viral entry. The newly formed virions are released from the host cell by NA. Viral shedding typically continues for 5 to 10 days and may last longer in children and immunocompromised individuals [142].

The common symptoms of influenza such as headaches and fever are the result of the release of large amounts of cytokines and chemokines produced by infected cells. These cytokines include IL-6, TNF-α, IL-1β and IL-8 that induce the immune system to defend the host against the infection. The over-activation of immune cells results in an uncontrolled immune response and the excessive production of cytokines and chemokines [143, 144]. This 'cytokine storm' is strongly associated with influenza-induced mortality and was thought to be responsible for the death of young adults in the 1918 Spanish flu and the 2009 swine flu outbreaks [144]. Influenza virus infection also leads to AHR and secondary bacterial pneumonia [145-147]. Secondary bacterial pneumonia causes significant mortality and morbidity and is thought to be the major cause of death in the 1918 Spanish flu due to the lack of antibiotics at that time [148]. Disruption of epithelial cell barrier and decreased mucocilliary clearance during influenza have been associated with increased risk of bacterial infection [149, 150].

1.3.3.1 Innate immune responses to influenza

Innate immunity is the body's first line of defence against influenza virus infection. Viral RNAs are the main pathogen-associated molecular patterns (PAMPs) of influenza A viruses and their binding to host PRRs leads to the initiation of an immune response against the virus.

Several PRRs have been identified to play a role in mediating innate immune responses against influenza virus infection, namely Toll-like receptors (TLR)3 and TLR7, retinoic acid-inducible gene I (RIG-I), and NOD-like receptor family pyrin domain containing 3 (NLRP3) [151-154]. Binding of TLR3 activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), leading to the expression of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α [152]. Studies using TLR3$^{-/-}$ mice infected with influenza A virus show improved host survival and reduced chemokine expression in the lungs [155]. Similarly, TLR7 signalling *via* MyD88 activates NF-κB and IFN-regulatory 7 (IRF7) that stimulates the expression of type 1 IFNs [153]. TLR7 signalling has also been shown to be important in the induction of adaptive immunity, in particular the activation of B cell responses in response to influenza virus infection [156, 157].

RIG-I, the primary PRR involved in detecting influenza viruses, plays an important role in type I IFN production. [158]. RIG-I recognises the 5'-triphosphate viral single stranded RNA generated intracellularly during influenza replication. Binding of viral RNA to RIG-I exposes the caspase activation and recruitment domain (CARD), and this allows the domain to interact with IFN-β promoter stimulator 1 (IPS-1) [151, 159-161]. Interaction between IPS-1 and TNF receptor associated factor-3 (TRAF3) results in the activation of TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK-1) and inhibitor of κB (IκB) kinase-i (IKKi) that subsequently phosphorylates IRF7 and IRF3 to induce the production of type 1 (IFNα/β) and type III IFNs (IFN λ 1/2/3) [162, 163]. The production of these IFNs induces the production of more than 300 IFN-stimulated genes (ISGs) that are able to disrupt the viral life cycle by inhibiting protein synthesis, cleaving viral RNA and preventing viral assembly and egress [164]. In addition to disrupting the viral life cycle, ISGs are also able to promote antiviral signalling [165]

Activation of NLRP3 receptor converts pro-IL-1β to IL-1β that is involved in the expansion of $CD4^+$ T cells [166]. Mice deficient in NLRP3 and caspase 1 were more susceptible to influenza infection, highlighting the role of inflammasome in immunity against influenza virus [167, 168].

1.3.3.2 Adaptive immune responses to influenza

The uptake of influenza virus antigens by DCs leads to their presentation to $CD8^+$ T cells by the MHC class I molecules. Type I IFNs are able to enhance the expression of MHC class I and co-stimulatory molecules such as CD80 and CD86 [169]. The proliferation and activation of $CD8^+$ T cells subsequently leads to the apoptosis and elimination of infected cells through the activity of released perforins and granzymes [170]. Presentation of viral antigens by DCs through MHC class II molecules leads to priming of $CD4^+$ T cells that also play a role in controlling influenza virus infections. $CD4^+$ T cells expressing IFN-γ are important in antiviral responses [171, 172]. In addition, $CD4^+$ T cells aid in the production of neutralising antibodies in particular anti-HA and anti-NA by B cells [173]. Production of influenza specific immunoglobulins such as IgM, IgG and IgA play important roles in adaptive immunity against influenza viruses. IgM is involved in viral clearance while IgA has the ability to neutralise influenza virus intracellularly [174, 175]. IgG antibodies facilitate NK cell-mediated clearance and opsonise virus for uptake by alveolar macrophages [176].

1.3.4 Animal models of influenza

Animal models of influenza are crucial in understanding the various factors (both host and viral) that contribute to disease pathogenesis as well as in the development of antiviral drugs and vaccines. Mouse models are the most widely used animal model in influenza research due to their cost and ease of husbandry. A typical influenza model involves intranasal infection with the mouse adapted H1N1 A/Puerto

Rico/8/1934 (A/PR/8/34) or A/WSN/1933 (WSN) influenza viruses. These models represent human infection and induce similar clinical signs (inflammation, mucus production), histopathological changes and viral recovery [177]. As some influenza viruses such as the 2009 H1N1 pandemic strains can also cause disease in mice without any adaptation, this allows investigators to determine the efficacy of treatments/vaccines against these specific strains [178, 179]. These mouse models can also be modified to mimic other disease features in human such as secondary bacterial pneumonia. Mouse models of influenza are valuable tools in investigating the underlying mechanism of this disease.

1.4 Important factors that control immune responses in the lung

There are many different pathways and key factors that are important in the development and protection against respiratory diseases. The pathways and factors described subsequently are those relevant to this thesis. These include the NF-κB signalling pathway, with a particular role for RelB, PP2A, the UPS and the anti-inflammatory molecule, TTP.

1.5 NF-κB signalling pathway

The NF-κB signalling pathway controls the transcription of deoxyribonucleic acid (DNA) and plays an important role in the induction of inflammatory and immune responses [180]. The mammalian NF-κB family comprises of five proteins; NF-κB1 (p50/p105), NF-κB 2 (p52/p100), RelA (p65), RelB and cRel. All members of the NF-κB family share a conserved Rel homology domain in their N-terminus that is crucial for dimerisation and interaction with proteins of the IκB family. NF-κB proteins exist as homo- or heterodimers bound to IκB family proteins in the cytoplasm of most cell types [181]. Binding to IκB prevents the translocation of the NF-κB:IκB complex to the nucleus, ensuring NF-κB remains in an inactive state.

NF-κB signalling occurs through the classical (canonical) or alternative (non-canonical) pathways. The classical pathway is activated by signals from TLR ligands and cytokines such as TNF-α that subsequently recruits adaptors such as TRAF to the cytoplasmic domain of the receptor. These adaptors activate the IκB kinase (IKK) complex, which phosphorylates IκB proteins leading to its ubiquitination and degradation by the proteasome system. This frees the NF-κB /Rel complexes, thereby allowing their entry into the nucleus to switch on target genes involved in inflammation and cell survival [182, 183]. The alternative pathway is stimulated by the TNF family of receptors such as CD40 or lymphotoxin beta receptor (LTβR). This results in the activation of NF-κB inducing kinase (NIK), which then phosphorylates and activates the IKKα complex. This subsequently results in the phosphorylation and proteasomal degradation of p100, thereby releasing the p52/RelB complex to translocate into the nucleus (**Figure 1.5**). The alternative pathway plays a vital role in the development of lymphoid organs [180, 181].

Over activation of the NF-κB transcription factors has been linked to several diseases such as inflammatory bowel disease, skin diseases and cancers [184-186].

Dysregulation of the NF-κB signalling pathway has also been implicated in autoimmune disorders such as rheumatoid arthritis and type I diabetes [187, 188]. NF-κB also plays a crucial role in asthma due to its ability to transcribe the genes encoding the various cytokines and chemokines involved in the pathogenesis of the disease [189, 190]. High levels of NF-κB activity have been observed in the serum and airways of asthmatic patients [191, 192]. A recent study also demonstrated the involvement of NF-κB in CS-induced lung inflammation [193]. Given the involvement of NF-κB in a wide variety of diseases, targeting this pathway, when it is aberrantly activated, may be beneficial in inflammatory diseases.

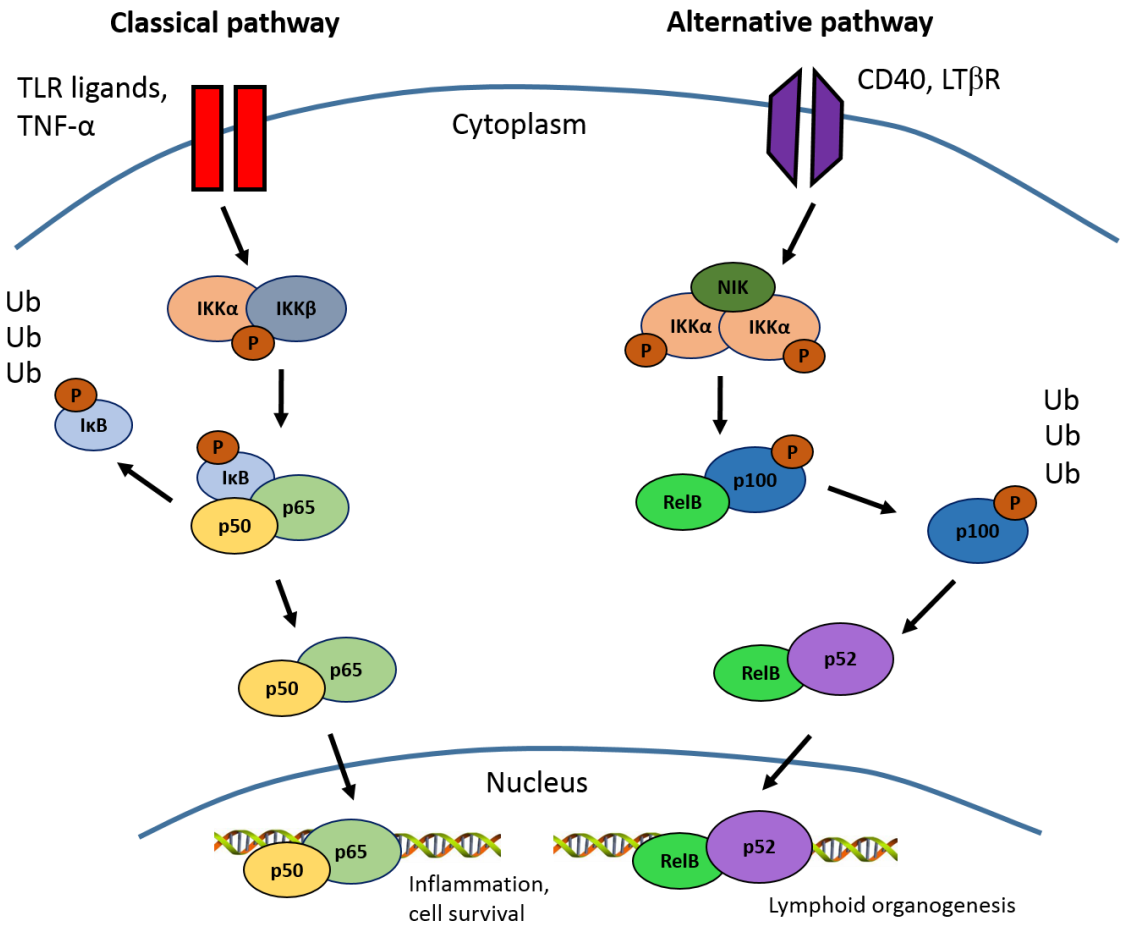


Figure 1.5: The classical and alternative NF-κB signalling pathways

The classical pathway is initiated by signals from activated Toll-like receptor (TLR) ligands and cytokines such as tumour necrosis factor alpha (TNF-α) that subsequently recruit various signalling adaptors to activate IκB kinase (IKK) complexes, which phosphorylate inhibitor of κB (IκB) proteins leading to its ubiquitination and degradation by the proteasome system. This frees NF-κB /Rel complexes to enter the nucleus and switch on target genes involved in inflammation and cell survival. Conversely, the alternative pathway is activated by the TNF family of receptors such as CD40 or lymphotoxin beta receptor (LTβR) that subsequently activate NF-κB inducing kinase (NIK), thereby phosphorylating IKKα complexes. This results in the phosphorylation and proteasomal degradation of p100, thereby releasing p52/RelB complexes to translocate into the nucleus and control gene expression involved in lymphoid organogenesis. *TLR:*

toll-like receptor, TNF-α: tumour necrosis factor alpha, IKK: IκB kinase, IκB: inhibitor of κB, Ub: ubiquitination, LTβR: lymphotoxin beta receptor, NIK: NF-κB inducing kinase.

1.5.1 RelB

RelB is an important member of the NF-κB family that plays a vital role in the development of lymphoid organs and in DC function and maturation [194, 195]. RelB is able to form heterodimers with p50 or p52 [196]. The RelB/p50 complex controls the expression of genes involved in DC function and maturation while the RelB/p52 complex controls genes involved in thymic function and the development of lymphoid organs [197]. RelB is constitutively expressed in human and mouse DCs, but is only expressed at low levels in other immune cells such as monocytes and neutrophils [195, 198].

Studies of genetic deletions of RelB highlight its crucial role in immune and inflammatory responses. RelB-deficient ($^{-/-}$) mice develop multi organ inflammation and have impaired cellular immunity, demonstrated by impaired delayed-type hypersensitivity and increased susceptibility to infection with *Listeria monocytogenes* [199, 200]. RelB-deficient kidney fibroblasts stimulated with lipopolysaccharide (LPS) persistently induced chemokines production while over expression inhibited CS-induced inflammation in the lungs [201, 202]. Collectively, these studies highlight the role of RelB as a negative regulator of inflammatory responses.

RelB has also been implicated in regulating xenobiotic responses in the lung [196]. RelB can form a dimer with the aryl hydrocarbon receptor (AhR), a transcription factor involved in the regulation of cellular responses to inflammation and oxidative stress [203]. The interaction of RelB-AhR is able to limit toxicity and promote detoxification of CS in the lung [204]. Other emerging roles of RelB include regulation of circadian rhythm by

binding to aryl hydrocarbon receptor nuclear translocator-like/clock circadian regulator (BMAL/CLOCK) that is involved in the expression of clock-controlled genes [205].

1.5.2 RelB and DCs

RelB plays a crucial role in DC differentiation and maturation and this is promoted by signalling through RelB-p50 dimers [206, 207]. The lack of RelB prevents the differentiation of DCs by up-regulation of B7 co-stimulatory molecules and IL-12 upon stimulation with LPS [208]. Alterations in DC maturation due to silencing of RelB expression by small interfering RNA (siRNA) result in reduced antigen-specific T cell responses [209]. These findings indicate that RelB-deficient DCs have reduced capability to present antigens.

Interestingly, RelB-deficient DCs are tolerogenic and have the ability to stimulate the development of regulatory T cells (Tregs) [209, 210]. The ability of RelB-silenced DCs to prevent allograft rejection in mice upon heart transplantation suggests the potential clinical use of RelB-silenced DCs [209]. Other studies showed the ability of RelB-silenced DCs to increase Treg number and subsequently supress experimental autoimmune myasthenia gravis [211].

While there is increasing evidence demonstrating the potential use of immature or co-stimulation deficient DCs in promoting peripheral tolerance, autoreactive T cells are able to resist regulation by these DCs due to the failure of controlling the expansion of autoreactive memory T cells [208, 212]. O'Sullivan *et al*., demonstrated that RelB and sufficient co-stimulation DCs are required to restore tolerance and prevent the development of autoimmune disease in $RelB^{-/-}$ mice, a mouse model that spontaneously develop multi organ inflammation and autoimmune disease as a result of defects in central tolerance [199, 208].

1.5.3 RelB$^{-/-}$ mice

RelB$^{-/-}$ mice serve as a valuable tool to investigate the significance of DCs in a defective immune response. One strain of loss of function mutant mice were generated by chance as a result of integration of class I MHC genomic clone as a transgene, which disrupted RelB expression [213]. Another complete deletion mouse strain was developed by targeting the mouse *relB* locus [199]. This disruption of the *relB* locus led to the complete ablation of RelB protein and reduced heterodimeric κB-binding activity in lymphoid tissues.

The disruption of the *relB* locus resulted in mice with a significant inflammatory phenotype and haemopoietic abnormalities. Multi-organ inflammation was observed in these mice, specifically in the skin, lung and liver with mixed inflammatory cell infiltration [199]. These mice also have myeloid hyperplasia and splenomegaly due to extramedullary haemopoiesis and the lack of lymph nodes [199, 214, 215]. Thymic atrophy in these mice leads to impaired negative selection in the thymus and subsequently the development of autoimmunity [216]. These mice also develop skin lesions resembling atopic dermatitis with high levels of IL-4 and IL-5 in the skin and high serum IgE levels [213]. They are highly susceptible to infection by *L.monocytogenes* due to impaired production of TNF-α by macrophages and a defective cytotoxic T cell response [200]. Interestingly, these mice have an increased proportion of CD4^{+} FoxP3^{+} Tregs in the periphery, but their regulatory function is significantly reduced possibly due to the lack of co-stimulatory signals from DCs. The regulatory function of these Tregs is restored when RelB-sufficient DCs from RelB heterozygous ($^{+/-}$) are transferred into RelB$^{-/-}$ mice [208].

Collectively, these findings highlight the importance of RelB in various developmental and immune responses. These mice provide a means of elucidating the

role of RelB in the pathogenesis of various diseases, in particular allergy and autoimmune diseases as well as examining DC function in these situations.

1.6 PP2A

The functions of proteins are typically regulated by phosphorylation. The phosphorylation status of a protein is regulated by kinases and phosphatases. The study of kinases has dominated the field due to their involvement in the progression of cancer. However, research over the past decade has revealed the importance of phosphatases. Phosphatases are divided into two major classes; 1) protein tyrosine phosphatase (PTP) and 2) protein serine/threonine phosphatases, which can be further classified into three families that include phosphoprotein phosphatases (PPPs) that consists of PP1, PP2A, PP2B and PP4, metal dependent protein phosphatases and DxDxT phosphatases [217]. Here, we examined the role of PP2A in respiratory diseases.

PP2A is the most abundant serine/threonine phosphatase with diverse cellular functions including the promotion of DNA replication and apoptosis [218-220]. PP2A also controls cell metabolism by regulating enzymatic activity in the metabolism of lipids and glycolysis [221]. PP2A is expressed ubiquitously in mammalian cells and is the main contributor to soluble phosphatases activity [222].

1.6.1 PP2A subunits

PP2A consists of three subunits; scaffold/structural (A), regulatory (B) and catalytic (C) that function as a holoenzyme. PP2A can exist in two different forms, the dimeric form ($PP2A_D$), which is the core enzyme that consist of the catalytic and scaffold subunit and the trimeric form ($PP2A_T$) that is composed of the core dimer bound to the variable B regulatory subunit (**Figure 1.6**) [223-225].

The scaffold/structural subunit, consists of two isoforms Aα and Aβ that are encoded by two separate genes, *PP2R1A* and *PP2R1B*. Both isoforms are ubiquitously

expressed and share 87% sequence homology [226]. However, despite the high sequence similarity, they cannot substitute for each other. Mutations in the Aβ subunit dampen the ability of Aβ to bind to B or C subunits and have been associated with breast, colorectal and lung cancer [227-229]. Albeit at a lower rate compared to Aβ, mutations in Aα have been found in melanoma and in lung and breast cancer [228, 230].

The regulatory subunits consist of four families that include B/PR55, B'/PR61, B''/PR72/PR130 and B'''/PR93/PR110. The B/PR55 family consists of four isoforms (α, β, γ and δ) and is known to be involved in cytoskeletal dynamics and nuclear translocation [222]. The B'/PR61 family consists of 5 isoforms (α, β, γ, δ and ε) that can be phosphorylated and has the ability to bind directly to the core enzyme. The highly acidic surface of the holoenzyme structure is able to recruit substrate proteins [231, 232]. The B''/PR72/130 is involved in regulating Rb phosphorylation during ultraviolet radiation and cell cycle progression while the B'''/PR93/PR110 family acts as a calmodulin binding protein and can interact with PP2AC to enhance calcium-dependent signalling [222, 233].

The catalytic subunit PP2AC has a globular structure and is ubiquitously expressed with its expression highest in the heart and brain [222]. It is made up of 309 amino acids and exists as 2 different isoforms; α and β [234]. Although both isoforms share a 97% sequence homology, the loss of one is not able to compensate for the other. This is evident in $PP2AC_{\alpha}$ deficient mice that are embryonic lethal [235].

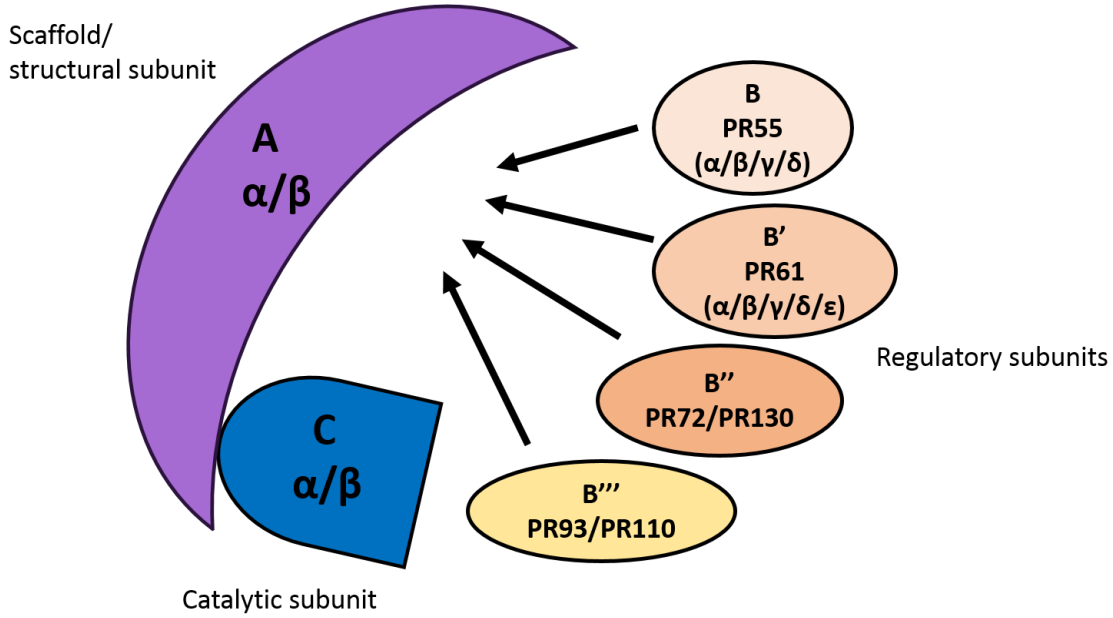


Figure 1.6: Schematic representation of protein phosphatase 2A (PP2A) complex

PP2A consists of three subunits; core scaffold/structural (A) and catalytic (C) subunit that is bound to the variable regulatory (B) subunits. The scaffold/structural and catalytic subunits consist of two isoforms, α and β. The regulatory subunit is further divided into four families that include B/PR55, B'/PR61, B''/PR72/PR130 and B'''/PR93/PR110. The B/PR55 family consists of four isoforms α, β, γ and δ while the B'/PR61 family consists of 5 isoforms α, β, γ, δ and ε.

1.6.2 Regulation and modification of PP2A

Due to the diverse and widespread functions of PP2A, it is crucial that it is tightly regulated. One level of regulation of PP2A activity and function involves the post-translational modification of its PP2A subunits. Activated epidermal growth factor receptors and insulin receptors can phosphorylate PP2AC on Tyr^{307}located in the conserved C-terminus of PP2AC [236]. Phosphorylation leads to inactivation of the enzyme, which enhances transmission of signals through kinase cascades. Interestingly, phosphorylation of PP2AC can be enhanced by the phosphatase inhibitor, okadaic acid, while PP2A can re-activate itself through an auto-dephosphorylation reaction [237]. Besides

PP2AC, the B regulatory subunits are also subjected to phosphorylation. For example, phosphorylation of B'δ by protein kinase A *in vitro* is able to change the substrate specificity of the enzyme and subsequently increased its activity [238]. Methylation of PP2AC on the carboxyl group of the C-terminal residue Leu^{309} by leucine carboxyl methyltransferase 1 (LCMT1) is another form of post-translational modification that alters PP2A activity [239, 240]. Methylation influences the binding affinity of the AC subunit to different B regulatory subunits. Increased phosphorylation and reduced methylation of PP2AC have been implicated in the pathogenesis of Alzheimer's disease [241]. More recently, ubiquitination of PP2AC and α4, a PP2AC-interacting protein, *via* its interaction with the E3 ubiquitin ligase midline-1 (MID1), has been identified as another post-translational modification that regulates PP2A activity and function [242]. The binding of α4 to PP2AC inactivates its enzymatic activity and allows its assembly into functional PP2A phosphatase complexes [243]. The α4:PP2AC interaction also prevents PP2AC ubiquitination and proteasomal degradation. Importantly, α4 deletion leads to the loss of the PP2A complex, highlighting the importance of α4 in regulating PP2A activity [244].

1.6.3 Dysregulation of PP2A

Among its many functions, PP2A is involved in regulating multiple kinase cascades such as the mitogen-activated protein kinase (MAPK) and Wnt signalling [245]. PP2A is also involved in apoptotic signal transduction pathways by activating or inhibiting apoptotic proteins [220]. It is also involved in the initiation of DNA replication and in maintaining cell adhesion and cytoskeletal dynamics [222]. Dysfunction or dysregulation of PP2A therefore would impact the various physiological and biological processes it controls. Dysregulation of PP2A has been implicated in various diseases including cancer and asthma.

1.6.3.1 Cancer

Mutations or aberrant expression of the different PP2A subunits has been implicated in different types of cancer. Mutations of the A structural subunit have been reported in lung carcinoma, melanoma and colon cancer [227-230]. Similarly, mutations in B regulatory subunits have been observed in different cancers. Mutations in B56γ inhibits its interaction with p53 leading to cell proliferation in lung cancer [246]. The expression of PP2A B55α was reduced in acute myeloid leukaemia patient [247]. This leads to persistent activation of Akt to increase cell proliferation [247].

1.6.3.2 Asthma

Dysregulation of PP2A activity and function have also been implicated in asthma [248-251]. PP2A is able to dephosphorylate MAPK and IκBβ to limit the activity of p38 MAPK and NF-κB, both of which have been associated with the development of AAD [252-255] . Reduced PP2A activity has also been observed in animal models of allergen and rhinovirus induced AAD [248, 249]. PP2A inhibition by okadaic acid resulted in increases in the expression of pro-inflammatory cytokines expression such as IL-6 and IL-8 *in vitro* [256]. Notably, PP2A expression and activity were significantly reduced in peripheral blood mononuclear cell (PBMC) from patients with severe asthma [251]. Collectively, these findings suggest that enhancing PP2A activity would be beneficial in asthma.

1.6.4 PP2A activators

The implication of impaired PP2A in the pathogenesis of various diseases highlights the potential of targeting PP2A as a therapy. Several PP2A activating drugs that enhance the activity and function of PP2A have been used in cancer and respiratory diseases.

Fingolimod (FTY720) has been approved for the treatment of relapsing multiple sclerosis [257]. FTY720 is internalised by sphingosine-1-phosphate receptor (SIPR1) upon

phosphorylation by sphingosine kinase 2 and sequesters lymphocytes in the lymph nodes, preventing them from taking part in autoimmune reactions [258, 259]. FTY720 is able to induce apoptosis of neoplastic cells and have been suggested as a treatment of multiple myeloma, breast and prostate cancer [260]. Enhancing PP2A activity with FTY720 promoted the degradation of BCR-ABL1 oncoprotein and inhibited the extracellular signal-regulated kinase (ERK) and Akt pathways in chronic myelogenous leukaemia [261, 262]. In animal models of acute myeloid leukaemia, the use of FTY720 restored PP2A activity and subsequently delayed tumour growth and prolonged survival [263, 264]. Enhancing PP2A activity with FTY720 also abrogated the development of inflammation and AHR in mouse models of AAD [265].

2-amino-4-(4-heptyloyphenol)-2-methylbutanol ($AAL_{(S)}$) that is devoid of sphingosine 1-phosphate agonism is a newer drug that is a more specific PP2A activator than FTY720 [266]. The use of this activator has been shown primarily in asthma and AAD. Activation of PP2A by $AAL_{(S)}$ reduced inflammation, Th2 cytokines, NF-κB activity and AHR in mouse models of HDM induced AAD [248]. Increasing PP2A activity with $AAL_{(S)}$ also abrogated rhinovirus-induced eosinophil influx and AHR *in vivo* [249]. *In vitro* studies using A549 cells show that PP2A activation with $AAL_{(S)}$ is able to reverse okadaic acid-mediated inhibition of basal PP2A activity and reduce the expression of IL-6 and IL-8 [256]. Importantly, increasing PP2A activity with $AAL_{(S)}$ is able to restore steroid sensitivity to AHR in mouse models of steroid resistant asthma [250].

1.7 UPS

In order to maintain homeostasis, most intracellular and extracellular proteins undergo degradation and are replaced by newly synthesised molecules. The degradation process is tightly controlled and regulated by the UPS. Thus, the UPS plays a crucial role in degrading key proteins and is essential for the regulation of multiple cellular processes

including cell cycle division, DNA transcription and repair, antigen processing, inflammation and immune responses. Dysregulation of the UPS may lead to malignancies and neurodegenerative diseases. Therefore, targeting this system may be an approach to treat inflammatory diseases.

1.7.1 Ubiquitin and ubiquitination

Degradation of proteins *via* the UPS involves two processes; the attachment of ubiquitin to target proteins and the subsequent degradation of the protein by the proteasome. Ubiquitin is a highly conserved protein that is 76 amino acids long and is found in all eukaryotes [267, 268]. It consists of a C-terminal tail and seven lysine residues that link the ubiquitin to the target protein *via* branched isopeptide bonds [269]. The seven lysine residues also allows the ligation of other ubiquitin, resulting in the emergence of polyubiqutinated chains that are involved in numerous processes. For example, chains that are linked to lysine 48 of another ubiquitin play a role in degradation of the target protein by the proteasome.[270].

The ubiquitination process involves three key steps; activation by ubiquitin-activating enzymes (E1), conjugation by ubiquitin-conjugating enzymes (E2) and ligation by ubiquitin ligases (E3). The initial step of ubiquitination involves the activation of the carboxyl-terminal glycine residue of ubiquitin by E1 enzymes through the formation of a thio-ester bond in an adenosine triphosphate (ATP)-dependent manner. Next, the ubiquitin is transferred to a cysteine site of the E2 enzyme *via* a trans(thio) esterification reaction. The final step involves the transfer and linking of ubiquitin to the lysine residue of the target protein [269]. This process is catalysed by diverse E3 ligases that recognises specific protein motifs. This feature is responsible for the high specificity and selectivity of the UPS [271].

1.7.2 Proteasomes

Upon ubiquitination, the target protein is recognised and degraded by proteasomes. Proteasomes are found in the nucleus and cytosol of eukaryotes and play a crucial role in degrading targeted proteins by proteolysis in an ATP-dependent manner [272].

1.7.2.1 20S proteasome

The barrel-shaped 20S proteasome is involved in the proteolytic activity of the proteasome. It is comprised of four stacked ring structures with 2 outer α rings and two inner β rings. The two α rings consist of 7 α subunits, each serving as a docking site for regulatory particles. The inner rings consist of 7 β subunits that contain multiple catalytic sites that are crucial for proteolysis reactions [273]. The catalytic functions identified in eukaryotes include trypsin-like, caspase-like and chymotrypsin-like activities, which are often the target for proteasome inhibitors [274]. The 20S proteasome can associate with a 19S regulatory protein to form a 26S complex or with 11S regulatory particle to promote the degradation of short peptides. The generation of small peptides by 11S often induced by IFN-γ responses can result in the binding of MHC to initiate an immune response [269].

1.7.2.2 26S proteasome

The 26S proteasome is composed of a 20S core particle and two 19S regulatory particles that caps the 20S proteasome on both ends. The 19S regulatory particle plays a crucial role in stimulating protein degradation by the 20S proteasome. It is able to recognise, cleave and translocate polyubiquitinated chains from target proteins to 20S proteasome for degradation in an ATP-dependent manner [269]. Besides degrading abnormal/damaged proteins, the 26S proteasome has also been implicated in antigen processing and activation and degradation of transcription factors [275-277].

1.7.3 UPS and inflammatory disorders

The UPS plays a significant role in the immune system due to its ability to generate peptide antigens that are recognised by MHC [278]. It also plays a key role in regulating T cell receptor and costimulatory CD28 signalling. The degradation of the E3 ubiquitin ligase, Casitas B lineage lymphoma b (Cbl-b), as a result of CD28 signalling leads to increased expression of pro-inflammatory cytokines [279]. Importantly, the UPS has a significant role in the regulation of NF-κB activity through IκB degradation [269]. Due to its involvement in the immune system and protein turnover, dysregulation of the UPS could lead to abnormal homeostasis and the development of inflammatory and autoimmune diseases.

1.7.3.1 Asthma

The E3 ligase Itch mediates immune tolerance by degrading the Th2-transcription factor, phosphorylated JunB. Itch deficient mice develop allergic inflammation due to increased JunB levels, which results in increased levels of Th2 cytokines [280]. In other studies, mice deficient in another E3 ligase, Cbl-b were more susceptible to the induction of experimental asthma with heightened Th2 and Th9 responses [281]. More recently, it was discovered that the E3 ligase MID1 was upregulated in mouse models of AAD and in pBECs upon HDM exposure [248].

1.7.3.2 COPD

Exposure to CS significantly impairs proteasome activity with accumulation of polyubiquitinated proteins in human alveolar epithelial cells and in the lungs of CS-exposed mice [282]. It was also reported that subjects with emphysema had increased ubiquitinated proteins and the deubiquitinating enzyme, ubiquitin carboxyl-terminal hydrolase L1 (UCHL-1), suggesting an increase in damaged proteins [283, 284]. Increased expression of valosin-containing protein (VCP) that plays a role in ubiquitin proteasome

mediated protein degradation have been observed in the lungs of COPD patients and mice exposed to CS [284]. Increased levels of VCP decreased nuclear factor erythroid 2-related factor and histone deacetylase 2 expression due to their degradation by VCP-mediated proteasomal pathway. This subsequently results in the attenuation of the protective oxidative stress response and aberrant inflammatory gene transcription, which contribute to the pathogenesis of emphysema [284].

1.7.4 Proteasome inhibition

The UPS plays a crucial role in multiple cellular processes and dysregulation of the UPS has been linked to many diseases. This suggests that targeting the UPS could be a potential therapeutic option for multiple diseases. Proteasome inhibition in particular has been shown to be a useful therapy, especially in cancer [285]. Numerous synthetic and natural proteasome inhibitors that target the proteolytic sites of the proteasome have been developed. These inhibitors have the ability to block protein degradation by the UPS.

The first proteasome inhibitor to enter clinical trial was Bortezomib (BORT). It has been approved by the Food & Drug Administration for the treatment of multiple myeloma [286]. BORT is a dipeptidyl boronic acid that potently inhibits the activity of the 26S proteasome. It promotes apoptosis, enhances the activity of chemotherapy and radiation and reverses chemo resistance in malignancy models. The protective effect in multiple myeloma was in part due to the inactivation of NF-κB as a result of inhibition of IκB degradation [287]. It was also shown to stabilise the tumour suppressors p21, p27 and p53 [288]. Administration of BORT in a mouse model of prostate cancer led to decrease in tumour volume [289]. The use of BORT was able to inhibit the growth of lung cancer cell lines both *in vivo* and *in vitro* [290]. It has also been reported that BORT can arrest cell cycle progression and induce apoptosis in pancreatic cancer [291].

The only other proteasome inhibitor that has been approved for use in humans is carfilzomib. It has been approved for use as a second line therapy for multiple myeloma. The mechanism of action involves inhibiting the chymotrypsin-like activity of the 20S proteasome, resulting in the accumulation of polyubiquitinated proteins that may lead to apoptosis and inhibition of tumour growth.

Besides cancer, proteasome inhibition is currently emerging as a therapy for other inflammatory diseases. In asthma, the use of proteasome inhibitor PS-519 significantly reduced the infiltration of eosinophils into the lung of a rat model of OVA-induced pulmonary eosinophilia [292]. It was also reported that proteasome inhibition by lactacystin and carbobenzoxy-L-leucyl-L-leucinal (MG-132) prevented dopaminergic neurogeneration and apormorphine-induced rotational asymmetry in a rat model of β-hydroxyl dopamine induced Parkinson's disease [293].

1.8 TTP

The stability, transcription and translation of mRNAs play a central role in controlling gene expression. Loss of post-transcriptional regulation underlie disease pathogenesis due to the overexpression of various genes that encode growth factors, proto-oncogenes and inflammatory cytokines [294, 295]. One of the mechanisms of regulating mRNA turnover involves the cis-acting regulatory element adenosine and uridine (AU)-rich elements (ARE) present within the 3'-untranslated region (3'-UTR) of mRNAs. AREs target mRNAs for decay and regulate the stability and translation of mRNAs [296]. These effects are largely dependent on the interaction with trans-acting RNA binding protein. This thesis will focus on one of the best characterised ARE binding protein, TTP.

1.8.1 Structure and function of TTP

TTP is a 34 kDA molecule that belongs to the family of cysteine-cysteine-cysteine-histidine (CCCH) tandem zinc finger proteins [297]. The TTP protein is made up

of three groups of four consecutive prolines and is encoded by the zinc finger protein 36 (*Zfp36*) gene in mouse and human [298]. TTP is also known as TPA-induced sequence 11 (TIS11), nuclear protein 475 (Nup475) and G0S24 [299-301]. Under quiescent growth conditions, TTP protein levels are typically low and predominantly found in the nucleus of cells [302]. However, its expression is induced upon stimulation with insulin, growth factors or bacterial products and this leads to its translocation to the cytoplasm [300, 303, 304]. TTP is ubiquitously expressed with highest levels observed in the lymph nodes, thymus and spleen [304]. TTP contains two tandem zinc finger domains that are required for mRNA decay functions and nuclear import [305, 306]. Mutations of the zinc finger domains lead to impaired cytoplasmic localisation of TTP [307]. Both the N-terminal and C-terminal portions of TTP protein contain sequences that promote mRNA degradation [308]. The binding of 14-3-3 proteins, which regulate various processes in cells such as cell growth control, to TTP promotes its stability and cytoplasmic localisation [309, 310].

The role of TTP as an mRNA decay factor was identified during the characterisation of $TTP^{-/-}$ mice. These mice develop a complex inflammatory phenotype characterised by cachexia, dermatitis, erosive arthritis and myeloid hyperplasia [311]. The development of these features is largely due to the systemic overexpression of TNF-α. Administration of anti-TNF-α antibodies to newborn $TTP^{-/-}$ mice abrogated the inflammatory phenotype. The elevated levels of TNF-α was attributed to the increased stability of its mRNA transcripts. Subsequent studies determined that TNF-α is a target of TTP due to its ability to bind to the ARE within the 3'-UTR of TNF-α mRNA [312]. This then leads to the recruitment of catabolite repression protein 4/carbon catabolite repression 4-associated factor 1 deadenylase complex that shortens the poly (A) tail of TNF-α mRNA, leading to its degradation. Further research on $TTP^{-/-}$ mice identified GM-CSF as another target of TTP. GM-CSF mRNA expression was stabilised in the bone

marrow-derived stromal cells from TTP$^{-/-}$ mice compared to wild-type (WT) mice [313]. Since then, many TTP targets have been identified (**Table 1.2**).

Table 1.2: Known tristetraprolin targets

Gene	Gene
IL-2 [314]	IL-1β [315]
IL-3 [316]	IFN-γ [317]
IL-6 [318]	CXCL1 [319]
IL-8 [320]	CXCL2 [318]
IL-13 [321]	CCL20 [318]
IL-17A [322]	MMP-1 [323]
IL-23 [324]	MMP-9 [325]

IL: interleukin, CXCL: Chemokine (C-X-C motif) ligand, CCL: Chemokine (C-C motif) ligand, MMP: matrix metalloproteinase

1.8.2 Regulation of TTP

TTP can be regulated at the transcriptional, post-transcriptional and post-translational levels. At the transcriptional level, TTP mRNA expression in human and mouse cell lines can be upregulated by several factors including insulin, LPS, dexamethasone, growth factors (fibroblast and nerve growth factors) and cytokines (TNF-α, IL-6, IL-11, IFN-β and IFN-γ) [299, 303, 304, 312, 326-337]. TTP mRNA transcription can also be regulated by the NF-κB signalling pathway. Inhibitors of NF-κB such as BAY 11-7082 and parthenolide reduced LPS-induced TTP expression in RAW264.7 cells [338]. This study highlights the ability of NF-κB to bind to the *Zfp36* promoter to activate TTP expression. The protein kinase C (PKC) pathway has also been implicated in the regulation of TTP mRNA. PKC inhibitors such as LY333531 downregulate TTP

expression in mouse J774 macrophages in response to LPS and the tumour promoter TPA [339].

At the post-transcriptional level, the stability of TTP mRNA is regulated by the p38 MAPK pathway [340-342]. The use of the p38 inhibitor SB203580 in RAW264.7 cells stimulated with LPS significantly destabilised TTP mRNA, suggesting that this pathway is important in stabilising the transcript [341, 342]. Interestingly, the stability of TTP mRNA is regulated by TTP itself due to its ability to bind to its own 3'-UTR [341]. Recent studies have also highlighted roles for micro RNAs (miRNAs) in regulating TTP mRNA stability. TTP transcripts were reported to be a target of miR-29a as over expression of miR-29a destabilised TTP mRNA in cancer models [343, 344]. Similarly, miR-346 was reported to control levels of TNF-α in rheumatoid arthritis by regulating TTP mRNA stability [345].

Post translational regulation of TTP protein involves phosphorylation and plays a crucial role in maintaining its stability and activity. TTP protein exists in two forms, the phosphorylated form (inactive) that stabilises mRNA of cytokines and the unphosphorylated form (active) that induces mRNA decay. Unphosphorylated TTP is less stable and therefore is subjected to degradation by the UPS [346, 347]. The p38 MAPK pathway plays a critical role in the phosphorylation of TTP protein. Upon stimulation, p38 MAPK activates the downstream MAPK-activated protein kinase 2 (MK2) that phosphorylates TTP at serines 52 and 178 in mice and serines 60 and 186 in humans [348, 349]. This results in the formation of complexes with the adaptor 14-3-3 proteins that inhibits mRNA decay of cytokines and prevents dephosphorylation of TTP, hence protecting it from proteasomal degradation [310, 350]. Conversely, PP2A is able to mediate dephosphorylation of TTP protein, leading to an increase in the active form (unphosphorylated) to induce mRNA decay of cytokines (**Figure 1.7**) [310]. Inhibition of

PP2A by okadaic acid or siRNA leads to increased p38 MAPK-MK2 phosphorylation of TTP protein, thereby increasing the stability of TNF-α mRNA in mouse alveolar macrophage cell lines [310]. The PP2A inhibitor calyculin A led to the accumulation of inactive form (phosphorylated) of TTP in RAW 264.7 cells [346]. These studies highlight the importance of PP2A in regulating the activity of TTP to induce mRNA decay of cytokines and suggest that enhancing PP2A activity could be an important therapeutic approach to control inflammatory responses through TTP.

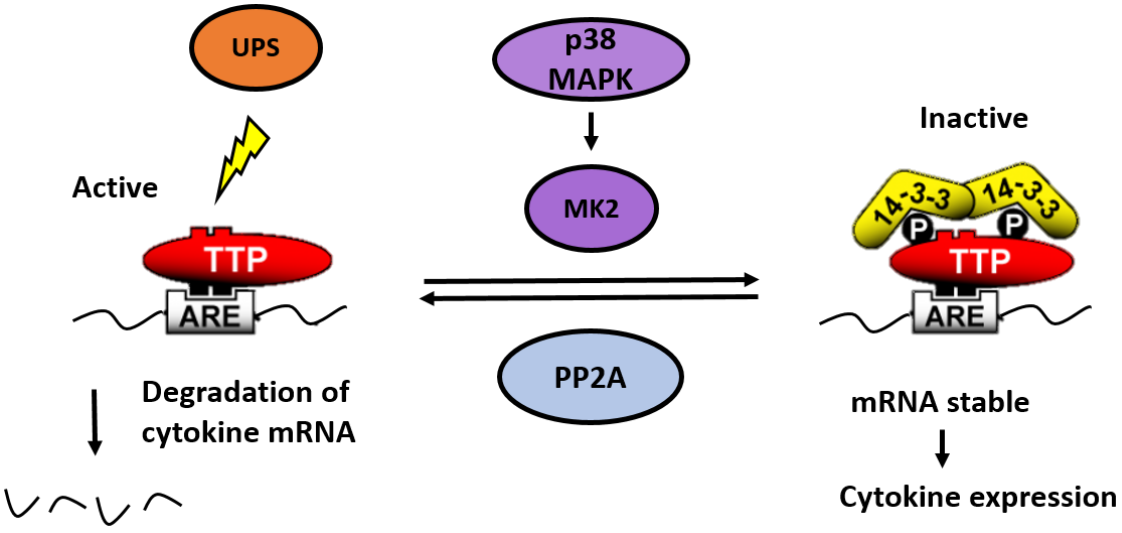


Figure 1.7: Post translational regulation of tristetraprolin (TTP)

When the immune system is stimulated, p38 mitogen-activated protein kinase (MAPK) activates the downstream kinase MAPK-activated protein kinase 2 (MK2), which results in the phosphorylation of TTP. The phosphorylated (inactive) form of TTP leads to the stabilisation of cytokine mRNA and hence increased cytokine production. Protein phosphatase 2A (PP2A) acts as an antagonist to MK2 by dephosphorylating TTP. Dephosphorylated (active) TTP induces the decay of cytokine mRNA. However, this form is less stable than inactive TTP and is subjected to degradation by the ubiquitin proteasome system (UPS). *ARE: adenosine-uridine-rich element, TTP: tristetraprolin, MAPK: mitogen-activated protein kinase, MK2: MAPK-activated protein kinase 2, mRNA: messenger ribonucleic acid, PP2A: protein phosphatase 2A, UPS: ubiquitin proteasome system*

1.8.3 Role of TTP in inflammatory diseases

As TTP regulates the expression of multiple pro-inflammatory cytokines, altered TTP activity could influence the development of a range of inflammatory diseases.

1.8.3.1 Rheumatoid arthritis

TTP expression in synovial tissue was inversely related to the degree of inflammation and serum levels of C-reactive protein in rheumatoid arthritis patients [351]. Interestingly, polymorphisms in *ZFP36* have been associated with rheumatoid arthritis. The presence of a single nucleotide polymorphism (SNP) *ZFP36*8*, a C to T transition in the protein coding domain was associated with high incidence of rheumatoid arthritis in African-American individuals [352]. Another similar study identified a SNP359 A/G within the *ZFP36* promoter region that impaired TTP promoter activity and was suggested to modulate disease activity in rheumatoid arthritis [353].

1.8.3.2 Respiratory diseases

The role of TTP in respiratory diseases remains poorly understood. As many of these diseases are driven by cytokine-induced inflammation, some of which are destabilised by TTP, we postulate that enhancing TTP function would have beneficial effects. A recent study identified the ability of corticosteroids to increase TTP activity, which subsequently suppressed the levels of IL-6 in airway smooth muscle cells [354]. Another study showed that inhibition of PP2A activity by okadaic acid in A549 lung epithelial cells led to the upregulation of phosphorylated (inactive) TTP and increased IL-6 and IL-8 expression [256]. Interestingly, these effects were reversed by the PP2A activators, FTY720 and $AAL_{(S)}$ [256]. A more recent study proposed that enhancing PP2A activity with FTY720 and $AAL_{(S)}$ increased the anti-inflammatory function of TTP by shifting the equilibrium from phosphorylated to unphosphorylated form of TTP [355].

1.8.4 TTP knock-in mice (*Zfp36*$^{aa/aa}$)

While TTP$^{-/-}$ mice provide a valuable tool to investigate the function of TTP, the profound inflammatory phenotype observed in these mice hinders the ability to use these mice in inflammatory disease models [311].

Zfp36$^{aa/aa}$ mice were generated by substituting the murine serines 52 and 178 with the unphosphorylatable alanine residues [356]. This resulted in a phenotype with the active (unphosphorylated) form of TTP that cannot be inactivated (phosphorylated). The expression of several inflammatory cytokines including TNF-α, CXCL1, CXCL2 and IL-1β were significantly reduced in LPS-treated BMMs of *Zfp36*$^{aa/aa}$ mice compared to WT mice. LPS injection resulted in significantly lower levels of CXCL1, CXCL2 and IL-12p70 in the serum of *Zfp36*$^{aa/aa}$ and heterozygous (*Zfp36*$^{+/aa}$) mice compared to WT mice. These findings highlight the ability of the active form of TTP to degrade target mRNAs. Interestingly, TTP protein expression is significantly lower in *Zfp36*$^{aa/aa}$ mice compared to WT mice [356]. This could potentially be due to autoregulation of TTP by degrading its own mRNA or due to proteasomal degradation as the unphosphorylated form of TTP is unstable. Nevertheless, these mice are gain of function transgenic animals [357]. The expression of inflammatory mediators in LPS-treated BMMs from *Zfp36*$^{+/aa}$ mice was as low as that in *Zfp36*$^{aa/aa}$ mice, suggesting that even a small shift towards the unphosphorylated form is sufficient to promote an anti-inflammatory response. These findings highlight the possibility of modulating the equilibrium of TTP as potential therapeutic approach in inflammatory diseases. These mice provide a valuable tool to delineate the role of TTP and its equilibrium in inflammatory diseases.

1.9 Study rationales

The studies described in this thesis were designed to determine the role of selected immune pathways and factors that may underpin the pathogenesis of the respiratory diseases asthma, COPD and influenza. We propose that targeting specific factors, such as RelB and TTP, and immune pathways such as PP2A and UPS, will suppress the pathogenesis of these respiratory diseases.

To investigate these mechanisms *in vivo*, murine models of AAD, COPD and influenza virus infection were used. These models have been established in our laboratory and exhibit hallmark features of the human diseases.

We used these models in novel studies to:

a) Investigate the roles of RelB in asthma-related AAI using $RelB^{-/-}$ mice and RelB-sufficient DCs

b) Investigate the potential of enhancing PP2A activity and inhibiting proteasome activity in asthma using mouse models of acute OVA and chronic HDM-induced AAD, PP2A activators (FTY720 and $AAL_{(S)}$) and the proteasome inhibitor (BORT).

c) Investigate the roles of active TTP in CS-induced experimental COPD using *Zfp36*$^{aa/aa}$ mice

d) Investigate the roles of active TTP in influenza using *Zfp36*$^{aa/aa}$ mice

CHAPTER 2:

RelB-deficient dendritic cells promote the development of spontaneous allergic airway inflammation

In this chapter, we show that the lack of RelB expression in DCs promotes the development of AAI that include increased inflammatory cell influx, chemokine levels, recruitment of Th2-associated cytokines and airway remodelling. This is independent of any allergen exposure. We also demonstrate that adoptive transfer of RelB-sufficient DCs is able to ameliorate disease features. Our study highlights the importance of RelB in DCs in controlling features of AAI and the potential of DC-targeted therapy for AAI.

This chapter is currently in preparation for submission as an original research article to the *Journal of Immunology*.

The study in this chapter was conducted in collaboration with Professor Ranjeny Thomas and Dr Brendan O'Sullivan from the Translational Research Institute, University of Queensland. I conducted part of the work at the University of Queensland, and part of it at the Hunter Medical Research Institute, University of Newcastle. I conducted the majority of the work and was heavily involved in experimental design, data interpretation and wrote the manuscript.

2.1 Abstract

RelB is a member of the NF-κB family that is essential for DC function and maturation. However, the contribution of RelB to the development of AAI is unknown. Here, we identify a pivotal role for RelB in the development of spontaneous AAI, independent of exogenous allergen exposure. We assessed AAI in two strains of $RelB^{-/-}$ mice, one with a targeted deletion and another with a mutant expressing a MHC transgene. Both strains had increased pulmonary inflammation compared to their respective wild-type ($RelB^{+/+}$) and heterozygous ($RelB^{+/-}$) controls. $RelB^{-/-}$ mice also had increased levels of inflammatory cell influx into the airways, levels of chemokines CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CXCL9, CXCL10 and CXCL13 and Th2-associated cytokines, IL-4 and IL-5 in lung tissues, serum IgE and airway remodelling (MSC numbers, collagen deposition and epithelial thickening). Transfer of RelB-sufficient $CD11c^{+}$ DCs to $RelB^{-/-}$ mice decreased pulmonary inflammation, with reduced lung chemokine (CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CXCL9, CXCL10, CXCL13) and Th2-associated cytokine (IL-4, IL-5, IL-13, IL-25, IL-33, TSLP) levels, serum IgE, numbers of ILC2s, myeloid (m)DCs, and gamma delta (γδ) T cells, and airway remodelling. These data indicate that RelB deficiency is a key pathway underlying AAI and that DC-encoded RelB is sufficient to restore control.

2.2 Introduction

RelB is a member of the NF-κB family that is involved in the canonical and non-canonical NF-κB signaling pathways [206]. It plays a crucial role in the development of lymphoid organs and in DC biology [194]. RelB has also been implicated in regulating circadian rhythm and xenobiotic responses in the lung [196].

RelB is highly expressed in human and mouse DCs and is critical for the function and maturation of these cells [195]. It is also expressed at low levels in innate-immune cells and requires certain stimuli to induce its expression [198]. This suggests that the effects of RelB activation on the immune response is cell-type specific. A lack of RelB prevents the differentiation of DCs and up-regulation of co-stimulatory molecules and IL-12 [208]. $RelB^{-/-}$ DCs are tolerogenic and stimulate the development of Tregs [358]. However, Langerhans cell differentiation is not affected in the absence of RelB [216, 359, 360]. Silencing of RelB in DCs has the potential to be an immunomodulatory therapy for autoimmune diseases [209].

In contrast, RelB also has the potential to act as a negative regulator of inflammatory responses [202, 204, 361, 362]. $RelB^{-/-}$ mice develop multi-organ inflammation [199]. RelB has been shown to regulate chemokine expression in mouse kidney fibroblasts and to have beneficial effects in human immunodeficiency virus-1-induced neuro-inflammation by inhibiting TNF-α release from microglial cells [201, 363]. In the lungs, overexpression of RelB inhibits CS-induced inflammation and this is largely due to the suppression of cyclooxygenase-2 (COX-2), prostaglandin E2 and intracellular adhesion molecule-1 (ICAM-1) levels that in turn reduced pro-inflammatory cytokine and chemokine production [202]. We showed previously that RelB-sufficient DCs control memory T cells and suppress established autoimmune disease in $RelB^{-/-}$ mice in an indoleamine-2,3-dioxygenase (IDO)-dependent fashion [208]. $RelB^{-/-}$ mice also develop a T cell-dependent skin disease resembling human atopic dermatitis, associated with DC and

mast cell infiltration, Th2 cytokine overexpression in the dermis and elevated serum IgE [200, 213].

In the lungs, peri-bronchial and peri-vascular inflammation occur in histological sections of RelB$^{-/-}$ mice [213]. However, little is known about its role in asthma and AAI. Nevertheless, it is likely important as a recent multi-ancestry genome-wide association study identified signals in genomic regions relevant to both the control of RelB expression and RelB-mediated regulation of immune responses [364].

Asthma is predominantly an AAD that has doubled in prevalence over the past three decades [365]. Th2 cells are critical players in the pathogenesis of allergic asthma [67]. An early phase response occurs within minutes of exposure to allergens and causes the activation of mast cells following the cross-linking of IgE to FcεRI [29]. Mast cell activation causes the release of several mediators such as histamine, type 2 cytokines such as IL-4, IL-5 and IL-13, chemokines such as eotaxin, prostaglandins and leukotrienes. This response results in mucus hypersecretion and AHR [366]. A late phase response then results in the activation and infiltration of leukocytes, consisting mainly of eosinophils, into the site of the allergic reaction [29]. Together with memory Th2 cells, there is persistent IgE and cytokine production, leading to the continuous recruitment and activation of mast cells and eosinophils [367]. ILC2s have been recently identified as mediators of type 2 immune responses in the lung. Upon exposure to allergens, the pulmonary epithelium produces IL-25, IL-33 and TSLP that drive the expansion of ILC2s [368]. This in turn leads to the production of type 2 cytokines, eosinophil influx, airway mucus hypersecretion and remodelling [369]. Other cells, including mDCs and γδ T cells are implicated in promoting type 2 immune responses [370, 371]. Current mainstay treatments for asthma, inhaled corticosteroids and long-acting β-agonists, generally suppress the symptoms of disease

but do not treat the underlying causes. Understanding the underlying mechanisms of disease pathogenesis is necessary to identify potential therapeutic targets for asthma.

In this study, we investigated the mechanisms by which RelB is involved in the development of spontaneous inflammation in the lung. We demonstrate that in the absence of RelB in $RelB^{-/-}$ mice, features of AAI develop independent of exogenous allergen exposure. Furthermore, adoptive transfer of RelB-sufficient DCs ameliorates this pathology.

2.3 Materials and Methods

2.3.1 Ethics statement

This study was performed in strict accordance with the recommendations in the Australian code of practise for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All protocols were approved by the Animal Ethics Committees of The University of Newcastle and University of Queensland.

2.3.2 Mice

All mice were maintained in specific pathogen-free conditions. RelB total knockout mice were kindly provided by Dr. Falk Weih, Leibniz-Institute for Age Research, Fritz-Lipmann-Institute, Jena, Germany [199]. Mice homozygous for an insertional mutation in the RelB gene were generated as previously described [213]. Both strains of mice were on a C57BL/6 background and were used for experimentations at 6-9 weeks of age together with their respective wild-type (WT; $^{+/+}$) and heterozygous ($^{+/-}$) controls.

2.3.3 Assessment of airway and parenchymal inflammation

BAL was performed as previously described, and differential leukocyte counts were determined from a total of 250 cells [372, 373]. Longitudinal sections were stained with

hematoxylin and eosin (H&E) and parenchymal inflammation was assessed by enumerating the numbers of inflammatory cells in 20 randomised, high powered fields [113].

2.3.4 Histopathology

Lungs were formalin fixed, embedded and sectioned. Longitudinal sections were stained with H&E (for histopathology) and periodic acid-Schiff (PAS-for MSC numbers). Histopathology was scored in a blinded fashion according to a set of custom-designed criteria (**Table 2.1**) and MSC numbers were enumerated in inflamed airways as previously described [374].

Table 2.1: Histopathological scoring system

Score 1: Airways inflammation score (/4)
0= Lack of inflammatory cells around airways- Absent
1= Some airways have small numbers of inflammatory cells- Mild
2= Some airways have significant inflammation- Moderate
3= Majority of airways have some inflammation- Marked
4= Majority of airways have significant inflammation- Severe
Score 2: Vascular inflammation score (/4)
0= Lack of inflammatory cells around blood vessels- Absent
1= Some blood vessels have small numbers of inflammatory cells- Mild
2= Some blood vessels have significant inflammation- Moderate
3= Majority of blood vessels have some inflammation- Marked
4= Majority of blood vessels have significant inflammation- Severe
Score 2: Parenchymal inflammation (at 10x magnification) (/5)
0= <1 % affected
1= 1-9 % affected
2= 10-29 % affected
3= 30-49 % affected
4= 50-69 % affected
5= >70 % affected
Total score= Score 1+ Score 2+ Score 3

2.3.5 Protein isolation

Protein was extracted from whole lungs. Briefly, tissues were homogenised in 500 µl of sterile Dulbecco's phosphate-buffered saline (Life Technologies, Mulgrave, Victoria, Australia) supplemented with PhoSTOP phosphatase and Complete ULTRA protease inhibitors cocktails (Roche Diagnostics, Manheim, Germany) using a Tissue Tearor stick homogeniser (BioSpec Products, Bartesville, Oklohama, USA) on ice. Homogenates were then centrifuged (8000x*g*, 10 minutes, 4 ^{o}C). [375]. Supernatants were collected and stored at -20 ^{o}C for assessment by enzyme-linked immunosorbent assay (ELISA) or multi-analyte flow assay kit.

2.3.6 Chemokine and cytokine concentrations in lungs

Protein concentrations for CCL2, CCL3, CCL4, CCL5, CCL11 CCL17, CXCL9, CXCL10, CXCL13, IL-4, IL-5 and IL-10 were determined in lung homogenates using a LEGENDplex multi-analyte flow assay kit (Biolegend, San Diego, California, USA) according to manufacturer's instructions. IL-13, IL-17A, IL-25, IL-33, TSLP and IFN-γ were determined using mouse DuoSet ELISA kits (R&D systems, Minneapolis, USA) according to manufacturer's instructions.

2.3.7 Serum antibodies

Total IgE in serum was determined by ELISA as previously described [70].

2.3.8 Airway remodelling

Lungs were formalin fixed, embedded and sectioned. Longitudinal sections were stained with H&E or Masson's Trichrome. Airway epithelial area (µm^2) and area of collagen deposition (µm^2) was assessed in a minimum of four small airways (basement membrane (BM) perimeter <1000 µm) per section [113, 375, 376]. Data were normalised to BM perimeter (µm) and quantified using ImageJ software (Version 1.49h, NIH, New York City, USA).

2.3.9 DC transfer

$RelB^{+/+}$ or $RelB^{+/-}$ lymphatic and splenic $CD11c^{+}$ DCs were purified by magnetic-activated cell sorting (MACS). Up to 2 x 10^6 MACS-purified DCs (>90% $CD11c^{hi}$) were injected intravenously into each RelB $knockout^{-/-}$ or RelB $mutant^{-/-}$ recipients. The lungs of untreated and treated mice were analysed 5 or 14 days after adoptive transfer.

2.3.10 Flow cytometry

Numbers of ILC2s, mDCs, γδ T cells, $CD4^{+}$ T cells, $CD8^{+}$ T cells, alveolar macrophages and Tregs in lung homogenates were determined based on surface marker expression using flow cytometry (**Table 2.2**) [373, 375, 377]. Cells were permeabilised and stained intracellularly for FoxP3 according to the manufacturer's instructions (eBioscience, San Diego, California, USA). Flow cytometric analysis was performed using a FACSAriaIII with FACSDiva software (BD Biosciences, North Ryde, Australia). Flow cytometry antibodies were from Biolegend (Karrinyup, WA, Australia) or eBioscience (**Table 2.3**).

Table 2.2: Characterisation of inflammatory cells

Cell type	**Cell-surface antigens**
ILC2	$CD45^{+}$ Lin^{-} $CD90.2^{+}$ $CD2^{-}$ IL-$7R\alpha^{+}$ $CD25^{+}$ IL-$33R\alpha^{+}$
mDCs	$CD45^{+}$ $CD11c^{+}$ $CD11b^{+}$ $F4/80^{-}$
γδ T cells	$CD45^{+}$ $CD3^{+}$ $CD4^{-}$ $CD8^{-}$ $\gamma\delta TCR^{+}$
CD4+ T cell	$CD45^{+}$ $CD3^{+}$ $CD4^{+}$ $CD8^{-}$ $\gamma\delta TCR^{-}$
CD8+ T cell	$CD45^{+}$ $CD3^{+}$ $CD4^{-}$ $CD8^{+}$ $\gamma\delta TCR^{-}$
Tregs	$CD45^{+}CD3^{+}$ $CD4^{+}$ $CD25^{+}$ $FoxP3^{+}$
Alveolar macrophages	$CD45^{+}$ $F4/80^{+}$ $CD11c^{+}$ $CD11b^{-}$

ILC2, type 2 innate lymphoid cell; mDCs, myeloid dendritic cells; γδ T cells, gamma delta T cells; Tregs, regulatory T cells

Table 2.3: Antibody used in flow cytometry analysis

Cell surface antigens	Clone	Fluorophore	Company
CD2	RM2-5	PE	Biolegend
CD25	PC61	FITC	Biolegend
Lineage (CD3/Ly-6G/CD11b/CD45R/ Ter119)	17A2/RB6-8C5/MI/70/ RA3-6B2/Ter-119	AF700	Biolegend
CD90.2	30-H12	APC/Cy7	Biolegend
cKit	2B8	BV421	Biolegend
IL-33Rα	DIH9	APC	Biolegend
CD45	30-F11	PerCPCy5.5	Biolegend
IL-7Rα	A7R34	BV605	Biolegend
CD3	17A2	AF700	Biolegend
CD80	16-10 A1	PE	Biolegend
CD86	GL-1	FITC	Biolegend
CD45	30-F11	AF700	Biolegend
Gr1	RB6-8C5	APC-Cy7	Biolegend
CD11c	N418	BV510	Biolegend
F4/80	BM8	BV605	Biolegend
I-A/I-E	M5/114.15.2	APC	Biolegend
CD11b	M1/70	PerCPCy5.5	Biolegend
FoxP3	FJK-16s	PE	eBioscience
CD4	RM4-5	APC-Cy7	Biolegend
γδ TCR	GL3	BV421	Biolegend
CD8a	53-6.7	BV510	Biolegend
CD3ε	145-2C11	APC	Biolegend

2.3.11 Lung function

Assessment of transpulmonary resistance was performed by whole body invasive plethysmography (Buxco electronics, Sharon, Connecticut, USA) as previously described [377].

2.3.12 Statistical analysis

Data are presented as means ± s.e.m. with 6-10 mice in each group and are representative of at least 2 independent experiments. Statistical significance for multiple comparisons was determined by non-parametric one-way analysis of variance (ANOVA) with Dunn's post-test or by one-way ANOVA with Bonferroni post-test, where appropriate. The two-tailed Mann-Whitney test was used to determine differences between two groups. AHR was analysed using two-way repeated measures ANOVA with Tukey's post-test. Analyses were performed using GraphPad Prism V.6 Software (San Diego, California, USA).

2.4 Results

2.4.1 Spontaneous pulmonary cellular inflammation develops in RelB$^{-/-}$ mice

To investigate the role of RelB in the spontaneous development of AAI, two different strains of RelB$^{-/-}$ mice were used; a targeted-deletion total knockout (open symbols) and a RelB mutant expressing an MHC class I transgene with a disruption in the *RelB* locus (closed symbols). RelB$^{-/-}$ mice and their respective RelB$^{+/+}$ and RelB$^{+/-}$ controls were sacrificed at 6-8 weeks of age and pulmonary inflammation was assessed in BAL fluid and lung tissue sections. RelB$^{-/-}$ mice had increased numbers of total leukocytes, eosinophils, lymphocytes, macrophages and neutrophils in BAL fluid (**Figure 2.1a-e**) compared to RelB$^{+/+}$ and RelB$^{+/-}$ controls. RelB$^{-/-}$ mice also had increased total, airway, vascular and parenchymal inflammatory scores (**Figure 2.1f-j**).

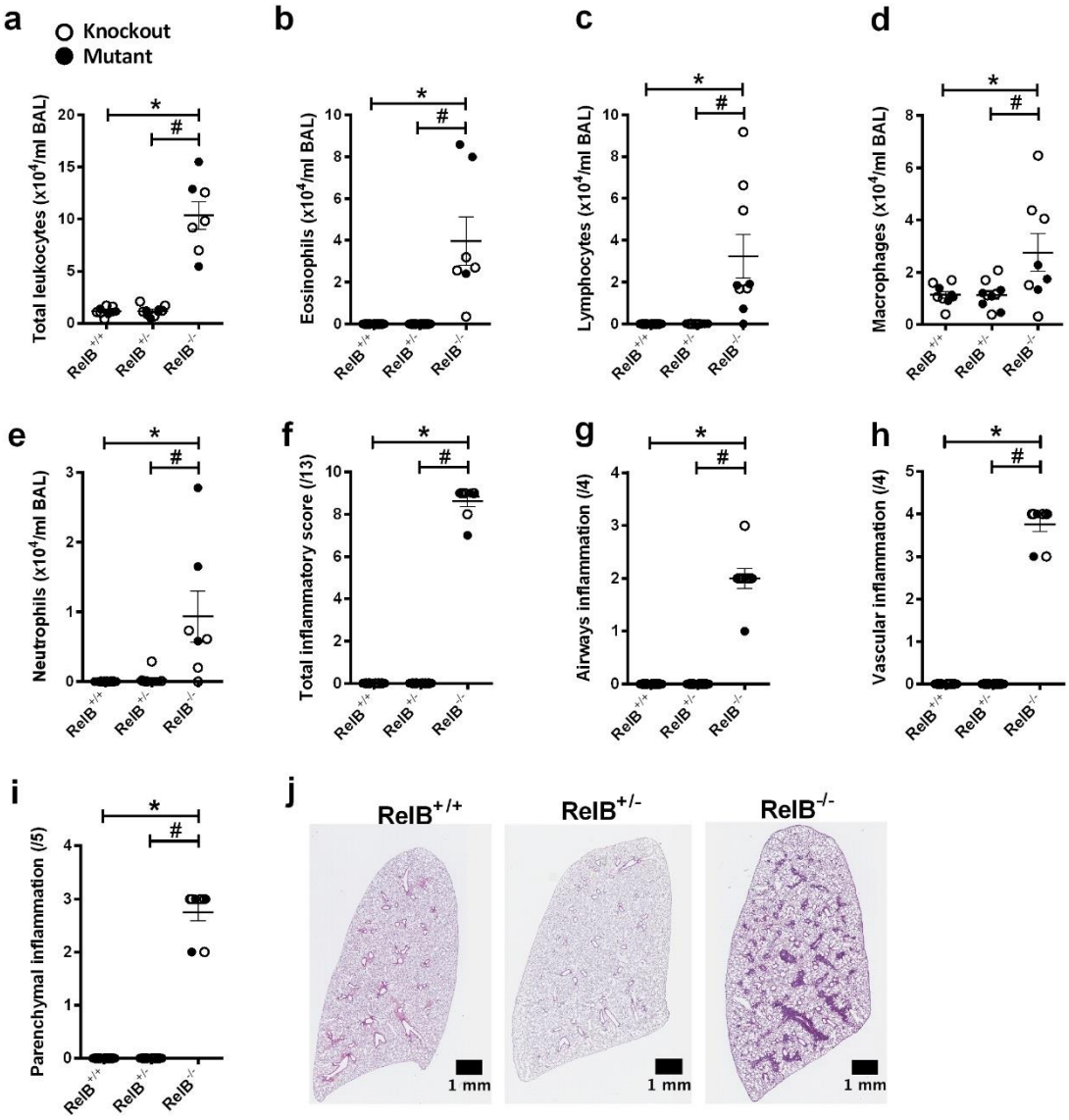


Figure 2.1: Spontaneous pulmonary cellular inflammation develops in RelB-deficient mice. Wild-type ($^{+/+}$), heterozygous ($^{+/-}$) and RelB-deficient ($^{-/-}$) knockout and mutant C57BL/6 mice were sacrificed at 6-9 weeks of age and pulmonary inflammation was assessed. **(a)** Total leukocytes, **(b)** eosinophils, **(c)** lymphocytes, **(d)** macrophages and **(e)** neutrophils in bronchoalveolar lavage (BAL) fluid. **(f)** Total, **(g)** airway, **(h)** vascular and **(i)** parenchymal inflammation scores in hematoxylin and eosin stained lung sections. **(j)** Representative images of lung sections. Scale bars = 1mm. Data (n=8-10) are presented as means ± s.e.m from two independent experiments. * represents $P \leq 0.05$

compared to $RelB^{+/+}$ mice, # represents $P \leq 0.05$, compared to $RelB^{+/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.

2.4.2 Increased chemokine and Th2-associated cytokine levels in the lungs of $RelB^{-/-}$ mice

Given that $RelB^{-/-}$ mice had increased pulmonary inflammation, we next assessed the levels of inflammatory chemokines and cytokines in the lung that may be mediating these effects. $RelB^{-/-}$ mice had increased levels of CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CXCL9, CXCL10 and CXCL13 (**Figure 2.2a-i**) in the lungs compared to $RelB^{+/+}$ and $RelB^{+/-}$ controls. Interestingly, $RelB^{-/-}$ mice also had increased levels of IL-4 and IL-5 (**Figure 2.3a and b**), but not IFN-γ and IL-17A (**Figure 2.3c and d**). As shown previously, total IgE was also increased in the serum of $RelB^{-/-}$ mice (**Figure 2.3e**) [200].

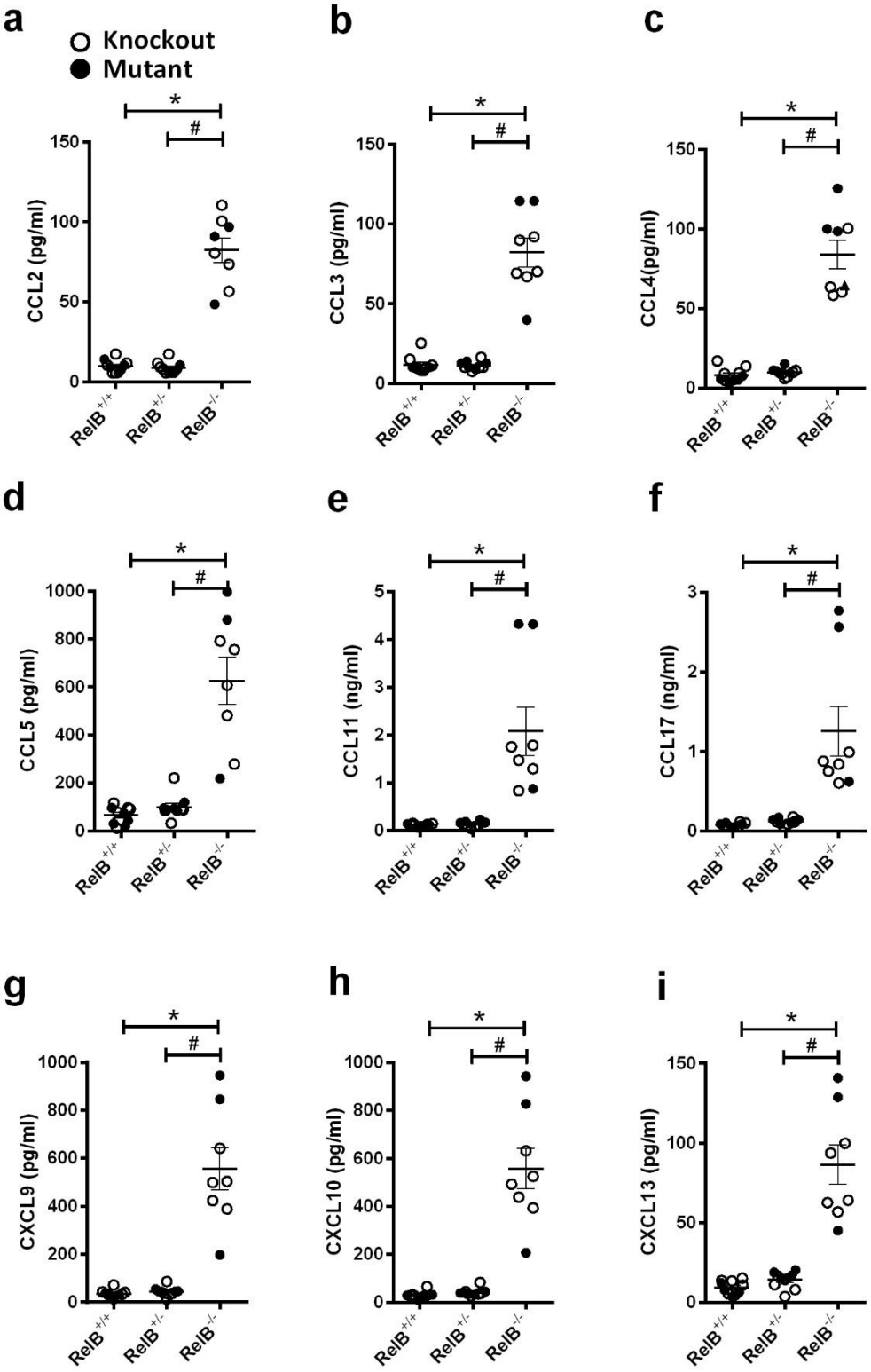

a
Knockout
Mutant
CCL2 (pg/ml)
b
CCL3 (pg/ml)
c
CCL4(pg/ml)
d
CCL5 (pg/ml)
e
CCL11 (ng/ml)
f
CCL17 (ng/ml)
g
CXCL9 (pg/ml)
h
CXCL10 (pg/ml)
i
CXCL13 (pg/ml)
RelB+/+
RelB+/-
RelB-/-

Figure 2.2: Increased chemokine levels in the lung of RelB-deficient mice. Wild-type ($^{+/+}$), heterozygous ($^{+/-}$) and RelB-deficient ($^{-/-}$) knockout and mutant C57BL/6 mice were sacrificed at 6-9 weeks of age. Protein was extracted from lung homogenates and chemokine levels assessed by multiplex assay. **(a)** Chemokine (C-C motif) ligand (CCL)2, **(b)** CCL3, **(c)** CCL4, **(d)** CCL5, **(e)** CCL11, **(f)** CCL17, **(g)** Chemokine (C-X-C motif) ligand (CXCL)9, **(h)** CXCL10 and **(i)** CXCL13 levels in lung homogenates. Data (n=8-10) are presented as means ± s.e.m from two independent experiments * represents $P \leq 0.05$ compared to $RelB^{+/+}$ mice, # represents $P \leq 0.05$, compared to $RelB^{+/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.

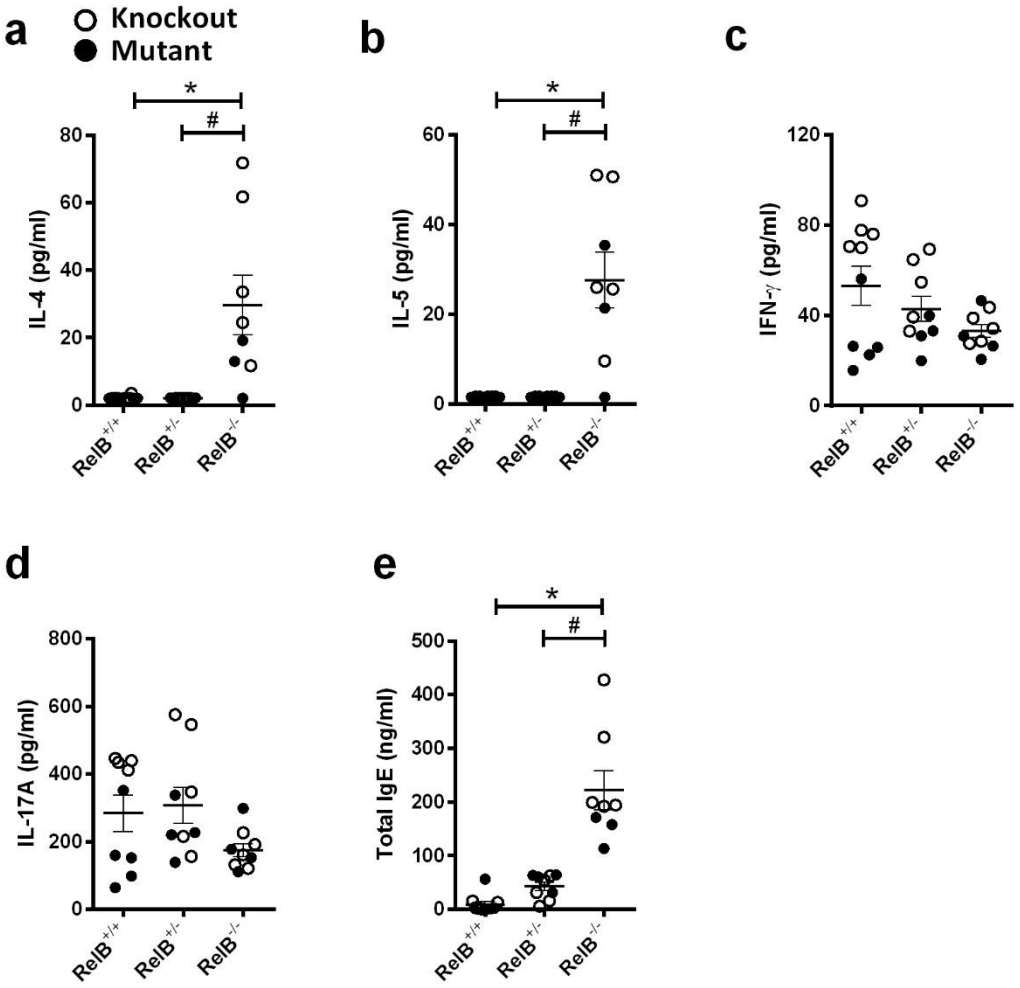


Figure 2.3: Increased interleukin (IL)-4, IL-5 and immunoglobulin (Ig)E levels in RelB-deficient mice. Wild-type ($^{+/+}$), heterozygous ($^{+/-}$) and RelB-deficient ($^{-/-}$) knockout and mutant C57BL/6 mice were sacrificed at 6-9 weeks of age. Protein was extracted from lung homogenates and cytokine levels assessed by multiplex assay or enzyme-linked immunosorbent assay (ELISA). **(a)** IL-4, **(b)** IL-5, **(c)** Interferon (IFN)-γ, and **(d)** IL-17A levels in lung homogenates. Total IgE in serum was determined by ELISA. **(e)** Total IgE in serum. Data (n=8-10) are presented as means ± s.e.m from two independent experiments * represents $P \leq 0.05$ compared to RelB$^{+/+}$ mice, # represents $P \leq 0.05$, compared to RelB$^{+/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.

2.4.3 Spontaneous airway remodelling develops in RelB$^{-/-}$ mice

The loss of control of inflammation can have damaging consequences on lung tissues. Thus, we next assessed features of airway remodelling in RelB$^{-/-}$ mice. These mice had increased MSC numbers around the airways, small airway epithelial cell area and collagen deposition compared to RelB$^{+/+}$ and RelB$^{+/-}$ controls (**Figure 2.4a-c**). Notably, both small airway epithelial cell area and collagen deposition were increased in RelB$^{+/-}$ compared to RelB$^{+/+}$ controls but there were no differences in MSC numbers (**Figure 2.4a-c**). Interestingly, RelB$^{+/-}$ mice had increased AHR, characterised by increased transpulmonary resistance in response to increasing doses of methacholine, compared to RelB$^{+/+}$ and RelB$^{-/-}$ mice (**Supplementary figure 2.10**).

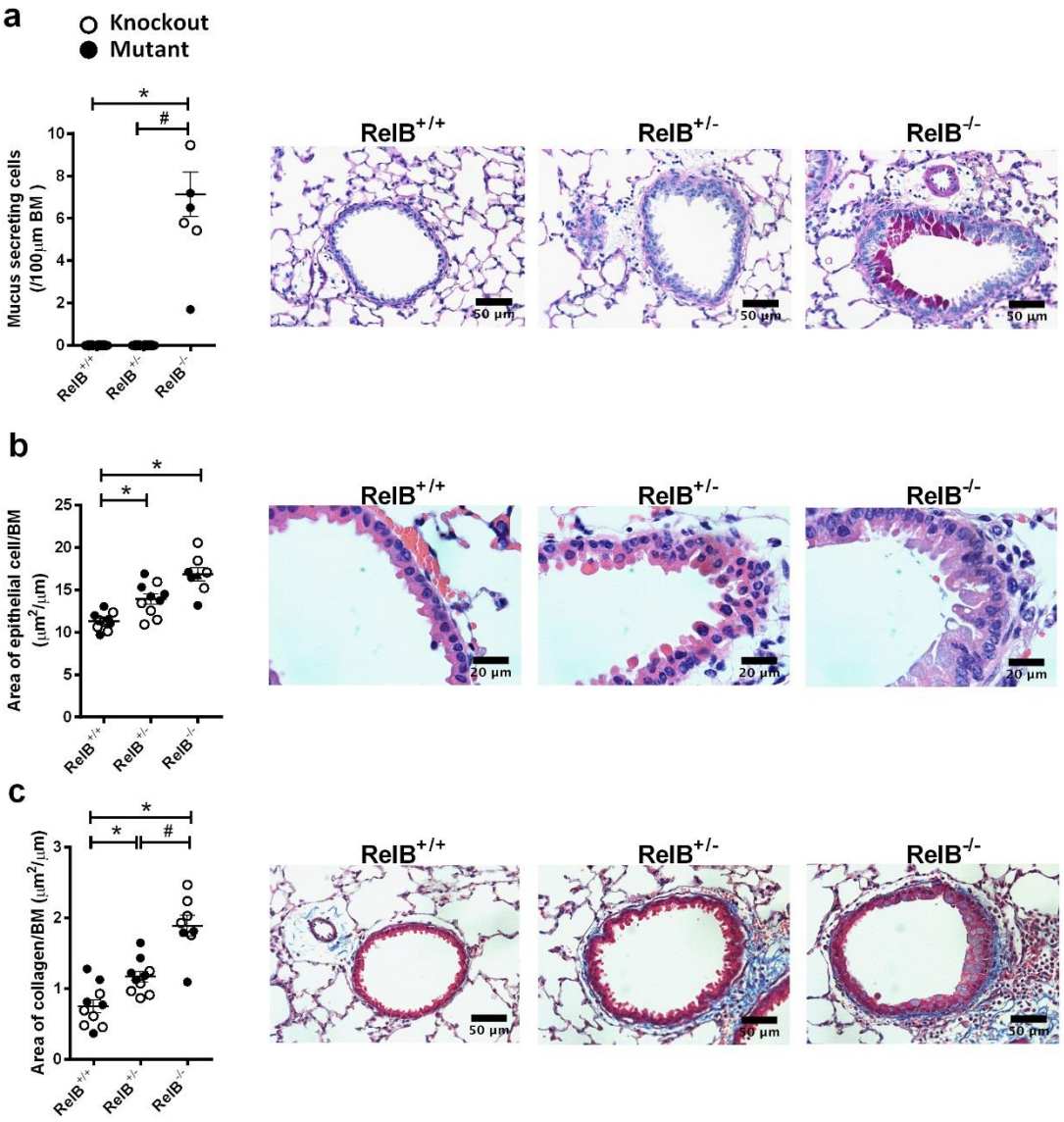


Figure 2.4: Spontaneous airway remodelling in RelB-deficient mice. Wild-type ($^{+/+}$), heterozygous ($^{+/-}$) and RelB-deficient ($^{-/-}$) knockout and mutant C57BL/6 mice were sacrificed at 6-9 weeks of age and airway remodelling was assessed. **(a)** Mucus secreting cell numbers per 100μm basement membrane (BM) in periodic acid-Schiff stained lung sections. Scale bars = 50 μm. **(b**) Small airway epithelial thickness in terms of epithelial cell area (μm^2) per basement BM perimeter in hematoxylin and eosin stained lung sections. Scale bars = 20 μm **(c)**. Area of collagen deposition (μm^2) per BM perimeter in Masson's Trichrome stained lung sections. Scale bars = 50 μm. Data (n=8-10) are

presented as means ± s.e.m from two independent experiments * represents $P \leq 0.05$ compared to $RelB^{+/+}$ mice, # represents $P \leq 0.05$, compared to $RelB^{+/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.

2.4.4 Adoptive transfer of RelB-sufficient DCs to $RelB^{-/-}$ reduces inflammation in BAL fluid and lungs

To assess the importance of RelB expression in DCs in the control of spontaneous AAI, purified lymphatic and splenic RelB-sufficient ($RelB^{+/+}$ or $RelB^{+/-}$) $CD11c^{+}$ DCs were adoptively transferred intravenously into $RelB^{-/-}$ mice. The lungs of untreated and treated mice were analysed 5 or 14 days after adoptive transfer.

We first examined the effects of RelB-sufficient DCs on pulmonary inflammation in BAL fluid. Adoptive transfer of RelB-sufficient DCs significantly reduced total leukocytes and eosinophils in the BAL fluid of $RelB^{-/-}$ mice while neutrophils were increased (**Figure 2.5a and b**). Notably, the influx of eosinophils was completely inhibited with numbers equivalent to those in $RelB^{+/-}$ mice. Transfer had no effect on the numbers of lymphocytes and macrophages. Next, we determined the effect of RelB-sufficient DCs on tissue inflammation. In DC-treated $RelB^{-/-}$ mouse lungs, parenchymal but not total, airway or vascular inflammatory score was decreased (**Figure 2.5c-g**) and there were fewer inflammatory cells per high-powered field in the parenchyma compared to untreated $RelB^{-/-}$ mice (**Figure 2.5h**).

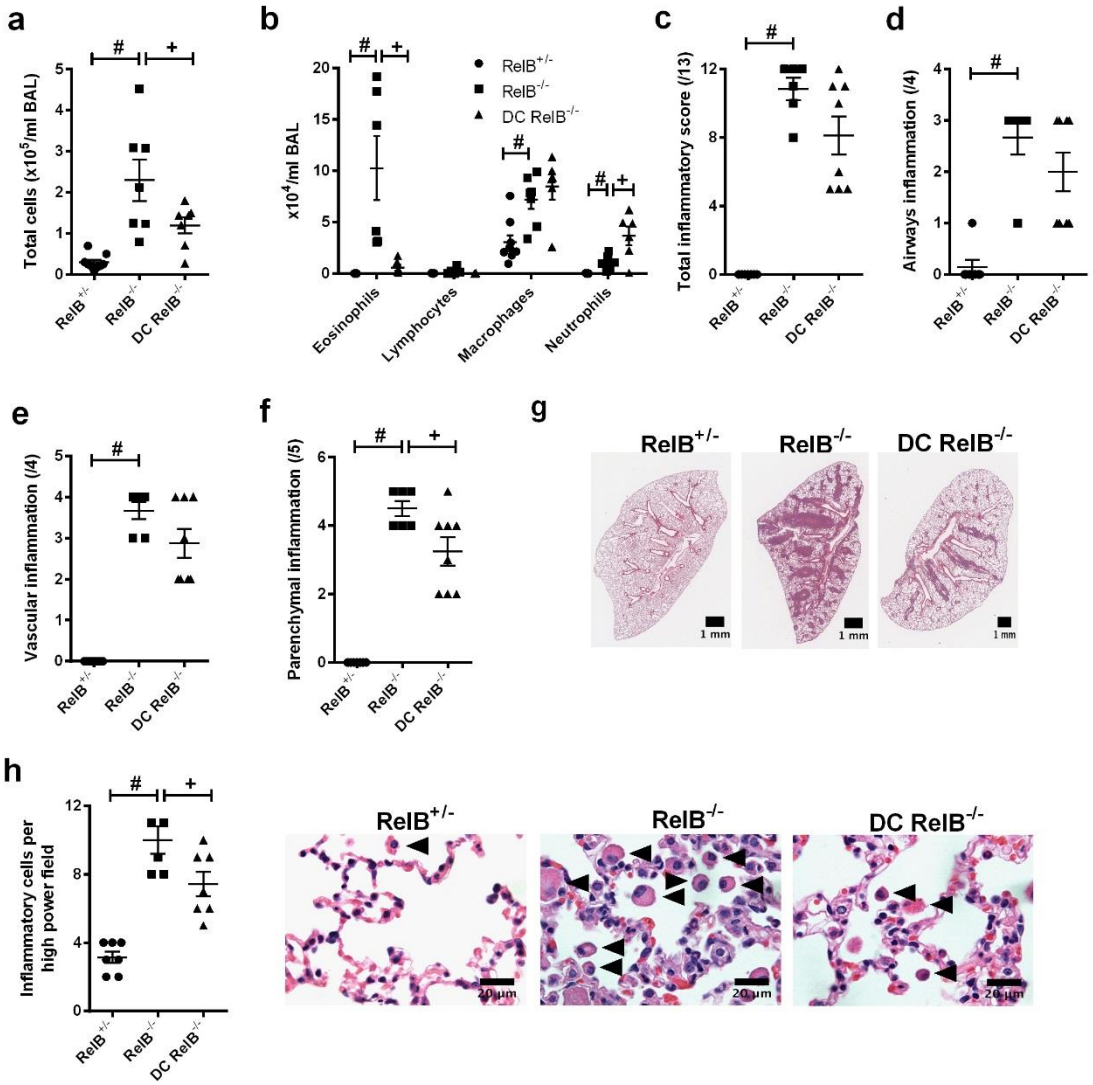


Figure 2.5: Adoptive transfer of RelB-sufficient dendritic cells (DCs) to RelB-deficient mice decreased pulmonary inflammation. Purified lymphatic and splenic $CD11c^+$ DCs from RelB heterozygous ($^{+/-}$) mice were adoptively transferred intravenously into RelB-deficient ($^{-/-}$) mutant C57BL/6 mice and pulmonary inflammation were assessed 5 days post DC transfer. **(a)** Total leukocytes and **(b)** eosinophils, lymphocytes, macrophages and neutrophils in bronchoalveolar lavage (BAL) fluid. **(c)** Total, **(d)** airways, **(e)** vascular and **(f)** parenchymal inflammation scores in hematoxylin and eosin (H&E) stained lung sections. **(g)** Representative images of lung sections. Scale bars= 1mm. **(h)** Numbers of parenchymal inflammatory cells

(arrowheads) were determined in H&E stained lung sections. Scale bars= 20 μm. Data (n=6-9) presented as means ± s.e.m from 3 independent experiments. # represents $P \leq 0.05$ compared to $RelB^{+/-}$ mice, + represents $P \leq 0.05$, compared to $RelB^{-/-}$ mice. Closed symbols represent RelB mutant mice.

2.4.5 Adoptive transfer of RelB-sufficient DCs to RelB-deficient mice reduced the numbers of ILC2s, γδ T cells and mDCs in the lung

To further explore the influence of RelB on inflammation we next assessed the effects on inflammatory leukocyte subsets in more detail. This was achieved by examining what subsets infiltrate into the lung in the absence of RelB by flow cytometry and if these could be regulated by adoptive transfer of RelB-sufficient DCs. Consistent with increases in the major inflammatory cell subtypes, $RelB^{-/-}$ mice had increased numbers of ILC2s, γδ T cells, mDCs and $CD8^+$ T cells in the lung (**Figure 2.6a-c, Supplementary figure 2.11b**). Also in line with our previous treatment data, all of these subsets were significantly reduced by adoptive transfer of RelB-sufficient DCs. We also observed increases in $CD4^+$ T cells and $CD4^+CD25^+FoxP3^+$ Tregs and reduced alveolar macrophages in $RelB^{-/-}$ mice, however, adoptive transfer of RelB-sufficient DCs did not alter the number of these cells (**Supplementary figure 2.11a, c and d**).

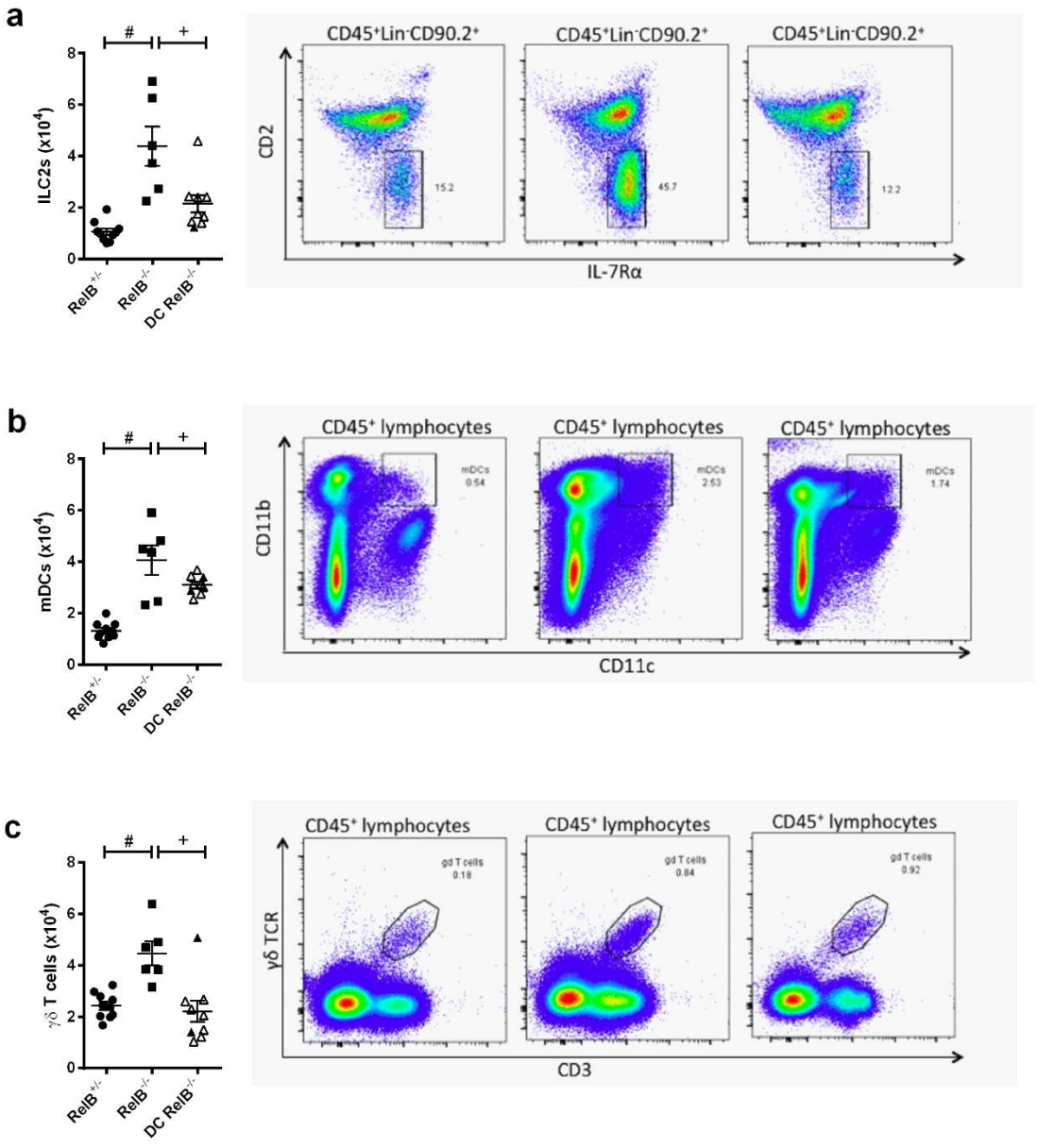


Figure 2.6: Adoptive transfer of RelB-sufficient dendritic cells (DCs) to RelB-deficient mice decreased the numbers of ILC2s, mDCs, and γδ T cells in the lung. Purified lymphatic and splenic CD11c^{+} DCs from RelB wild-type (WT, $^{+/+}$) or RelB heterozygous ($^{+/-}$) mice were adoptively transferred intravenously into RelB-deficient ($^{-/-}$) knockout or mutant C57BL/6 mice. Data from RelB$^{+/+}$ transfer into knockout (n=8) and RelB$^{+/-}$ into mutant mice (n=2) are combined. The numbers of inflammatory cell subsets in the lungs were assessed by flow cytometry 14 days post DC transfer. **(a)** Type 2 innate

lymphoid cells (ILC2s) were gated as $CD45^+$ Lin^- $CD90.2^+$ $CD2^-$ $IL7R\alpha^+$ $CD25^+$ $IL33R\alpha^+$, **(b)** myeloid dendritic cells (mDCs) were gated as $CD45^+$ $CD11c^+$ $CD11b^+$ $F4/80^-$, and **(c)** gamma delta (γδ) T cells were gated as $CD45^+$ $CD3^+$ $CD4^-$ $CD8^-$ $\gamma\delta TCR^+$. Data (n=2-8) are presented as means ± s.e.m from 2 independent experiments. # represents $P \leq 0.05$ compared to $RelB^{+/-}$ mice, + represents $P \leq 0.05$, compared to $RelB^{-/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.

2.4.6 Adoptive transfer of RelB-sufficient DCs to RelB-deficient mice reduced chemokine and Th2-associated cytokine levels in the lung

The reduction in pulmonary inflammatory cells after adoptive transfer of RelB-sufficient DCs suggested that the production of inflammatory chemokines and cytokines might be reduced. Adoptive transfer of RelB-sufficient DCs significantly reduced the levels of CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CXCL9, CXCL10 and CXCL13 (**Figure 2.7a-i**) in the lung compared to untreated $RelB^{-/-}$ controls. We again showed that IL-4 and IL-5 were increased in the lungs of $RelB^{-/-}$ mice (**Figure 2.8a and 2.8b**). We furthered this observation by detecting elevated IL-13, IL-25, IL-33 and TSLP levels (**Figure 2.8c, 2.8e-g**). Adoptive transfer of RelB-sufficient DCs to $RelB^{-/-}$ mice also significantly reduced the levels of all of these cytokines (**Figure 2.8a-c and 2.8e-g**). Total serum IgE was also suppressed compared to untreated $RelB^{-/-}$ controls (**Figure 2.8i**). In contrast to these Th2-associated cytokines, the levels of IL-17A increased in the lung in response to adoptive transfer of RelB-sufficient DC to $RelB^{-/-}$ mice (**Figure 2.8d**). Levels of IFN-γ were reduced, while there was no difference in IL-10 levels (**Figure 2.8h, Supplementary Figure 2.12**).

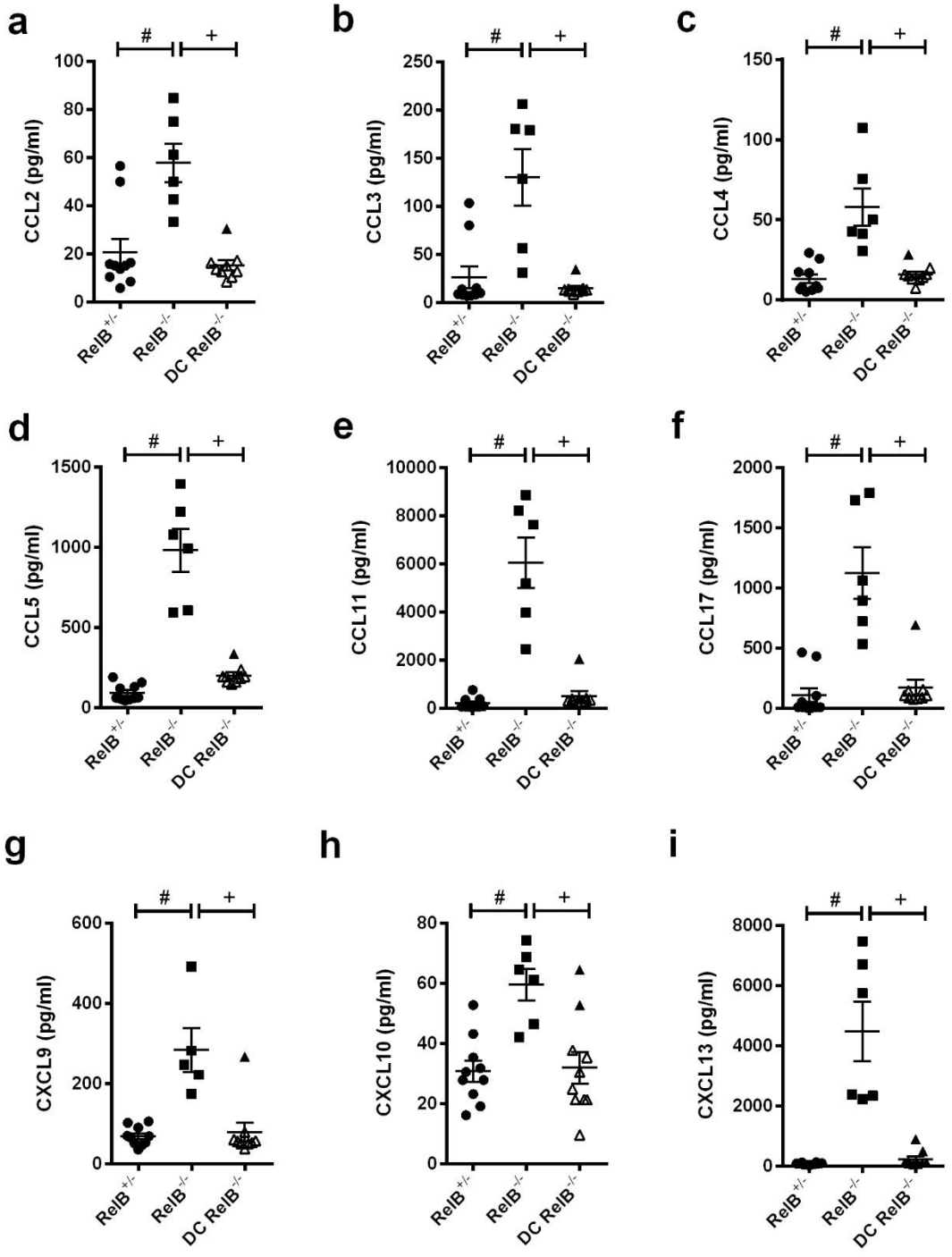


Figure 2.7: Adoptive transfer of RelB-sufficient dendritic cells (DCs) to RelB-deficient mice reduced chemokine levels in the lung. Purified lymphatic and splenic CD11c^{+} DCs from RelB wild-type ($^{+/+}$) or heterozygous ($^{+/-}$) mice were adoptively transferred intravenously into RelB-deficient ($^{-/-}$) knockout or mutant C57BL/6 mice. Data from RelB$^{+/+}$ transfer into knockout (n=8) and RelB$^{+/-}$ into mutant mice (n=2) are

combined. Protein was extracted from lung homogenates and chemokine levels assessed by multiplex assay 14 days post DC transfer. **(a)** Chemokine (C-C motif) ligand (CCL)2, **(b)** CCL3, **(c)** CCL4, **(d)** CCL5, **(e)** CCL11, **(f)** CCL17, **(g)** Chemokine (C-X-C motif) ligand (CXCL)9, **(h)** CXCL10 and **(i)** CXCL13 levels in lung homogenates. Data (n=2-8) are presented as means ± s.e.m from 2 independent experiments. # represents $P \leq 0.05$ compared to $RelB^{+/-}$ mice, + represents $P \leq 0.05$, compared to $RelB^{-/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.

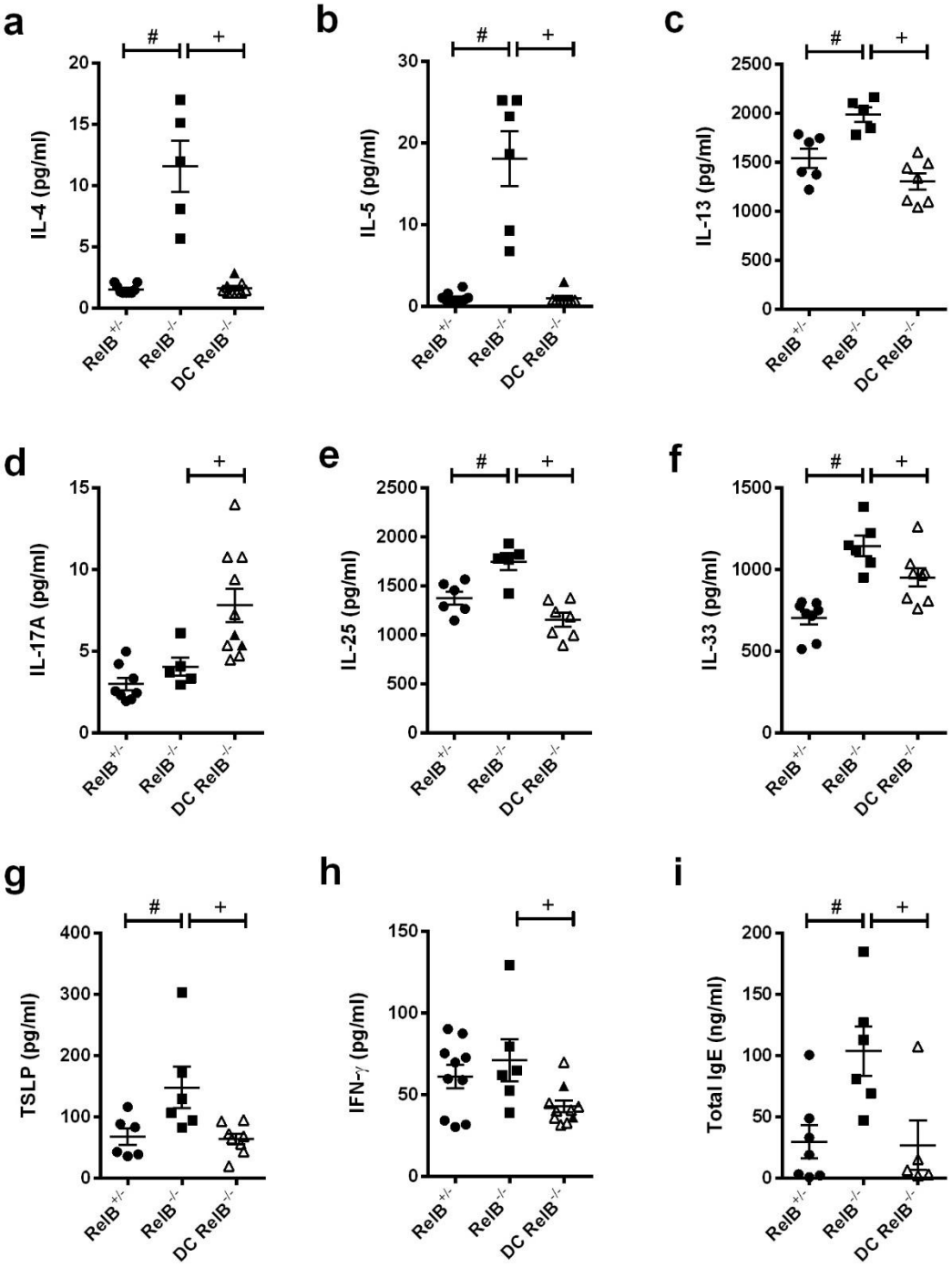


Figure 2.8: Adoptive transfer of RelB-sufficient dendritic cells (DCs) to RelB-deficient mice reduced levels of Th2-associated cytokines in the lung. Purified lymphatic and splenic CD11c^{+} DCs from RelB wild-type ($^{+/+}$) or heterozygous ($^{+/-}$) mice were adoptively transferred intravenously into RelB-deficient ($^{-/-}$) knockout or mutant C57BL/6 mice. Data from RelB$^{+/+}$ transfer into knockout (n=8) and RelB$^{+/-}$ into mutant

mice (n=2) are combined. Protein was extracted from lung homogenates and cytokine levels assessed by multiplex assay or enzyme-linked immunosorbent assay (ELISA) 14 days post DC transfer. **(a)** Interleukin (IL)-4, **(b)** IL-5, **(c)** IL-13, **(d)** IL-17A, **(e)** 1L-25, **(f)** IL-33, **(g)** thymic stromal lymphopoietin (TSLP) and **(h)** interferon (IFN)-γ levels in lung homogenates. Total IgE in serum was determined by ELISA 14 days post DC transfer. **(i)** Total IgE in serum. Data (n=2-8) are presented as means ± s.e.m from 2 independent experiments. # represents $P \leq 0.05$ compared to $RelB^{+/-}$ mice, + represents $P \leq 0.05$, compared to $RelB^{-/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.

2.4.7 Adoptive transfer of RelB-sufficient DCs to RelB-deficient mice decreased airway remodelling

We then assessed the effect of DC transfer on spontaneous airway remodelling. Adoptive transfer of RelB-sufficient DCs significantly suppressed airway remodelling with reduced MSC numbers, small airway epithelial cell area and collagen deposition around the small airways compared to $RelB^{-/-}$ controls (**Figure 2.9a-c**).

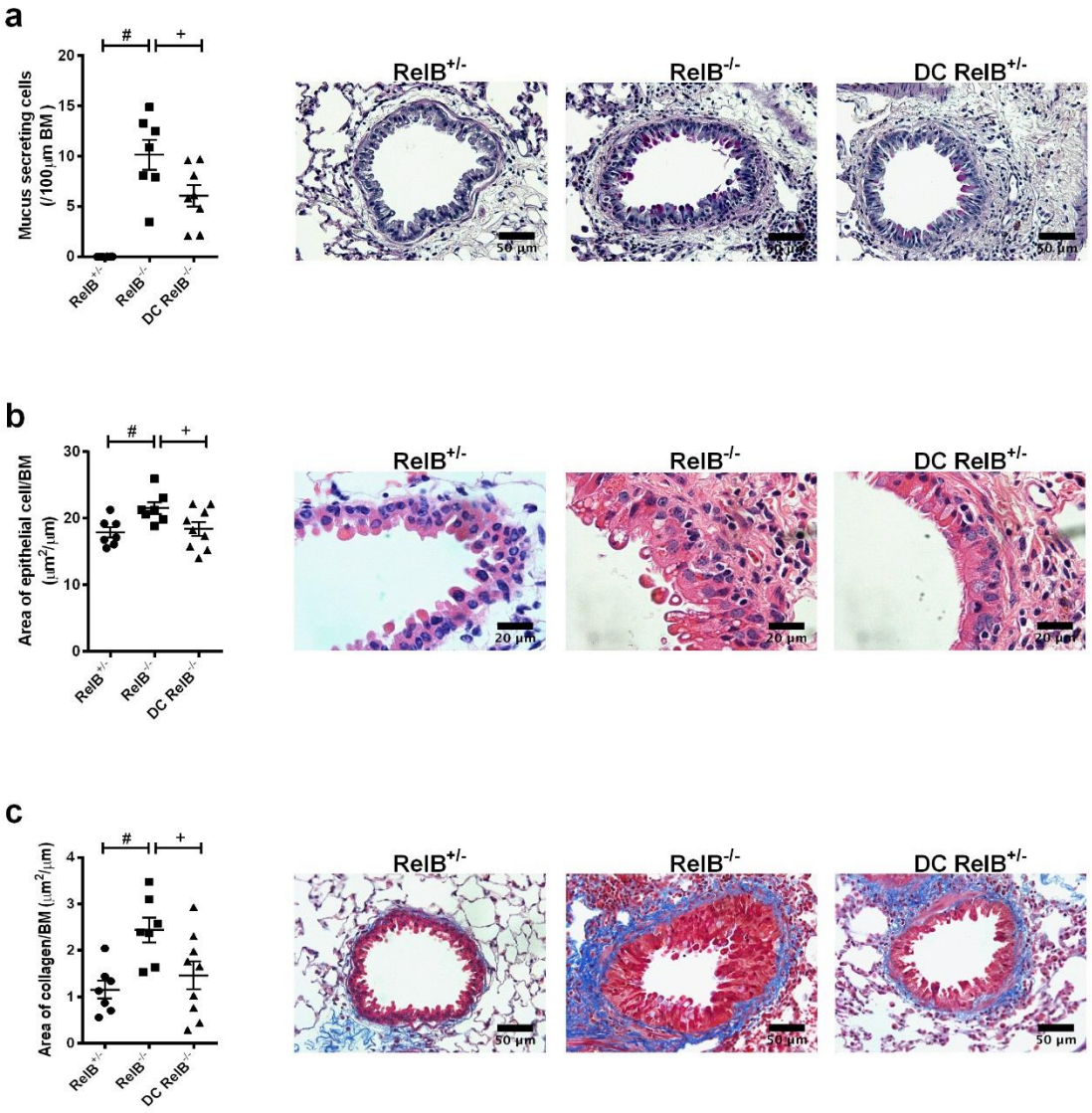


Figure 2.9: Adoptive transfer of RelB-sufficient dendritic cells (DCs) to RelB-deficient mice suppressed airway remodelling. Purified lymphatic and splenic $CD11c^{+}$ DCs from RelB heterozygous ($^{+/-}$) were adoptively transferred intravenously into RelB-deficient ($^{-/-}$) mutant C57BL/6 and airway remodelling was assessed 5 days post DC transfer. **(a)** Mucus secreting cell numbers per 100μm basement membrane (BM) in periodic acid-Schiff stained lung sections. Scale bars = 50 μm. **(b)** Small airway epithelial thickness in terms of epithelial cell area (μm^2) per BM perimeter in hematoxylin and eosin stained lung sections. Scale bars = 20 μm. **(c)** Area of collagen deposition (μm^2) per BM perimeter in Masson's Trichrome stained lung sections. Scale bars = 50 μm. Data (n=6-

9) are presented as means ± s.e.m from 3 independent experiments. # represents $P \leq 0.05$ compared to $RelB^{+/-}$ mice, + represents $P \leq 0.05$, compared to $RelB^{-/-}$ mice. Closed symbols represent RelB mutant mice.

2.5 Discussion

In this study, we showed that $RelB^{-/-}$ mice developed spontaneous AAI that is independent of exogenous allergen exposure. Spontaneous pulmonary inflammation occurred that was characterised by increases in eosinophils, lymphocytes, macrophages and neutrophils in BAL fluid and lung histopathology typical of AAI. These events were associated with increases in the levels of a range of chemokines and Th2-associated cytokines in the lung. Airway remodelling also developed with increasing numbers of MSCs around the airways, epithelial thickening and collagen deposition. Transfer of RelB-sufficient DCs to $RelB^{-/-}$ mice suppressed pulmonary inflammation, chemokines, Th2-associated cytokines and airway remodelling. Our data indicate that RelB deficiency in parenchymal and haemopoietic cells promotes AAI and remodelling. Importantly, in this RelB-deficient setting, DCs encoding RelB are sufficient to control pulmonary and Th2 inflammation and airway remodelling. These data have important implications for immunotherapy of allergic asthma.

In our study, we used two different strains of $RelB^{-/-}$ mice; a targeted total knockout of the *RelB* gene [199], and a mutant that was generated by chance integration of a class I MHC genomic clone as a transgene[216]. Both strains were able to receive RelB-sufficient DCs from $RelB^{+/-}$ littermates.

Other studies have shown that $RelB^{-/-}$ mice have multi-organ inflammation, particularly in the lungs and liver [199, 213]. In the lungs of 10-day-old $RelB^{-/-}$ mice, inflammation was mainly focused around the blood vessels and by day 20, the

perivascular infiltrate had extended into the parenchyma, leading to focal consolidation. Our analysis of adult RelB$^{-/-}$ mice demonstrated similar inflammation around the airways and blood vessels, and in the lung parenchyma, as well as infiltration into the airway lumen.

Consistent with the conclusion that RelB deficiency leads to AAI, we observed that chemokines associated with allergic inflammation (CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CXCL9, CXCL10 and CXCL13) were increased in the lungs of RelB$^{-/-}$ mice. Relevant to humans, the levels of CCL2, CCL3, CCL5, and CCL11 have been shown to be elevated in the BAL fluid of patients with allergic asthma [378-380]. CCL2 enhances the secretion of IL-4, an important Th2 cytokine in asthma, by T cells [381, 382]. CCL3, CCL5 and CCL11 are potent eosinophil chemoattractants [383-385]. Although not specifically shown in asthmatic patients, CCL4 was increased in nasal secretions of patients with allergic rhinitis [386]. CCL17 promotes development of eosinophilia through the recruitment of Th2 type CD4^{+} T lymphocytes [387]. Increased levels of CXCL9 and CXCL10 have been observed in the plasma of asthmatic children with acute exacerbations and elevated levels of CXCL13 have also been shown in the BAL fluid from patients with asthma and a murine model of OVA-induced allergic inflammation [388, 389]. The increase in each of these chemokines is consistent with the increases in eosinophils observed in the BAL fluid of RelB$^{-/-}$ mice. RelB$^{-/-}$ parenchymal cells are particularly prone to chemokine production due to the lack of transcriptional repression imposed by RelB. Upon stimulation by LPS, RelB$^{-/-}$ kidney fibroblasts were found to produce high levels of chemokines *in vitro* and this was reduced by transfection with RelB complementary DNA (cDNA) [201]. In support of a key role for RelB-deficient parenchyma driving the development of AAI, transfer of RelB$^{-/-}$ bone marrow into

lethally irradiated WT controls does not induce AAI [208], even though stimulated DCs from these mice produce low levels of IL-12p70 *in vitro* [390].

The type of T cell-dependent immune response is determined by the production of specific cytokines. In addition to increased chemokines, we observed elevated levels of Th2-associated cytokines IL-4, IL-5 and IL-13 in the lungs of RelB$^{-/-}$ mice, but not the Th1-associated cytokine IFN-γ, or the Th17-associated cytokine, IL-17A. IL-4 plays a pivotal role in the differentiation of naïve T cells into Th2 cells [391]. IL-5 has been strongly implicated in the aetiology of AAI by regulating the growth, differentiation and activation of eosinophils [392]. In agreement with these properties, increased expression of IL-4 and IL-5 was associated with the accumulation of eosinophils in the airway lumen of RelB$^{-/-}$ mice. IL-13 is involved in the initiation of Th2 responses and mucus production and increased expression of IL-13 in RelB$^{-/-}$ was associated with increased MSC numbers [34]. These cytokines also promote Ig isotype switching to IgE [44, 393, 394]. The cross-linking of FcεRI on eosinophils and mast cells stimulates degranulation with the release of histamine and inflammatory mediators that together elicit AAI [395]. Our data suggest that type 2 responses and their inflammatory consequences dominate in the lungs of RelB$^{-/-}$ mice.

Flow cytometric analysis of lung homogenates from RelB$^{-/-}$ mice identified increased numbers of ILC2s, mDCs and γδ T cells in the lung. ILC2s have recently emerged as a key innate driver of type 2 immunity in the lungs of both mice and humans [58, 396]. In allergy, mDCs and γδ T cells skew T cells towards Th2-type immune responses [397]. Consistent with the increase in ILC2s, the cytokines IL-25, IL-33 and TSLP, which activate and expand ILC2s to produce IL-4 and IL-13, were also increased in RelB$^{-/-}$ lungs. IL-25 is secreted by eosinophils and epithelial cells and promotes airway eosinophil accumulation, mucus hypersecretion and AHR in mice [49, 50]. IL-33 is released

by the airway epithelium and stimulates the production of Th2 cytokines and serum IgE and IgA [398]. TSLP is also secreted by the airway epithelium, and plays a role in the initiation of allergic inflammation through the induction of OX40 ligand expression by DCs and subsequent differentiation of Th2 cells [51, 52]. TSLP also induces the secretion of CCL17 by mDCs, promoting the recruitment of Th2 cells to the airway [51]. These epithelium-derived cytokines activate and expand ILC2s to increase their production of IL-4, IL-5 and IL-13, which in turn promotes eosinophil recruitment and mucus hypersecretion. Transfer of RelB-sufficient DCs to $RelB^{-/-}$ mice significantly reduced the numbers of ILC2s, mDCs, γδ T cells, and Th2-associated cytokines IL-4, IL-5, IL-13, IL-25, IL-33 and TSLP as well as serum IgE. We have previously shown that the levels of IL-4 and IL-5 are reduced in the serum of DC-treated mice [208]. IL-25 and IL-33 also promote airway remodelling in allergic diseases [399, 400]. Reduction in the levels of these cytokines in mice receiving DC treatment was associated with the suppression of airway remodelling with reductions in MSC numbers, collagen deposition and small airway epithelial cell area.

Chronic airway inflammation promotes the development of airway remodelling. The increases in MSC numbers within the airways, collagen deposition around the small airways and airway epithelial cell area in $RelB^{-/-}$ mice are consistent with previous observations [213]. Surprisingly, despite the lack of inflammation in $RelB^{+/-}$ mice, we observed an increase in small airway epithelial cell area and collagen deposition compared to $RelB^{+/+}$ mice although the change was not as large as that in $RelB^{-/-}$ mice. These data suggest that even heterozygous RelB deficiency may predispose to mild to moderate airway epithelial changes. In support of this observation, $RelB^{+/-}$ mice had increased transpulmonary resistance in response to increasing doses of methacholine compared to $RelB^{+/+}$ and $RelB^{-/-}$ mice. We speculate that these differences in AHR result

from the epithelial alterations in RelB$^{+/-}$ mice that improves the permeability for methacholine, thus increasing responses to methacholine, while the massive inflammation and remodelling around the airways of RelB$^{-/-}$ mice may reduce methacholine permeability.

Recently, three patients with a homozygous C1191A mutation in the *relB* gene were identified within a consanguineous family [401]. This mutation prevented RelB protein production and caused immune deficiency and inflammatory disease. Some similarities to our observations in mice were observed; T cells were skewed to effector memory cells, IL-2 and IFN-γ production was markedly reduced *in vitro*, and the TNF-induced p65 response was increased in patient fibroblasts. B cell maturation was defective and T-cell dependent and independent antibody responses were low to absent [402]. Consistent with this immunodeficiency, each of the patients had persistent upper and lower respiratory tract infections with associated chronic cough and pneumonia. One patient had reactive airway disease from infancy, and the mother of 2 of the patients had psoriasis and eczema. Together these observations demonstrate that homozygous RelB-deficiency in mice and humans is associated with severe immunodeficiency and inflammatory disease, the severity of which is exacerbated by pathogen exposure [200, 403]. Heterozygous RelB-deficiency may predispose to inflammatory autoimmune or atopic disease, within the context of other genes and microbial exposure.

With respect to other contributory genes, a recent genome-wide association study and meta-analysis in 21,000 cases and 95,000 controls of multiple ancestry identified 10 new risk loci associated with atopic dermatitis, a disease clinically linked with asthma [364]. Interestingly, some loci are associated with several key cells and factors that are altered in RelB$^{-/-}$ mice, including Th2 and Tregs (*IL13*, *IL2RA*, *IL7R* and *IL2)*, pro-inflammatory cytokine signalling (*IL6R, IL18R, STAT3, STAT6, IL22, SOCS3)* and antigen

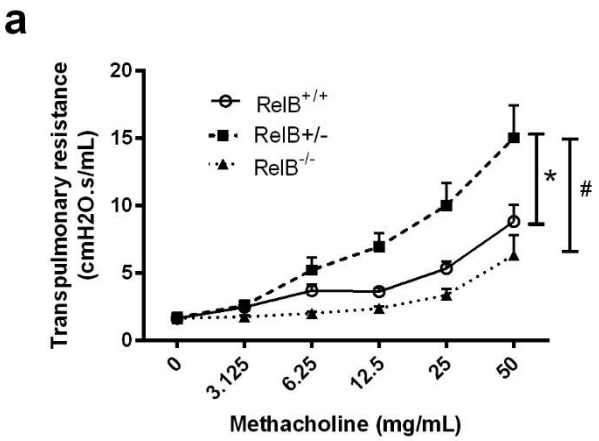
presentation/Langerhans cells (*HLA-DRB1*, *CD207*). Two loci (*TRAF3*, *PUS10*) highlight the potential role of the NF-κB pathway. In myeloid cells, TRAF3 regulates the NF-κB2 pathway and thus p52 [404], which partners with RelB in transcriptional regulation [405]. The *PUS10* locus lies very close to *REL*, encoding the c-Rel subunit of NF-κB. In a second study, using a systems biology approach, *NFKBIA* promoter polymorphisms were found to be associated with susceptibility to childhood inflammatory lung diseases [406]. *NFKB1A* encodes for the IKBα inhibitor of classical NF-κB activity [407]. While RelB has low affinity for IKBα, it regulates the stability in fibroblasts by mediating the phosphorylation steps involved in the degradation of IKBα [408]. Together these clinical studies, in the context of the current and previous studies in $RelB^{-/-}$ mice, strongly suggest that further translational insight will emerge from further functional studies of RelB and its regulation in human inflammatory airway diseases and atopic eczema.

RelB is highly expressed in DCs and is crucial for their maturation and DC-mediated immunity [195, 409]. DCs play critical roles in priming T cells and controlling subsequent immune responses. $RelB^{-/-}$ mice have defects in DCs but not Langerhans cell development [410]. In the current studies, adoptive transfer of RelB-sufficient DCs reduced pulmonary inflammation, chemokines, Th2-associated cytokines and small airway remodelling.

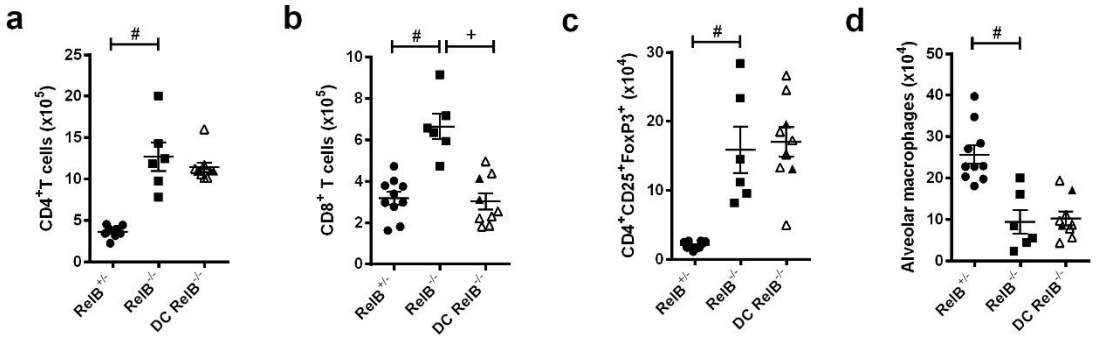
Previously, we showed that transfer of RelB-sufficient DCs achieved long-term suppression of autoimmune disease and suppressed organ (liver, spleen and pancreas) inflammation in $RelB^{-/-}$ mice in an IDO-dependent fashion [208]. IDO activity in RelB-sufficient DC-treated mice was CD80/CD86-dependent, and promoted IFN-γ and suppressed IL-4 and IL-5 production by T cells [208]. We speculate that this regulatory mechanism also suppresses the pro-inflammatory chemokines and cytokines driving AAI in DC-treated $RelB^{-/-}$ mice.

In summary, data in this and other studies indicate that RelB deficiency is a key pathway underlying AAI, and that DC-encoded RelB is sufficient to restore control through immune regulation of inflammation.

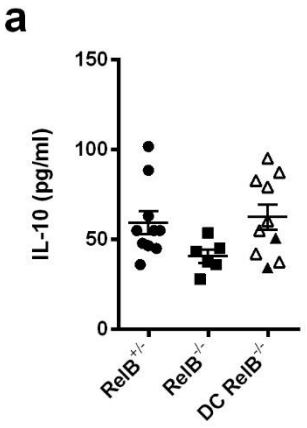
2.6 Supplementary data



Supplementary figure 2.10: Increased transpulmonary resistance in RelB-heterozygous mice. Wild-type ($^{+/+}$), heterozygous ($^{+/-}$) and RelB-deficient ($^{-/-}$) mutant C57BL/6 mice were sacrificed at 6-9 weeks of age and transpulmonary resistance was assessed. **(a)** Transpulmonary resistance. Data (n=8-10) are presented as means ± s.e.m from 2 independent experiments. * represents $P < 0.05$ compared to RelB$^{+/+}$ mice, # represents $P < 0.05$, compared to RelB$^{+/-}$ mice.



Supplementary figure 2.11: Numbers of $CD4^+$, $CD8^+$ T cells, regulatory T cells and alveolar macrophages in the lung. Purified lymphatic and splenic $CD11c^+$ dendritic cells (DCs) from RelB wild-type ($^{+/+}$) or heterozygous ($^{+/-}$) mice were adoptively transferred intravenously into RelB-deficient ($^{-/-}$) knockout or mutant C57BL/6 mice. Data from $RelB^{+/+}$ transfer into knockout (n=8) and $RelB^{+/-}$ into mutant mice (n=2) are combined. The numbers of inflammatory cell subsets in the lung were assessed by flow cytometry 14 days post DC transfer. **(a)** $CD4^+$ T cells were gated as $CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$ $\gamma\delta TCR^-$. **(b)** $CD8^+$ T cells were gated as $CD45^+$ $CD3^+$ $CD4^-$ $CD8^+$ $\gamma\delta TCR^-$. **(c)** Regulatory T cells were gated as $CD45^+$ $CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$. **(d)** Alveolar macrophages were gated as $CD45^+$ $F4/80^+$ $CD11c^+$ $CD11b^-$. Data (n=2-8) are presented as means ± s.e.m from 2 independent experiments. # represents $P < 0.05$ compared to $RelB^{+/-}$ mice, + represents $P < 0.05$, compared to $RelB^{-/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.



Supplementary figure 2.12: Levels of interleukin (IL)-10 in the lung. Purified lymphatic and splenic $CD11c^{+}$ dendritic cells (DCs) from RelB wild-type ($^{+/+}$) or heterozygous ($^{+/-}$) mice were adoptively transferred intravenously into RelB-deficient ($^{-/-}$) knockout or mutant C57BL/6 mice. Data from $RelB^{+/+}$ transfer into knockout (n=8) and $RelB^{+/-}$ into mutant mice (n=2) are combined. Protein was extracted from lung homogenates and levels of IL-10 were assessed by multiplex assay 14 days post DC transfer. **(a)** IL-10 levels in lung homogenates. Data (n=2-8) presented as means ± s.e.m from 2 independent experiments. # represents $P < 0.05$ compared to $RelB^{+/-}$ mice, + represents $P < 0.05$, compared to $RelB^{-/-}$ mice. Open symbols represent RelB total knockout mice, closed symbols represent RelB mutant mice.

CHAPTER 3:

Enhancing PP2A and inhibiting proteasome activity ameliorates features of allergic airway disease

In this chapter, we demonstrate the importance of targeting PP2A and the UPS in AAD. Enhancing PP2A activity with $AAL_{(S)}$ suppresses features of AAD, and to a greater extent than FTY720. Inhibiting proteasome activity with BORT also suppresses certain features of AAD. We show for the first time that the combination of enhancing PP2A and inhibiting proteasome activity at the same time have synergistic effects and ameliorates the major features of acute and chronic AAD. Our findings highlight the potential of therapeutically targeting PP2A and the proteasome system, individually and in combination, for the treatment of asthma.

This chapter is currently in preparation for submission as an original research article to the *European Respiratory Journal*.

The study in this chapter was conducted in collaboration with Dr Nikki Verrills from the University of Newcastle and Dr Jonathon Morris from the University of New South Wales, who provided us with $AAL_{(S)}$. I conducted the majority of the work and was heavily involved in experimental design, data interpretation and wrote the manuscript.

3.1 Abstract

Allergic asthma is caused by aberrant immune responses to allergens. PP2A is an abundant serine/threonine phosphatase with anti-inflammatory activity. The UPS controls many cellular processes by protein degradation, including those involved in the initiation of inflammatory responses. We assessed if enhancing PP2A activity with FTY720 or $AAL_{(S)}$, or inhibiting proteasome activity with BORT could suppress AAD. C57BL/6 mice were subjected to acute OVA- or chronic HDM-induced AAD. Mice with AAD were treated with FTY720, $AAL_{(S)}$, BORT or $AAL_{(S)}$+BORT. $AAL_{(S)}$ reduced the severity of acute AAD by suppressing tissue eosinophils and inflammation, MSC numbers, Th2-associated cytokines (IL-33, TSLP, IL-5 and IL-13), serum IgE, and AHR. FTY720 only significantly suppressed tissue inflammation and IgE. BORT reduced BAL and tissue eosinophils and inflammation, IL-5, IL-13, and AHR. Combined treatment with $AAL_{(S)}$ and BORT had synergistic effects and suppressed BAL and tissue eosinophils and inflammation, MSC numbers, and reduced the production of Th2-associated cytokines and AHR. $AAL_{(S)}$, BORT and $AAL_{(S)}$+BORT also reduced airway remodelling in chronic AAD. These findings highlight the potential of combination therapies that enhance PP2A and inhibit proteasome activity as novel therapeutic strategies for asthma.

3.2 Introduction

Asthma is a common chronic inflammatory AAD that is typically caused by aberrant inflammatory responses to innocuous allergens. Exposure of the airway epithelium to allergens induces the release of the cytokines IL-33 and TSLP [368]. This promotes the development and activation of Th2 cells that induce the development and progression of allergic asthma [67]. Th2 cells release their characteristic cytokines IL-4, IL-5 and IL-13, which cause airway eosinophil accumulation, systemic IgE production, MSC metaplasia and AHR [67, 411]. The chronic release of cytokines, remodelling factors, such as TGF-β, and histamine by eosinophils and Th2 cells damages the epithelial lining, which over time leads to airway remodelling [367]. Current mainstay asthma treatments are inhaled corticosteroids and long acting β-agonists. Whilst these agents reduce symptoms, they do not treat the underlying causes of the disease, and their use has numerous issues such as steroid resistance, waning efficacy over time and side effects [67]. Therefore, there is an urgent need for effective alternative treatments. Enhancing the activity of PP2A and/or inhibiting proteasome activity offer potential new therapeutic approaches.

PP2A, the most abundant serine/threonine phosphatase, is expressed ubiquitously and exists as a heterotrimeric enzyme consisting of structural (A), regulatory (B) and catalytic (C) subunits [219, 222]. PP2A is considered a tumour suppressor as it is inactivated in several types of cancer, and loss of function or mutations in its structural and regulatory subunits are detected in multiple cancers [222]. Recently, it has emerged that PP2A may be a potential therapeutic target in respiratory diseases [248]. Reduced PP2A activity has been reported in animal models of AAD and in patients with severe asthma [248, 249, 251]. Enhancing PP2A activity with FTY720 or ($AAL_{(S)}$) abrogated the development of inflammation and AHR in mouse models of AAD [248, 265]. While these studies suggest that these agents may be potential asthma therapies, it is currently unknown which is more

effective. Determining the efficiency of each of these agents in suppressing features of acute and chronic AAD would provide valuable information for the development of a more efficacious treatment plan for asthma.

The UPS plays a crucial role in regulating tissue homeostasis, primarily by degrading key proteins involved in cellular functions [289, 412]. In this process, ubiquitinated target proteins are selectively degraded by ubiquitin ligases. In the lungs, the ubiquitin E3 ligase Itch promotes immune tolerance by degrading the Th2-specific transcription factor, phosphorylated JunB. In a murine model of asthma, Itch deficiency led to the development of allergic inflammation [280]. Another E3 ligase, MID1, is upregulated in HDM-induced AAD and in pBECs upon HDM exposure [248]. The proteasome inhibitor, BORT has been approved for the treatment of multiple malignancies [286]. The protective effects are thought to be in part, due to the suppression of NF-κB signalling, which leads to the down regulation of anti-apoptotic target genes [287]. These findings indicate that BORT may also suppress airway inflammation driven by NF-κB and indicates the potential for targeting the UPS by proteasome inhibition as a therapeutic approach for asthma. The combination of drugs that increase PP2A activity and suppress proteasome activity have not been tested. Combined strategies may have synergistic or additive effects that may be more effective in controlling allergic inflammation and asthma.

Here, the effects of enhancing PP2A and inhibiting proteasome activity in multiple mouse models of acute and chronic AAD were investigated. Increasing PP2A activity with $AAL_{(S)}$ suppressed several features of acute AAD. Inhibiting proteasome activity with BORT suppressed some of the same as well as other features of acute AAD. Combined treatment with $AAL_{(S)}$ and BORT had synergistic effects and suppressed the hallmark features of acute AAD, with the inhibition of type 2 responses and AHR. Both drugs alone and in combination were able to supress airway remodelling induced by

chronic HDM exposure. Our study provides valuable evidence that therapeutically targeting the PP2A and proteasome activity, particularly in combination, may be effective as potential asthma therapies.

3.3 Methods

3.3.1 Ethics statement

This study was performed in strict accordance with the recommendations in the Australian code of practise for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All protocols were approved by the Animal Ethics Committee of the University of Newcastle.

3.3.2 Induction of AAD

Female, 6-8 weeks old, C57BL/6 mice were used throughout. Acute AAD was induced by intraperitoneal (i.p) sensitisation to OVA (100µg, Sigma-Aldrich, St Louis, Missouri, USA) with Alhydrogel (1mg, InvivoGen, San Diego, California, USA) in sterile saline (200µl, 0.9%) on day 0 and 7. Mice were then challenged intranasally (i.n) with OVA (10µg, 50µl in sterile saline) on days 12-15. AAD was assessed on day 16. Chronic AAD was induced by i.n challenge with crude HDM extract (*Dermatophagoides pteronyssinus*, Greer Labs, Lenoir, North Carolina, USA) five times a week for five weeks. AAD was assessed on day 35.

3.3.3 Drug treatment

FTY720 (0.8mg/kg in 200µl of PBS, Sapphire Bioscience, New South Wales, Australia), $AAL_{(S)}$ (0.8mg/kg in 200µl of PBS, synthesised in-house), BORT (0.2mg/kg in 200µl of PBS, LC laboratories, Woburn, USA) or combined treatments ($AAL_{(S)}$+BORT) were administered i.p on days 12-15 (30 minutes before each challenge) in the acute model. In the chronic model, drugs were administered everyday throughout the model.

3.3.4 Airway inflammation

BAL was performed, cells were cytocentrifuged (300 x*g*, 10 minutes), stained with May Grunwald-Giemsa and differential leukocyte counts were determined according to morphological criteria from a total of 250 cells as previously described [372, 413]

3.3.5 Histopathology

Lungs were formalin fixed, embedded and sectioned. Longitudinal sections were stained with H&E (for histopathology), Lendrum's Carbolchromotrope (for eosinophils) and PAS (for MSC numbers). Histopathology (tissue inflammation) was scored in a blinded fashion according to a set of custom-designed criteria as described previously (**Table 3.1**) [374]. Eosinophils and MSC numbers were enumerated in inflamed airways as described previously [377, 414].

Table 3.1: Histopathological scoring system

Score 1: Airways inflammation score (/4)
0= Lack of inflammatory cells around airways- Absent
1= Some airways have small numbers of inflammatory cells- Mild
2= Some airways have significant inflammation- Moderate
3= Majority of airways have some inflammation- Marked
4= Majority of airways have significant inflammation- Severe
Score 2: Vascular inflammation score (/4)
0= Lack of inflammatory cells around blood vessels- Absent
1= Some blood vessels have small numbers of inflammatory cells- Mild
2= Some blood vessels have significant inflammation- Moderate
3= Majority of blood vessels have some inflammation- Marked
4= Majority of blood vessels have significant inflammation- Severe
Score 2: Parenchymal inflammation (at 10x magnification) (/5)
0= <1 % affected
1= 1-9 % affected
2= 10-29 % affected
3= 30-49 % affected
4= 50-69 % affected
5= >70 % affected
Total score= Score 1+ Score 2+ Score 3

3.3.6 mRNA expression

Whole lungs were collected and stored in RNA Stabilisation Reagent, RNA*later* (Qiagen, Chadstone Centre, Australia). RNA was extracted by guanidinium thiocyanate phenol chloroform (TRIzol, Invitrogen, Mount Waverly, Victoria, Australia) extraction. Isolated RNA was treated with DNAse I (Sigma-Aldrich) and reverse-transcribed using Bioscript (Bioline, Alexandria, Australia) and random hexamer primers (Invitrogen, Mount Waverly, Australia). The relative abundance of cytokine cDNA was determined relative to the reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) by real-

time quantitative (q)PCR using a ViiA 7 Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, California, USA) [413]. Custom designed primers (IDT, Coralville, Iowa, USA) expression for Mucin 5AC (Muc5AC) For; 5‘- GCAGTTGTGT CACCATCATCTGTG- 3’ Rev; 5- ‘GGGGCAGTCTTGACTAACCCTCTT- 3’) and transforming growth factor beta (TGF- β) (For; 5’- TGTGGAACTCTACCAGAAATA TAGC- 3’ Rev; 5’- GAAAGCCCTGTATTCCGTCTC-3’).

3.3.7 Protein isolation

Protein was extracted from whole lungs. Briefly, tissues were homogenised in 500µl of sterile Dulbecco’s phosphate-buffered saline (Life Technologies, Mulgrave, Victoria, Australia) supplemented with PhoSTOP phosphatase and Complete ULTRA protease inhibitors cocktails (Roche Diagnostics, Manheim, Germany) using a Tissue Tearor stick homogeniser (BioSpec Products, Bartesville, OK) on ice. Homogenates were then centrifuged (8000x*g*, 10 minutes, 4 ^{o}C). Supernatants were collected and stored at -20 ^{o}C for assessment by ELISA [141].

3.3.8 ELISA

The concentrations of IL-33 and TSLP in lung homogenates as well as IL-5 and IL-13 in BAL supernatants were determined using mouse DuoSet ELISA kits (R&D systems, Minneapolis, USA) according to manufacturer’s instructions.

3.3.9 Serum antibodies

Total IgE was determined by ELISA as previously described [70].

3.3.10 Lung function

AHR was measured in anaesthetised mice using whole body invasive plethysmography (Buxco electronics, Sharon, Connecticut, USA) by determination of the peak of transpulmonary resistance in response to increasing doses of nebulised methacholine (Sigma-Aldrich) as previously described [377, 415] .

3.3.11 Airway remodelling

Lungs were formalin fixed, embedded and sectioned. Longitudinal sections were stained with Masson's Trichrome. Area of collagen deposition (μm^2) was assessed in a minimum of four small airways (BM perimeter <1,000 μm) per section. Data were normalised to BM perimeter (μm) and quantified using ImageJ software (Version 1.49h, NIH, New York City, USA) [375].

3.3.12 Statistical analysis

Data are presented as means ± s.e.m with 6-8 mice and are representative of at least two independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA with Bonferroni post-test, or non-parametric equivalent, where appropriate. AHR was analysed using two-way repeated measures ANOVA with Tukey's post-test. Analyses were performed using GraphPad Prism V.6 Software (San Diego, CA).

3.4 Results

3.4.1 $AAL_{(S)}$ but not FTY720 suppressed OVA-induced pulmonary tissue inflammation and MSC numbers

To induce acute AAD, mice were sensitised to OVA i.p (day 0 and 7) and challenged i.n on days 12-15 and outcomes assessed on day 16 (**Figure 3.1a**). Vehicle, FTY720 or $AAL_{(S)}$ were administered i.p 30 minutes before each i.n challenge. The development of AAD was associated with increases in total leukocytes, eosinophils, neutrophils, macrophages and lymphocytes in BAL fluid of allergic vehicle-treated (OVA+vehicle) mice compared to non-allergic vehicle-treated (Saline+vehicle) controls (**Figure 3.1b and c, Supplementary figure 3.8a-c**). Treatment of allergic mice with FTY720 (OVA+FTY720) or $AAL_{(S)}$ (OVA+ $AAL_{(S)}$) had no significant effects on BAL leukocytes compared to allergic vehicle-treated controls. There were increases in eosinophils, inflammation, and MSC numbers around the airways in lung tissues and mucin 5AC (Muc5AC) mRNA expression in lung homogenates in allergic vehicle-treated mice compared to non-allergic vehicle-treated controls (**Figure 3.1d-g**). Treatment with FTY720 reduced tissue inflammation but not tissue eosinophils, MSC numbers or Muc5AC mRNA expression compared to allergic vehicle-treated controls. Treatment with $AAL_{(S)}$ significantly reduced tissue eosinophils, tissue inflammation and MSC numbers, which was associated with reduced Muc5AC expression in lung homogenates (**Figure 3.1d-g**).

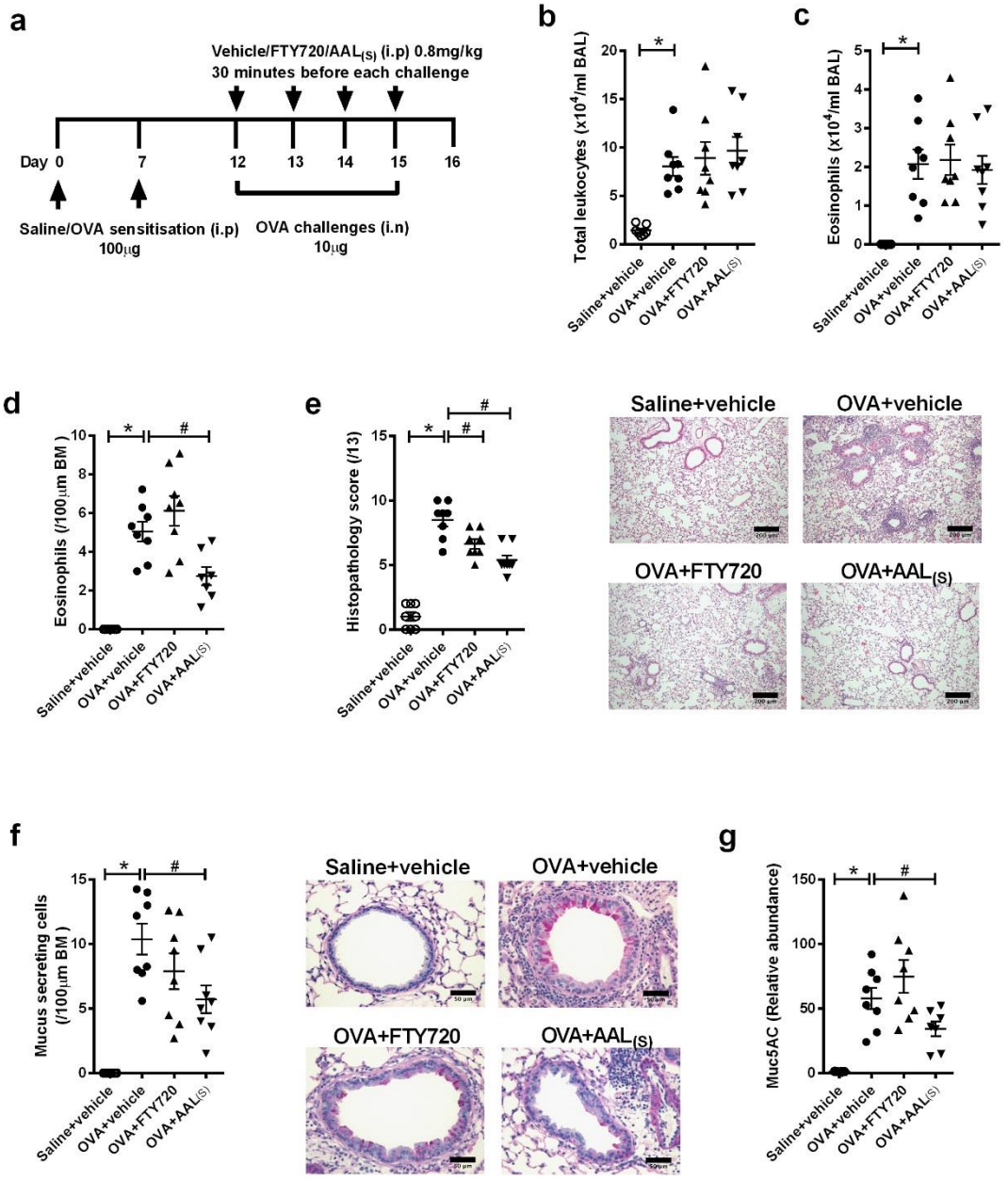


Figure 3.1: $AAL_{(S)}$ but not FTY720 suppressed pulmonary tissue inflammation and mucus secreting cell (MSC) numbers in ovalbumin (OVA)-induced acute allergic airway disease (AAD). (a) AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle, FTY720 or $AAL_{(S)}$ were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(b)** Total leukocytes and **(c)** eosinophils in bronchoalveolar lavage (BAL) fluid. **(d)** Numbers

of airway tissue eosinophils per 100μm basement membrane (BM) in Lendrum's Carbolchromotrope stained lung sections. **(e)** Histopathology score in haematoxylin and eosin stained lung sections. Scale bars= 200μm. **(f)** MSC numbers per 100μm BM in periodic acid-Schiff stained lung sections. Scale bars= 50μm **(g)** Mucin 5AC (Muc5AC) mRNA expression in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.

3.4.2 $AAL_{(S)}$ but not FTY720 suppressed Th2-associated cytokine levels and AHR

Next, the effects of FTY720 and $AAL_{(S)}$ treatment on Th2-associated cytokines, IgE and AHR were assessed. There were increased levels of IL-33 and TSLP in lung homogenates, IL-5 and IL-13 in BAL supernatants, and total IgE in the serum of allergic vehicle-treated mice compared to non-allergic vehicle-treated controls (**Figure 3.2a-e**). Treatment of allergic mice with $AAL_{(S)}$, but not FTY720, significantly reduced the levels of IL-33, TSLP, IL-5 and IL-13 compared to allergic vehicle-treated controls (**Figure 3.2a-d**). There were non-significant trends to a reduction in TSLP, IL-5 and IL-13 with FTY720 treatment. Both FTY720 and $AAL_{(S)}$ reduced serum total IgE compared to allergic vehicle-treated controls (**Figure 3.2e**). Allergic vehicle-treated mice also had AHR, characterised by exaggerated transpulmonary resistance in response to increasing doses of methacholine (**Figure 3.2f**). Treatment of allergic mice with $AAL_{(S)}$, but not FTY720 reduced AHR compared to allergic vehicle-treated controls (**Figure 3.2f**).

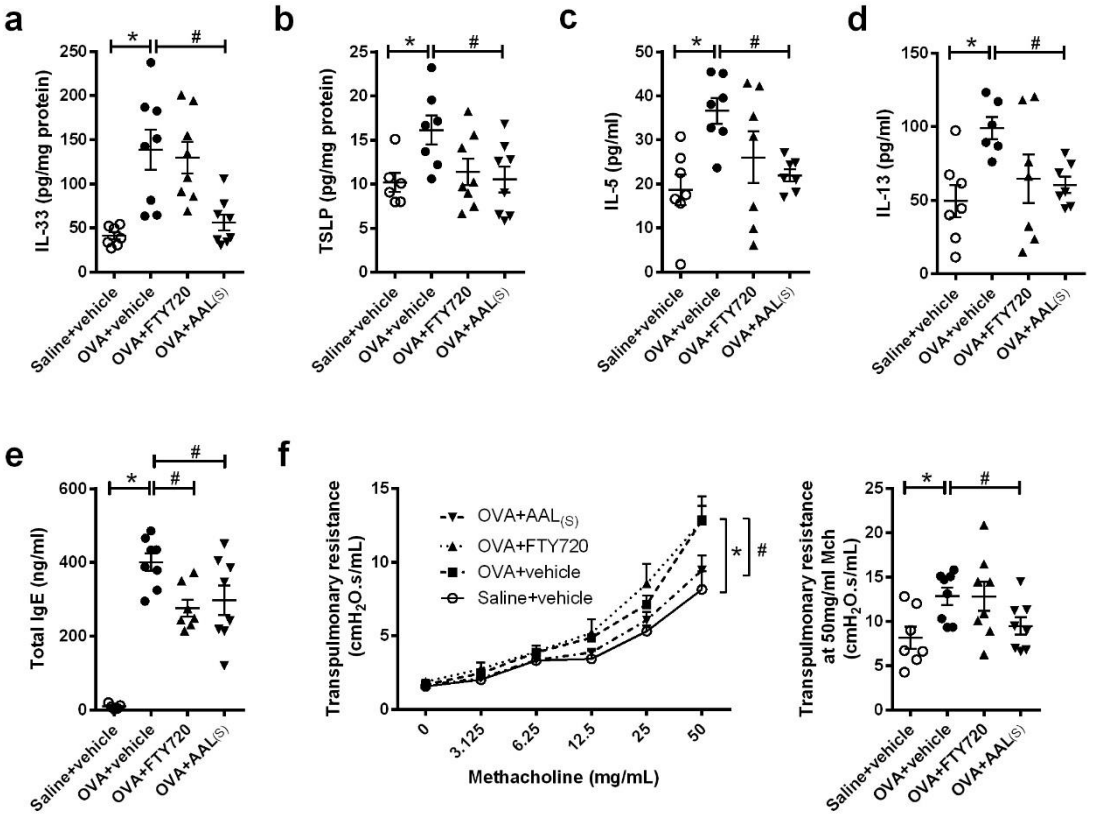


Figure 3.2. AAL$_{(S)}$ but not FTY720 suppressed Th2-associated cytokine levels and airway hyper-responsiveness (AHR) in OVA-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle, FTY720 or AAL$_{(S)}$ were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(a)** Interleukin (IL)-33 and **(b)** thymic stromal lymphopoietin (TSLP) in lung homogenates. **(c)** IL-5 and **(d)** IL-13 in bronchoalveolar lavage supernatants. **(e)** Total immunoglobulin (Ig)E in serum. **(f)** AHR in terms of transpulmonary resistance in response to increasing doses of methacholine (left) and at the maximal dose of methacholine (50mg/ml; right). Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.

3.4.3 Treatment with BORT reduced OVA-induced pulmonary inflammation and Muc5ac mRNA expression in the lung

Next, the effects of the proteasome inhibitor, BORT, on acute AAD were assessed. Treatment of allergic mice with BORT (OVA+BORT) significantly reduced total leukocytes, eosinophils, neutrophils, macrophages and lymphocytes in BAL fluid compared to allergic vehicle-treated controls (**Figure 3.3b and c, Supplementary figure 3.9a-c**). There were also reduced eosinophils in lung tissue (**Figure 3.3d**). Treatment did not affect tissue inflammation or MSC numbers, but significantly reduced Muc5AC mRNA expression in lung homogenates (**Figure 3.3e-g**).

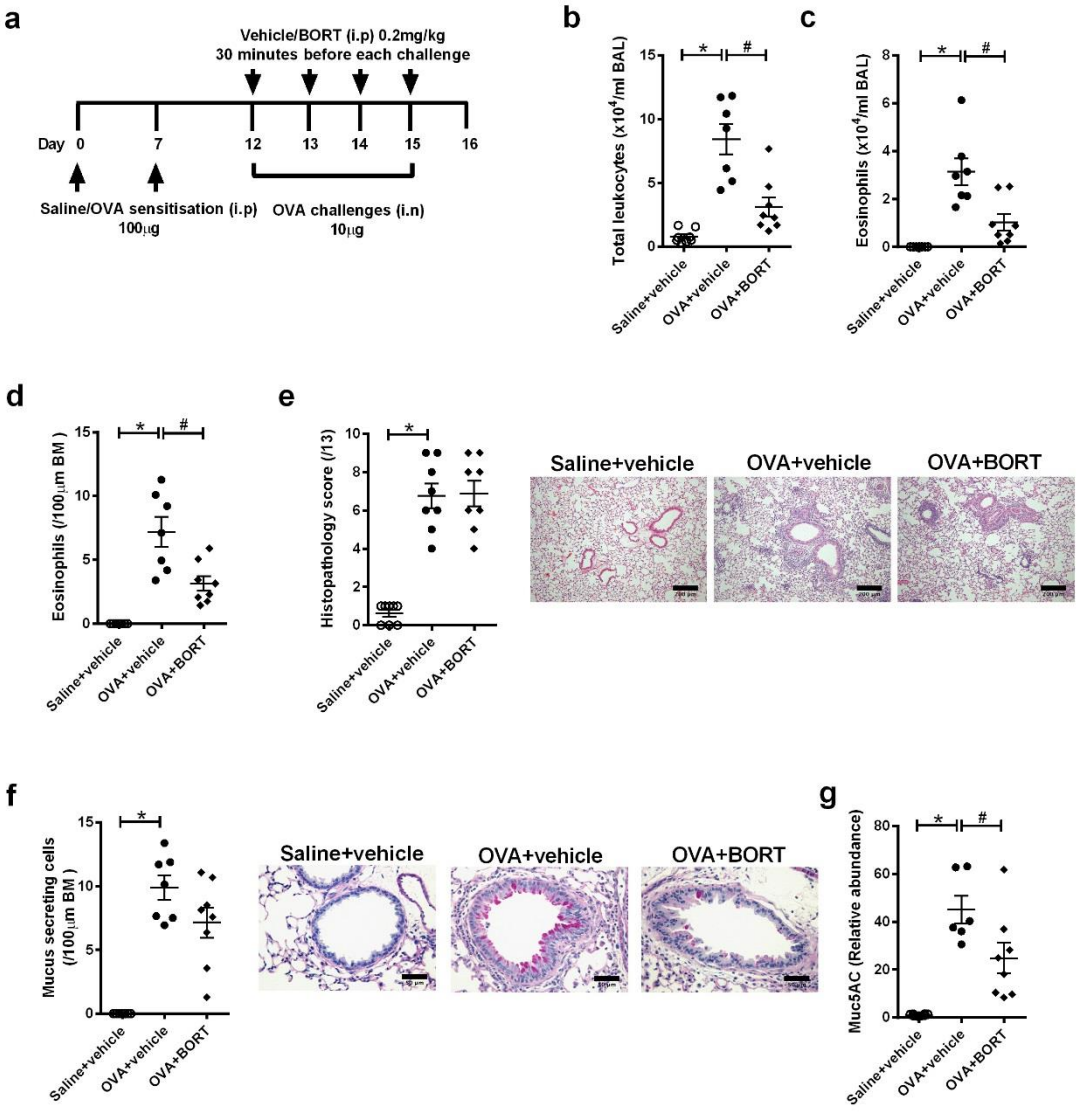


Figure 3.3: Treatment with BORT suppressed eosinophil influx into the airways and lung tissues as well as mucin 5AC (Muc5AC) expression in ovalbumin (OVA)-induced acute allergic airway disease (AAD). (a) AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle or BORT were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(b)** Total leukocytes and **(c)** eosinophils in bronchoalveolar lavage (BAL) fluid. **(d)** Numbers of airway tissue eosinophils per 100µm basement membrane (BM) in Lendrum's Carbolchromotrope stained lung sections. **(e)** Histopathology score in

haematoxylin and eosin stained lung sections. Scale bars= 200μm. **(f)** Mucus secreting cell numbers per 100μm BM in periodic acid-Schiff stained lung sections. Scale bars= 50μm. **(g)** Muc5AC mRNA expression in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, # represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.

3.4.4 Treatment with BORT reduced IL-5 and IL-13 levels in the lung and suppressed AHR

Treatment of allergic mice with BORT did not significantly affect the levels of IL-33 and TSLP (trend to a decrease) in lung homogenates compared to allergic vehicle-treated controls (**Figure 3.4a and b**). However, treatment reduced the levels of IL-5 and IL-13 in BAL supernatants compared to allergic vehicle-treated controls (**Figure 3.4c and d**). Treatment had no effect on serum total IgE (**Figure 3.4e**) but it did decrease AHR compared to allergic vehicle-treated controls (**Figure 3.4f**).

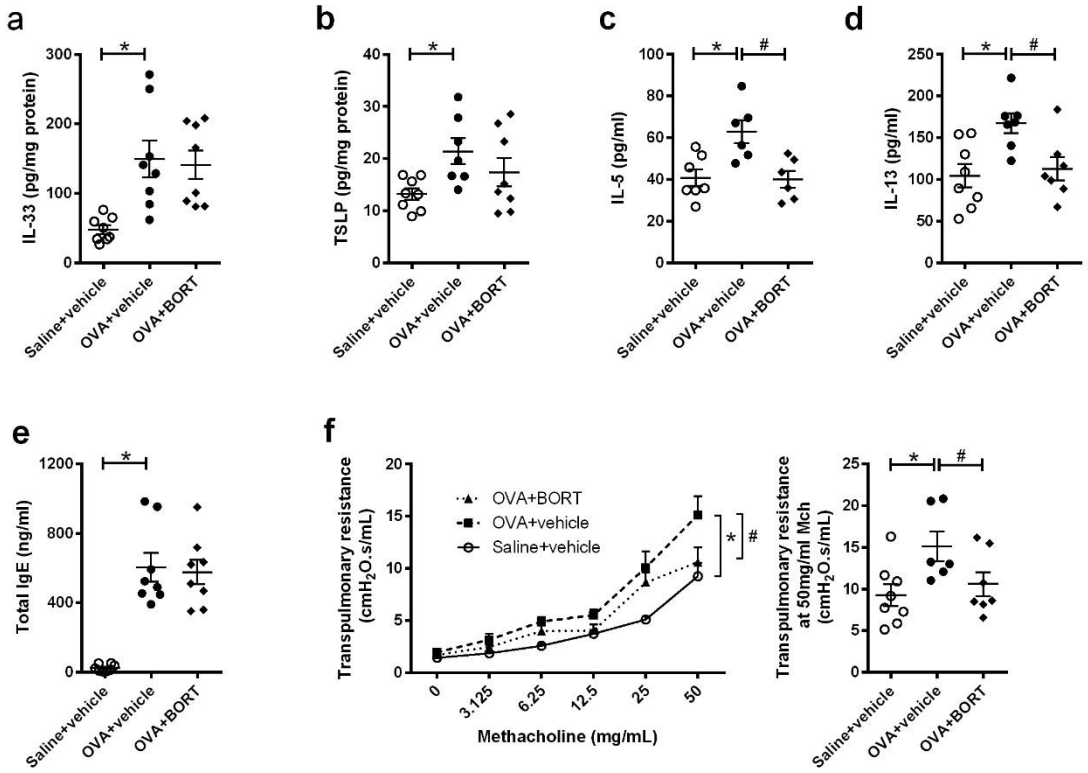


Figure 3.4: Treatment with BORT reduced interleukin (IL)-5 and IL-13 levels in the lung and airway hyper-responsiveness (AHR) in ovalbumin (OVA)-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle or BORT were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(a)** IL-33 and **(b)** thymic stromal lymphopoietin (TSLP) in lung homogenates. **(c)** IL-5 and **(d)** IL-13 in bronchoalveolar lavage supernatants. **(e)** Total immunoglobulin (Ig)E in serum. **(f)** AHR in terms of transpulmonary resistance in response to increasing doses of methacholine (left) and at the maximal dose of methacholine (50mg/ml; right). Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, # represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.

3.4.5 Combined treatment with $AAL_{(S)}$+BORT synergistically reduced OVA-induced eosinophilic pulmonary inflammation, MSC numbers and Muc5AC expression

Our data show that treatment with $AAL_{(S)}$ had beneficial effects on several features of AAD including tissue eosinophils and inflammation, MSC numbers, Muc5AC expression, levels of Th2-associated cytokines, IgE production and AHR. FTY720 had lesser effects. BORT had complimentary effects and suppressed the influx of inflammatory cells into the airways, tissue eosinophils, Muc5AC expression, IL-5 and IL-13 production and AHR (**Table 3.2**). This suggested that combined treatment with the PP2A activator $AAL_{(S)}$ and proteasome inhibitor BORT have complimentary and synergistic effects in AAD. Thus, we next assessed the effects of combining $AAL_{(S)}$ and BORT into one treatment for acute AAD. Treatment of allergic mice with $AAL_{(S)}$+BORT (OVA+$AAL_{(S)}$+BORT) had no effect on total leukocytes, neutrophils, macrophages, or lymphocytes in BAL fluid compared to allergic vehicle-treated controls (**Figure 3.5b, Supplementary figure 3.10a-c**)**.** However, combined treatment reduced eosinophils in both BAL fluid and lung tissue, tissue inflammation, MSC numbers and Muc5AC mRNA expression compared to allergic vehicle-treated controls (**Figure 3.5c-g**).

Table 3.2: Summary of the effects of different treatments on features of AAD

	FTY720	**AAL$_{(S)}$**	**BORT**	**AAL$_{(S)}$+BORT**
	Fig 1		**Fig 3**	**Fig 5**
All BAL cells	-	-	↓	↓ (eosinophils)
Tissue eosinophils	-	↓	↓	↓
Tissue inflammation	↓	↓	-	↓
MSCs	-	↓	-	↓
Muc5AC mRNA	-	↓	↓	↓
	Fig 2		**Fig 4**	**Fig 6**
IL-33, TSLP	-	↓↓	-	↓↓
IL-5, IL-13	-	↓	↓	↓↓
IgE	↓	↓	-	-
AHR	-	↓	↓	↓↓

- represents no effect, ↓ represents reduced, ↓↓ represents reduced to baseline

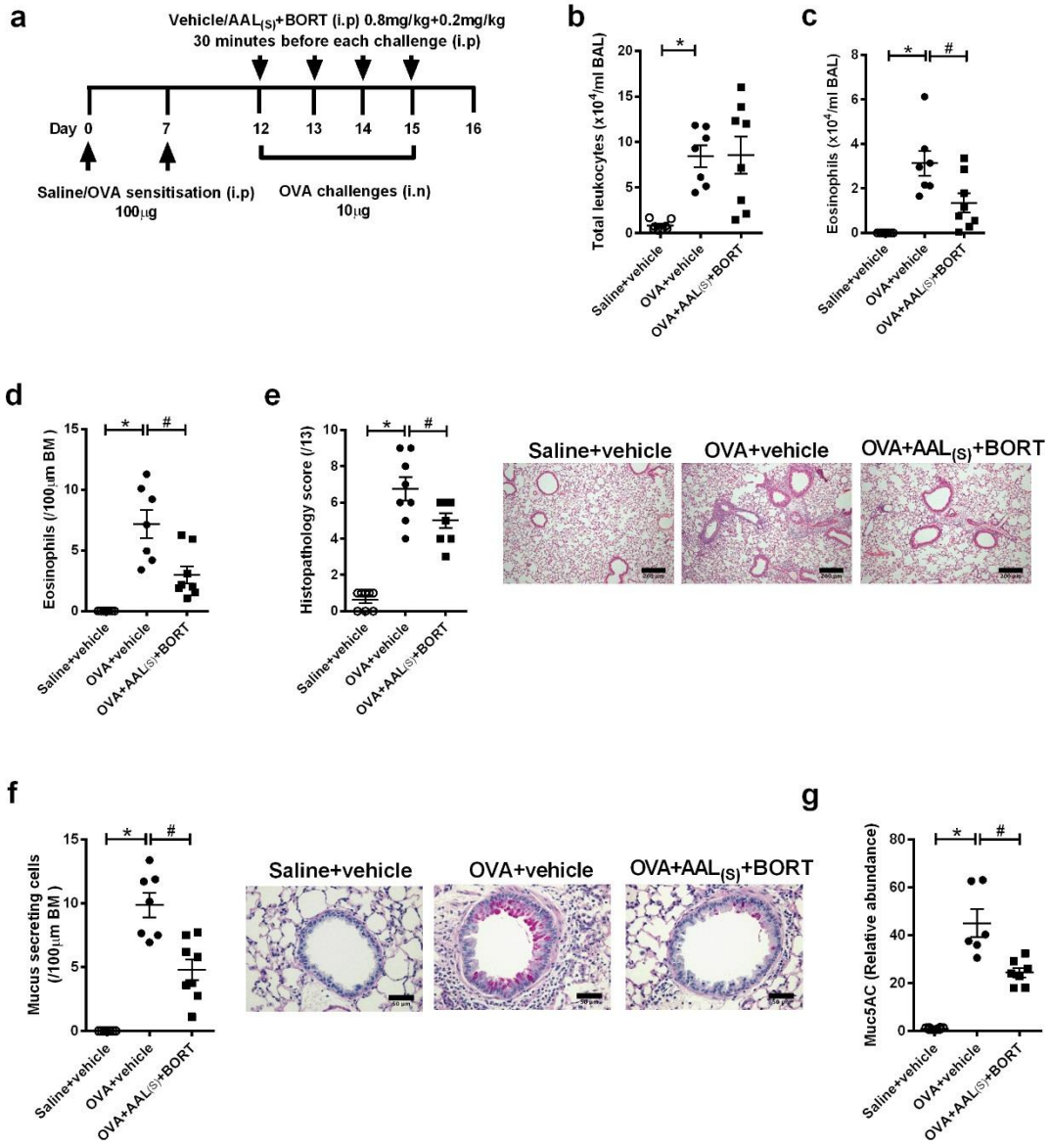


Figure 3.5: Combined treatment with AAL$_{(S)}$+BORT synergistically reduced eosinophilic pulmonary inflammation and mucus secreting cell (MSC) numbers and mucin 5AC (Muc5AC) expression in ovalbumin (OVA)-induced acute allergic airway disease (AAD). (a) AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle or AAL$_{(S)}$+BORT were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(b)** Total leukocytes and **(c)** eosinophils in bronchoalveolar lavage (BAL) fluid. **(d)** Numbers

of airway tissue eosinophils per 100μm basement membrane (BM) in Lendrum's Carbolchromotrope stained lung sections. **(e)** Histopathology score in haematoxylin and eosin stained lung sections. Scale bars= 200μm. **(f)** MSC numbers per 100μm BM in periodic acid-Schiff stained lung sections. Scale bars= 50μm. **(g)** Muc5AC mRNA expression in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. Data from controls (Saline+vehicle and OVA+vehicle) are recapitulated from Figure 3.3. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.

3.4.6 Combined treatment with $AAL_{(S)}$+BORT synergistically inhibited OVA-induced Th2-associated cytokines and AHR

Consistent with the reduction in eosinophilic pulmonary inflammation, MSC numbers and Muc5AC expression (**Figure 3.5c-f**), treatment of allergic mice with $AAL_{(S)}$+BORT significantly reduced levels of IL-33 and TSLP in lung homogenates and IL-5 and IL-13 in BAL supernatant compared to allergic vehicle-treated controls. The production of these factors was completely inhibited, with levels returning to similar levels to those of allergic vehicle-treated controls (**Figure 3.6a-d**). Combined treatment did not reduce serum total IgE, but inhibited the development of AHR compared to allergic vehicle-treated controls (**Figure 3.6e and f**).

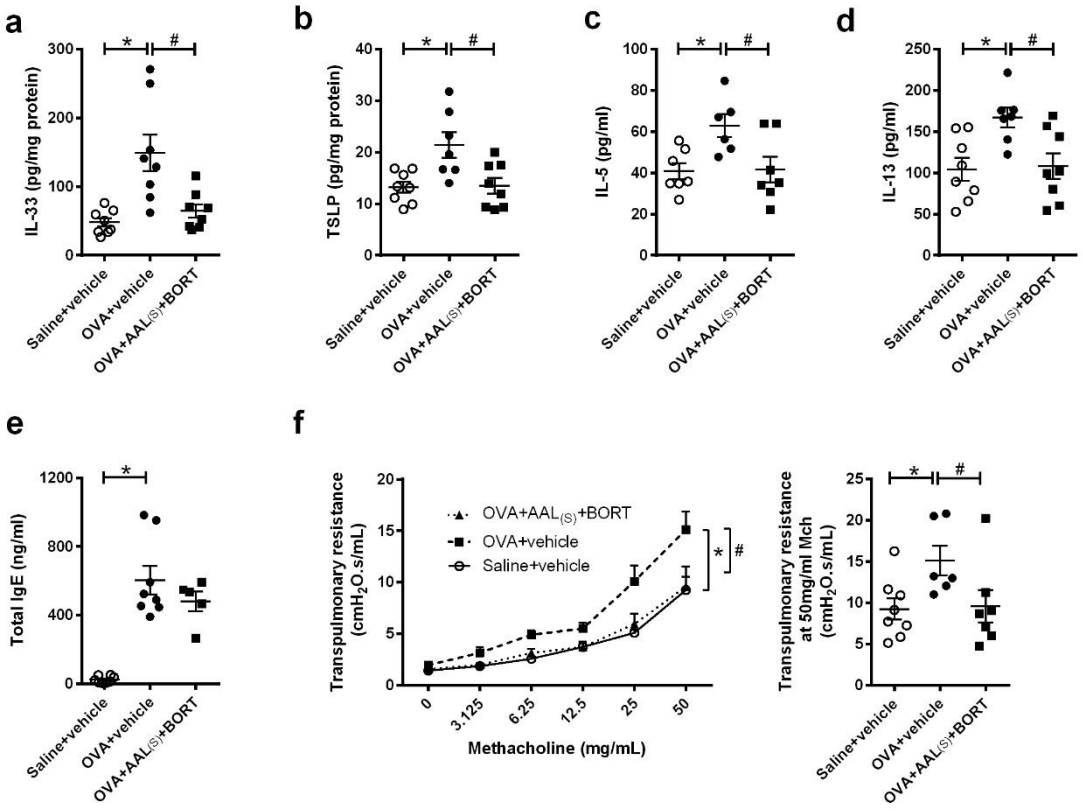


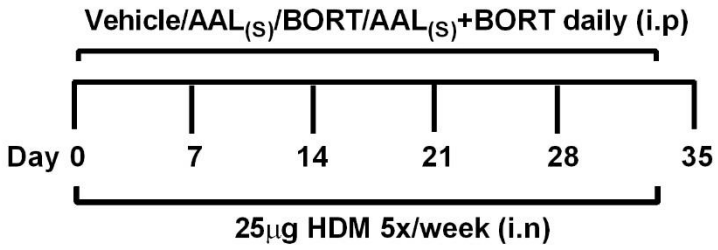
Figure 3.6: Combined treatment with AAL$_{(S)}$+BORT synergistically inhibited Th2-associated cytokines and airway hyper-responsiveness (AHR) in ovalbumin (OVA)-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle or AAL$_{(S)}$+BORT were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(a)** Interleukin (IL)-33 and **(b)** thymic stromal lymphopoietin (TSLP) in lung homogenates. **(c)** IL-5 and **(d)** IL-13 in bronchoalveolar lavage supernatants. **(e)** Total immunoglobulin (Ig)E in serum. **(f)** AHR in terms of transpulmonary resistance in response to increasing doses of methacholine (left) and at the maximal dose of methacholine (50mg/ml; right). Data are (n=6-8) presented as means ± s.e.m. Control mice (Saline+vehicle and OVA+vehicle) is recapitulated from Figure 3.4. * represents

$P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.

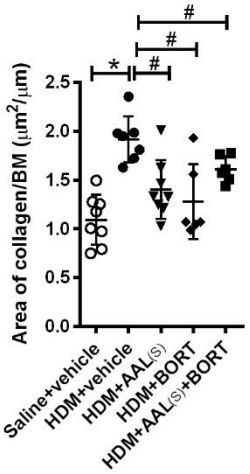
3.4.7 Treatment with $AAL_{(S)}$, BORT and $AAL_{(S)}$+BORT reduced airway remodelling in chronic AAD

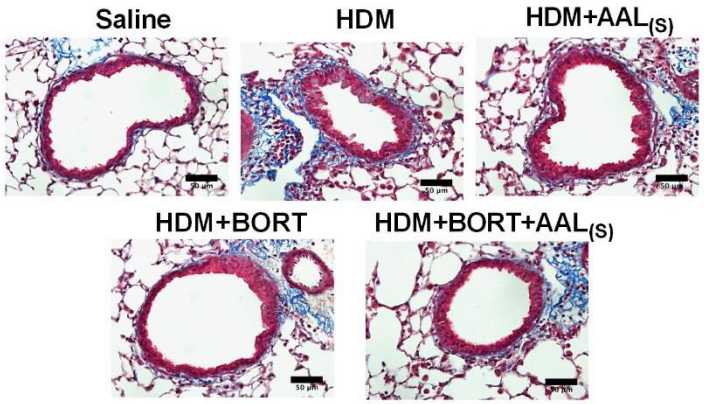
The acute OVA-induced AAD model does not involve chronic features of asthma such as airway remodelling. Thus, the effects of $AAL_{(S)}$ and BORT alone or combined on airway remodelling were assessed by exposing mice to HDM i.n, five times a week for five weeks (**Figure 3.7a**). Similar to acute AAD, chronic HDM exposure (HDM+vehicle) increased pulmonary inflammation, MSC numbers and AHR compared to non-allergic vehicle-treated controls (Saline+vehicle) (**Supplementary figure 3.11a-g**). Some of these features were reduced by treatment with $AAL_{(S)}$, BORT or $AAL_{(S)}$+BORT compared to allergic vehicle-treated controls (**Supplementary figure 3.11a-g**). Chronic HDM exposure also induced airway remodelling characterised by increased collagen deposition around the airways (**Figure 3.7b**). Treatment of allergic mice with $AAL_{(S)}$, BORT and $AAL_{(S)}$+BORT throughout HDM exposure significantly reduced collagen deposition. This was associated with reductions in TGF-β mRNA expression in the lungs (**Figure 3.7c**).

a


Vehicle/AAL(S)/BORT/AAL(S)+BORT daily (i.p)
Day 0
7
14
21
28
35
25μg HDM 5x/week (i.n)

b


Area of collagen/BM (μm²/μm)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
#
#
Saline+vehicle
HDM+vehicle
HDM+AAL(S)
HDM+BORT
HDM+AAL(S)+BORT


Saline
HDM
HDM+AAL(S)
HDM+BORT
HDM+BORT+AAL(S)

c

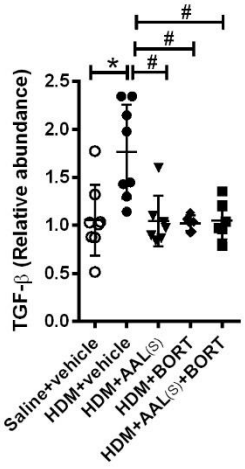

TGF-β (Relative abundance)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
#
#
Saline+vehicle
HDM+vehicle
HDM+AAL(S)
HDM+BORT
HDM+AAL(S)+BORT

Figure 3.7: Treatment with $AAL_{(S)}$, BORT and $AAL_{(S)}$+BORT reduced airway remodelling in house dust mite (HDM)-induced chronic allergic airway disease (AAD). (a) AAD was induced by administration of HDM intranasally five times a week for five weeks. Vehicle, $AAL_{(S)}$, BORT or $AAL_{(S)}$+BORT were administered intraperitoneally daily. Airway remodelling in terms of collagen deposition around the airways were assessed after 5 weeks. **(b)** Area of collagen deposition (μm^2) per basement membrane (BM) perimeter in Masson's Trichrome stained lung sections. Scale bars = 50μm. **(c)** Transforming growth factor beta (TGF-β) mRNA expression in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to allergic vehicle-treated (HDM+vehicle) controls.

3.5 Discussion

In this study, we assessed the effects of enhancing PP2A activity and inhibiting proteasome activity, either alone or in combination, as potential therapies for AAD. Enhancing PP2A activity with $AAL_{(S)}$ suppressed tissue eosinophils, tissue inflammation, MSC numbers and Muc5AC expression, Th2-associated cytokines in the lungs, IgE levels in serum and AHR, in acute OVA-induced AAD. FTY720 only significantly suppressed tissue inflammation and IgE levels. Inhibiting proteasome activity with BORT reduced eosinophils both in the airway lumen and in lung tissue, levels of IL-5 and IL-13, and AHR. Importantly, we show for the first time that combined treatment with $AAL_{(S)}$ and BORT had synergistic effects, which was superior to either treatment alone. Combined treatment reduced eosinophils influx both in the airway lumen and in lung tissue, tissue inflammation, MSC numbers and Muc5AC expression, Th2-associated cytokines and AHR. Notably, increases in cytokine levels and AHR were completely inhibited. Furthermore, both $AAL_{(S)}$ and BORT on their own and in combination suppressed airway remodelling in chronic HDM-induced AAD.

PP2A is one of the most abundant serine/threonine phosphatases in mammals [222]. Research on PP2A has primarily focused on cancer due to its role in maintaining normal cell division and its dysregulation being implicated in the development of malignancies [260]. Several studies have shown the importance of reduced PP2A activity in asthma [250, 251, 416]. PP2A activity was impaired in airway smooth muscle cells of asthmatics compared to non-asthmatics [416]. PP2A activity and expression was also significantly reduced in PBMCs from severe asthma patients and in animal models of steroid-resistant AHR [250, 251]. These studies highlight the potential for enhancing PP2A activity as a therapeutic approach for asthma. Whilst there have been studies that reported the use of the two

common PP2A activators, FTY720 and $AAL_{(S)}$, in mouse models of AAD [248, 265], it is unknown which of these activators is more effective.

Here, the effects of these PP2A activators on acute and chronic AAD were assessed and compared. In an acute OVA-induced AAD model, treatment with $AAL_{(S)}$ effectively inhibited eosinophil influx into the lung, but not the airways, and tissue inflammation and to a greater extent than FTY720. Its effects were associated with reduced levels of Th2-associated cytokines (IL-33, TSLP, IL-5 and IL-13). Treatment with $AAL_{(S)}$, but not FTY720, reduced MSC numbers, which was associated with reduced levels of IL-13 and Muc5AC mRNA expression in the lung. Both treatments reduced total IgE in serum. AHR was significantly reduced in allergic mice treated with $AAL_{(S)}$, but not FTY720, and this was associated with decreased levels of IL-13 [34]. Consistent with these findings, others have also shown that i.n treatment with $AAL_{(S)}$ reduced tissue inflammation, Th2-associated cytokines (IL-33, IL-5 and IL-13) and AHR in a model of acute HDM-induced AAD [248]. Enhancing PP2A activity with $AAL_{(S)}$ also reduced AHR and eosinophilic airway inflammation in a model of rhinovirus-induced AAD exacerbation [249]. In contrast to our findings, others have shown that oral treatment with FTY720 decreased airway inflammation, MSC numbers and AHR in T-cell transfer and OVA-induced AAD models [417]. This was postulated to be due to sequestration of T cells in lymphoid tissues. Another study showed that intratracheal administration of FTY720 during OVA-induced AAD reduced airway inflammation and Th2 cytokines (IL-5 and IL-13) by altering the function of lung DCs to stimulate T cells [265]. The differences in our findings with FTY720 that showed a lack of effect on Th2 cytokines, MSC numbers and AHR compared to previous studies could be partly due to the different routes of administration of FTY720 or different mouse strains used. In our study, FTY720 was administered systemically (i.p) compared to oral or intratracheal administration by others,

which may have a direct effect on the lung environment. Others used BALB/c mice that are more susceptible to developing Th2-driven AAD, while we used C57Bl/6 mice that are less susceptible to Th2-driven responses.

As $AAL_{(S)}$ was more effective at suppressing features of AAD than FTY720, its effect on airway remodelling was determined. Treatment with $AAL_{(S)}$ suppressed chronic HDM-induced collagen deposition around the airways. This was associated with reduced TGF-β mRNA expression in the lungs. Others have also shown $AAL_{(S)}$ treatment reduced collagen deposition around large conducting airways of mice chronically exposed to OVA [418]. Our study used a chronic HDM induced model, which is a more clinically relevant allergen compared to OVA [419]. In addition, our HDM model involves sensitisation solely *via* the airways instead of systemic sensitisation in the presence of an adjuvant used in OVA models [420]. This is consistent with allergen exposure in humans and results in a local, instead of systemic immune response.

Our study, for the first time, compares the different effects of the two different PP2A activators, $AAL_{(S)}$ and FTY720, on AAD. Treatment with $AAL_{(S)}$ is more effective at suppressing features of AAD than FTY720 in the acute model of AAD. This may be due to the off-target effects of FTY720. In addition to activating PP2A, FTY720 also binds to SIPR1 to cause lymphocyte trafficking. In contrast, $AAL_{(S)}$ does not bind to SIPR1, and is therefore more specific at increasing PP2A activity.

The UPS has important roles in regulating cell cycle and growth [421]. Even though the UPS has been studied extensively, few studies have focussed on asthma [248, 422, 423]. The E3 ubiquitin ligase Itch is involved in maintaining tolerance through the induction of anergy in Th2 cells [280]. Other E3 ubiquitin ligases such as Cbl-b and gene related to anergy in lymphocytes (GRAIL) have been implicated in the induction of T cell tolerance by targeting the Th2 transcription factors signal transducer and activator of transcription-6

(STAT6) and trans-acting T-cell-specific transcription factor GATA-3 for degradation [422, 423]. Recently, the E3 ligase MID1 has been shown to be upregulated in both mouse and pBECs after exposure to HDM [248]. Inhibition of the proteasome is currently emerging as a potential therapy in many diseases particularly cancer, whereas studies in asthma are only just emerging. The use of the proteasome inhibitor PS-519 in a rat model of OVA-induced pulmonary eosinophilia significantly reduced the infiltration of eosinophils into the lung [292]. Only one other study has used BORT, which was tested in a chronic OVA model in mice. Long term treatment with a high dose of BORT reduced OVA- specific IgE, but not airway inflammation or AHR [424]. However, the effects of BORT treatment in an acute model of AAD, or the clinically relevant chronic HDM model and features of airway remodelling have not been assessed.

In our study, the effects of inhibiting proteasome activity with BORT on both acute and chronic models of AAD were determined. Treatment with BORT suppressed some features of acute AAD, including eosinophil infiltration into both the airway lumen and lung tissue, which was associated with reduced levels of IL-5 and IL-13 in BAL supernatants. It also reduced AHR, which was consistent with reduced levels of IL-13. However, treatment did not alter tissue inflammation, MSC numbers, levels of IL-33 and TSLP or total IgE. Importantly, it did reduce airway remodelling in chronic AAD, which was associated with reduced TGF-β mRNA expression in the lung. The discrepancy in our and previous findings could be attributed to the dose of BORT that was used. We used an intermediate dose (0.2mg/kg) while a higher dose of BORT (0.75mg/kg) was used in the previous study. Others demonstrated that the attenuation of dextran sodium sulfate-induced colitis in mice by BORT treatment was dose dependent [425]. While a low dose (0.1mg/kg) reduced inflammation, it had no effect on cytokine or chemokine production. An intermediate dose (0.2 and 0.35mg/kg) attenuated colitis while a higher

dose (0.5mg/kg) caused mortality. It is important to note that BORT is a non-selective proteasome inhibitor, hence there is the possibility that the general reduction of proteasome activity is not optimal in attenuating all features of AAD, and specific inhibition may have greater effects.

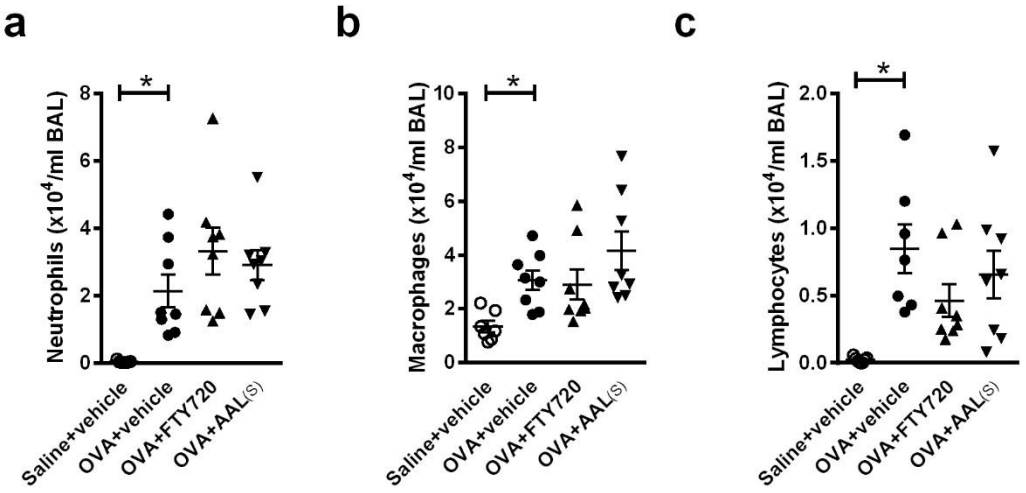
Several studies highlight the association between PP2A and the UPS. Inhibition of PP2A significantly augmented the proteolytic function of murine cardiac proteasomes [426]. Another study identified the mediation of PP2A/C ubiqutination and degradation by the E3 ligase Cullin-3 in response to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) signalling [427]. In HDM-induced AAD, MID1 protein decreases PP2A activity [248]. Given that both PP2A and the UPS have been implicated in the pathogenesis of asthma, and that $AAL_{(S)}$ and BORT inhibit different features of AAD, the synergistic effects of enhancing PP2A activity and inhibiting proteasome activity at the same time were determined. Treatment with $AAL_{(S)}$+BORT synergistically suppressed the major hallmark features of AAD including eosinophil infiltration into the airway lumen and tissue, tissue inflammation, MSC numbers, Th2-associated cytokines (IL-33, TSLP, IL-5, IL-13), AHR and collagen deposition. Notably Th2-associated cytokine production and AHR were completely inhibited, with levels returning to similar levels to those of allergic vehicle-treated controls. Interestingly, combined treatment reduced the levels of eosinophils in airway lumen, which was not observed with $AAL_{(S)}$ treatment alone. This is particularly important as anti-IL-5 treatment in humans is only effective when there is an effective suppression of eosinophil levels [428, 429]. Ours is the first study to show the synergistic effects of enhancing PP2A activity and inhibiting proteasome activity at the same time on acute and chronic features of AAD.

The exact mechanisms of how these immunomodulatory drugs suppress AAD remains to be fully elucidated. One possible mechanism is through the inhibition of NF-

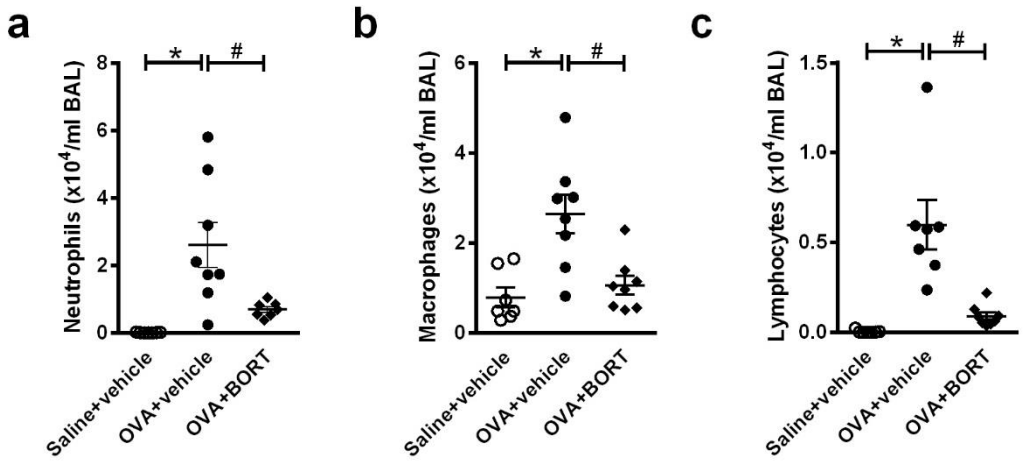
κB activity. NF-κB regulates the expression of multiple cytokines [430]. PP2A has been identified as a crucial regulator of NF-κB [253]. Inhibition of PP2A leads to increased activity of IKKβ, which subsequently leads to the proteasomal degradation of IκBα, allowing NF-κB to translocate into the nucleus to activate responsive genes[253]. The UPS is also known to control NF-κB activity through IκB degradation [431]. The inhibition of tumour growth in human T-cell lymphoma cell line by BORT has been postulated to be due to nuclear translocation of IκB that leads to the inactivation of NF-κB [432]. The mechanisms of action of these drugs clearly need further study.

In summary, this study demonstrates that enhancing PP2A activity and inhibiting proteasome activity, either alone or in combination has beneficial effects in multiple models of acute and chronic AAD. Enhancing PP2A activity with $AAL_{(S)}$ was more effective at suppressing hallmark features of AAD than FTY720 while inhibiting proteasome activity with BORT had some beneficial effects on features of AAD. Importantly, combining both $AAL_{(S)}$ and BORT had synergistic effects and was a better treatment option compared to treatment with $AAL_{(S)}$ or BORT alone. Our findings highlight the importance of PP2A and the UPS in mouse models of AAD and suggest that targeting these two systems synergistically may have therapeutic potential in asthma management.

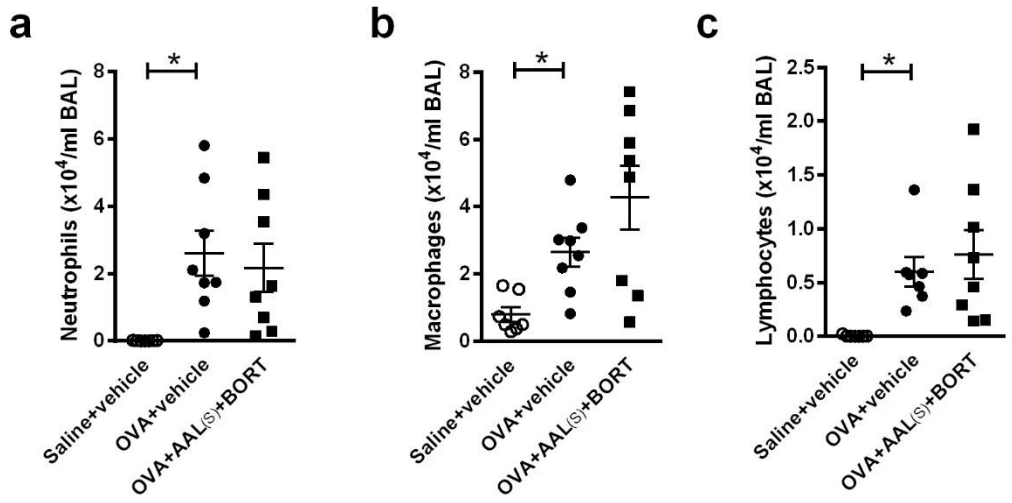
3.6 Supplementary data



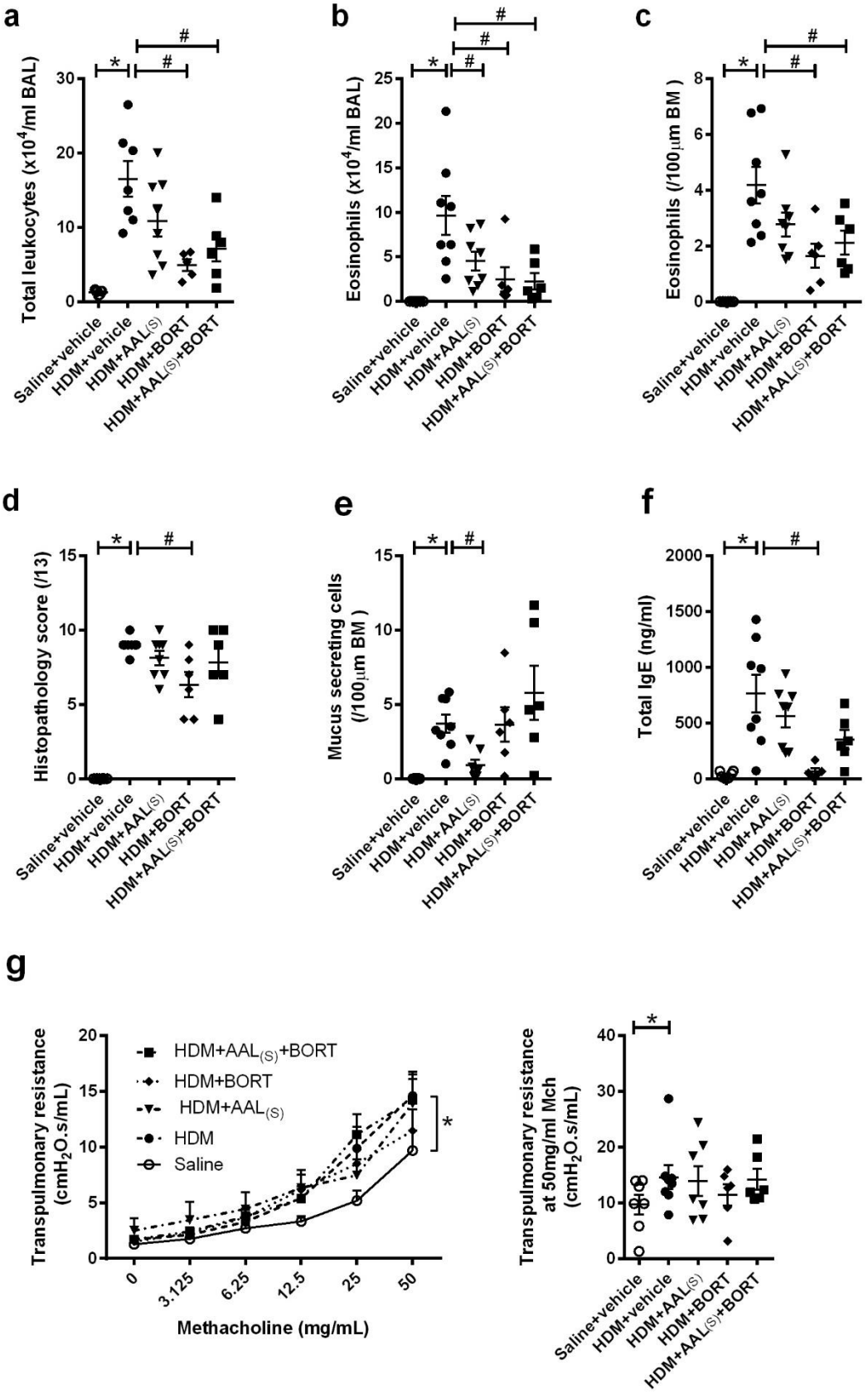
Supplementary figure 3.8: Treatment with FTY720 and $AAL_{(S)}$ had no effect on the numbers of neutrophils, macrophages or lymphocytes in bronchoalveolar lavage (BAL) fluid in ovalbumin (OVA)-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle, FTY720 or $AAL_{(S)}$ were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(a)** Neutrophils, **(b)** macrophages and **(c)** lymphocytes in BAL fluid. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.



Supplementary figure 3.9: Treatment with BORT reduced the numbers of neutrophils, macrophages or lymphocytes in bronchoalveolar lavage (BAL) fluid in ovalbumin (OVA)-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) ovalbumin OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle or BORT were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(a)** Neutrophils, **(b)** macrophages and **(c)** lymphocytes in BAL fluid. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.



Supplementary figure 3.10: Combined treatment with $AAL_{(S)}$+BORT had no effect on the numbers of neutrophils, macrophages or lymphocytes in bronchoalveolar lavage (BAL) fluid in ovalbumin (OVA)-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle or $AAL_{(S)}$+BORT were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. **(a)** Neutrophils, **(b)** macrophages and **(c)** lymphocytes in BAL fluid. Data (n=6-8) are presented as means ± s.e.m. Control data (Saline+vehicle and OVA+vehicle) is recapitulated from supplementary figure 3.9. * represents $P \leq 0.05$ compared non-allergic vehicle-treated (Saline+vehicle) controls, # represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.


a
b
c
d
e
f
g
Total leukocytes (x10⁴/ml BAL)
Eosinophils (x10⁴/ml BAL)
Eosinophils (/100μm BM)
Histopathology score (/13)
Mucus secreting cells (/100μm BM)
Total IgE (ng/ml)
Saline+vehicle
HDM+vehicle
HDM+AAL(S)
HDM+BORT
HDM+AAL(S)+BORT
HDM+AAL(S)+BORT
HDM+BORT
HDM+AAL(S)
HDM
Saline
Transpulmonary resistance (cmH2O.s/mL)
Methacholine (mg/mL)
0
3.125
6.25
12.5
25
50
Transpulmonary resistance at 50mg/ml Mch (cmH2O.s/mL)

Supplementary figure 3.11: Effects of treatment with $AAL_{(S)}$, BORT and $AAL_{(S)}$+BORT on chronic features of allergic airway disease (AAD). AAD was induced by administration of house dust mite (HDM) intranasally five times a week for five weeks. Vehicle, $AAL_{(S)}$, BORT or $AAL_{(S)}$+BORT were administered intraperitoneally daily. Features of AAD were assessed after 5 weeks. **(a)** Total leukocytes and **(b)** eosinophils in bronchoalveolar lavage (BAL) fluid. **(c)** Numbers of airway tissue eosinophils per 100μm basement membrane (BM) in Lendrum's Carbolchromotrope stained lung sections. **(d)** Total histopathology score in haematoxylin and eosin stained lung sections. **(e)** Mucus secreting cell numbers per 100μm BM in periodic acid-Schiff stained lung sections. **(f)** Total immunoglobulin (Ig)E in serum. **(g)** AHR in terms of transpulmonary resistance in response to increasing doses of methacholine (left) and at the maximal dose of methacholine (50mg/ml; right). Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to allergic vehicle-treated (HDM+vehicle) controls.

CHAPTER 4:

Enhancing tristetraprolin activity reduces severity of cigarette smoke-induced experimental chronic obstructive pulmonary disease

In this chapter, we demonstrate the importance of the anti-inflammatory molecule, TTP, in an experimental model of COPD. Using *Zfp36*$^{aa/aa}$ mice, we demonstrate that active TTP reduced the severity of CS-induced experimental COPD, by suppressing pulmonary inflammation, pro-inflammatory cytokine and chemokine expression, airway remodelling and improved lung function. This shows for the first time the novel role of TTP in suppressing the pathogenesis of experimental COPD. Our findings highlight TTP as a novel therapeutic option for COPD.

This chapter is currently in preparation for submission as an original research article to the *American Journal of Respiratory Cell and Molecular Biology*

The studies described were conducted in collaboration with Professor Alaina Ammit from the University of Sydney and Professor Andrew Clark from the University of Birmingham, who provided us with the *Zfp36*$^{aa/aa}$ mice used in this study. I conducted the majority of the work and was heavily involved in experimental design, data interpretation and wrote the manuscript.

4.1 Abstract

COPD is a progressive lung disease that causes significant mortality and morbidity worldwide, and is primarily caused by CS exposure in Western societies. The lack of effective treatment for COPD means that there is an urgent need to identify new therapeutic strategies that target the underlying mechanisms of disease pathogenesis. TTP, encoded by *Zfp36* gene, is an anti-inflammatory molecule that induces mRNA decay, especially of transcripts encoding inflammatory cytokines, including those that have been implicated in COPD. Here, we identify a novel protective role for TTP in experimental COPD using *Zfp36*$^{aa/aa}$ mice. TTP wild-type (*Zfp36*$^{+/+}$) and *Zfp36*$^{aa/aa}$ C57BL/6 mice were exposed to CS for 4 days to assess acute inflammatory responses or 8 weeks to assess hallmark pathological features of COPD. After 4 days of CS exposure, *Zfp36*$^{aa/aa}$ mice had reduced numbers of neutrophils in BAL fluid and mRNA expression of IL-6, TNF-α and CXCL2 compared to CS-exposed *Zfp36*$^{+/+}$ controls. After 8 weeks of CS exposure, *Zfp36*$^{aa/aa}$ mice had reduced numbers of neutrophils in BAL fluid and decreased CXCL2 mRNA expression compared to CS-exposed *Zfp36*$^{+/+}$ controls. CS-exposed *Zfp36*$^{aa/aa}$ also had reduced airway remodelling, characterised by decreased collagen deposition and airway epithelial cell thickness. Importantly, CS-exposed *Zfp36*$^{aa/aa}$ mice had decreased emphysema-like alveolar enlargement and improved lung function, characterised by decreased airway resistance and total lung capacity and increased forced expiratory volume in 100 seconds (FEV_{100})/FVC ratio. These findings provide novel evidence for the therapeutic potential of increasing the activity of TTP to control CS-induced inflammatory responses and development of hallmark features of experimental COPD in mice.

4.2 Introduction

COPD is a progressive lung disease that is characterised by chronic airway inflammation, airway remodelling, emphysema and impaired lung function [78]. It is the third leading cause of morbidity and death worldwide and imposes a substantial economic burden [72]. Cigarette smoking is a major risk factor for COPD [433]. Other factors such as occupational exposure to dust and chemicals and air pollution have also been associated with the aetiology of COPD [83]. Current treatments for COPD involve the use of bronchodilators and inhaled steroids, which are only generally effective in managing the symptoms of COPD and do not treat the underlying cause or halt the progression of the disease [76]. Hence, there is an urgent need to understand the underlying mechanisms of disease pathogenesis to identify new potential therapeutic targets.

TTP, encoded by the *Zfp36* gene is an anti-inflammatory protein that promotes mRNA decay of cytokines by binding to the AREs in the 3'-UTR of their transcripts [297, 434]. The activity of TTP is regulated by p38 MAPK, where phosphorylation of TTP by MK2 inactivates it, leading to stabilisation of target mRNA [348]. PP2A is as an antagonist of MK2 and can dephosphorylate TTP, hence enhancing its activity and promoting the de-stabilisation of target mRNA [310, 346]. However, active TTP is unstable and is targeted by the UPS for degradation [347]. Research on TTP has primarily focused on rheumatoid arthritis [311, 435]. Mice deficient in TTP have a severe inflammatory phenotype characterised by cachexia, autoimmunity and arthritis [311]. This is primarily due to increased mRNA stability of the pro-inflammatory cytokines, TNF-α and GM-CSF [313, 436]. A SNP was discovered in the promoter region of the TTP gene in patients with rheumatoid arthritis [353]. It was postulated that a small defect in the promoter activity of the TTP gene could lead to increased production of TNF-α, which subsequently resulted in disease progression.

The role of TTP in respiratory disease and lung inflammation remains poorly understood. In a cellular model of airway inflammation, inhibition of PP2A activity by okadaic acid led to accumulation of the inactive form of TTP, which in turn caused increased expression of IL-6 and IL-8 in A549 lung epithelial cell line [256]. Conversely, the activation of PP2A with $AAL_{(S)}$ led to the inhibition of IL-6 and IL-8 expression as a result of increases in the levels of the active form of TTP [355]. Given that TTP regulates the mRNA stability of multiple inflammatory genes, including those involved in COPD, such as IL-6, TNF-α and the chemokines CXCL1 and CXCL2 that are functional homologs of human IL-8, we hypothesised that enhancing the activity of TTP would be beneficial in COPD [311, 318, 319].

In the current study, the potential for increasing the active form of TTP in experimental CS-induced COPD was investigated using *Zfp36*$^{aa/aa}$ mice. As TTP$^{-/-}$ mice develop severe spontaneous and systemic autoimmune inflammation, we used *Zfp36*$^{aa/aa}$ mice that constitutively express the active (unphosphorylated) form of TTP [319]. These mice possess TTP that cannot be inactivated by the p38 MAPK-MK2 signalling, which leads to the accumulation of TTP in its active form and enabling degradation of target mRNAs. We demonstrate for the first that active TTP protects mice from hallmark features of experimental COPD by reducing pulmonary inflammation, small airway remodelling, emphysema-like alveolar enlargement and improving lung function. This study identifies a previously unrecognised role for TTP in the regulation of CS-induced inflammatory responses and in the pathogenesis of COPD. Thus, strategies that promote the activation and stabilisation of TTP may be a novel therapeutic option for COPD.

4.3 Methods

4.3.1 Ethics statement

This study was performed in strict accordance with the recommendations in the Australian code of practise for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All protocols were approved by the Animal Ethics Committee of The University of Newcastle.

4.3.2 Experimental COPD

Female, 6-8 weeks old, *Zfp36*$^{+/+}$ or *Zfp36*$^{aa/aa}$ C57BL/6 mice were exposed to normal air or CS through the nose only for 8 weeks as previously described [113, 375, 376].

4.3.3 Airway inflammation

BAL was performed, cells were cytocentrifuged (300 x*g*, 10 minutes) stained with May Grunwald-Giemsa and differential leukocyte counts were determined according to morphological criteria from a total of 250 cells as previously described [69, 372, 414].

4.3.4 mRNA expression

Whole lungs were collected and stored in RNA Stabilisation Reagent, RNA*later* (Qiagen, Chadstone Centre, Australia). RNA was extracted by guanidinium thiocyanate phenol chloroform (TRIzol) extraction. Extracted RNA was treated with DNAse I (Sigma, Castle Hill, Australia) and reverse-transcribed using Bioscript (Bioline, Alexandria, Australia) and random hexamer primers (Invitrogen, Mount Waverly, Australia) [375, 413]. The relative abundance of cytokine cDNA for IL-6, TNF-α, CXCL1, CXCL2, fibronectin and MMP-12 was determined relative to the reference gene HPRT by real-time qPCR using a ViiA 7 Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, USA). Custom-designed primers (IDT, Coralville, IA, USA) were used (**Table 4.1**).

Table 4.1: Custom-designed primers used in qPCR analysis

Primer	Primer sequence (5' → 3')
IL-6 forward	AGAAAACAATCTGAAACTTC CAGAGAT
IL-6 reverse	GAAGACCAGAGGAAATTTTCAATAGG
TNF-α forward	TCTGTCTACTGAACTTCGGGGTGA
TNF-α reverse	TTGTCTTTGAGATCCATGCCGTT
CXCL1 forward	GCTGGGATTCACCTCAAGAA
CXCL1 reverse	CTTGGGGACACCTTTTAGCA
CXCL2 forward	TGCTGCTGGCCACCAACCAC
CXCL2 reverse	AGTGTGACGCCCCCAGGACC
Fibronectin forward	TGTGGTTGCCTTGCACGAT
Fibronectin reverse	GCTATCCACTGGGCAGTAAAGC
MMP-12 forward	CCTCGATGTGGAGTGCCCGA
MMP-12 reverse	CCTCACGCTTCATGTCCGGAG
HPRT forward	AGGCCAGACTTTGTTGGATTTGAA
HPRT reverse	CAACTTGCGCTCATCTTAGGATTT

IL: Interleukin, TNF-α: Tumour necrosis factor alpha, CXCL: Chemokime (C-X-C motif) ligand, MMP: Matrix metalloproteinase, HPRT: hypoxanthine–guanine phosphoribosyltransferase

4.3.5 Parenchymal inflammation

Lungs were perfused, formalin fixed, embedded and sectioned. Longitudinal sections were stained with H&E and parenchymal inflammation was assessed by enumerating the numbers of inflammatory cells in 20 randomised high-powered fields [113, 375].

4.3.6 Airway remodelling

Lungs were perfused, formalin fixed, embedded and sectioned. Longitudinal sections were stained with H&E or Masson's Trichrome. The area of collagen deposition (μm^2),

airway epithelial area (μm^2) and cell (nuclei) number were assessed in a minimum of four small airways (BM perimeter <1000 μm) per section. Data were normalised to BM perimeter (μm) and quantified using ImageJ software (Version 1.49h, NIH, New York City, NY) [375, 376].

4.3.7 Alveolar enlargement

Lungs were perfused, formalin fixed, embedded and sectioned. H&E stained longitudinal sections were assessed for alveolar enlargement. Alveolar diameter was determined using the mean linear intercept method, and the percentage of alveolar destruction was determined using destructive index method [113, 377, 437].

4.3.8 Lung function

Lung function parameters were assessed using forced oscillation (Flexivent, Scireq, Scientific Respiratory Equipment, Montreal, Canada) and forced maneuver (Buxco electronics) techniques as previously described [113, 141].

4.3.9 Statistical analysis

Data are presented as means ± s.e.m. with 5-8 mice in each group and are representative of two independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA with Bonferroni post-test, or non-parametric equivalent, where appropriate using GraphPad Prism V.6 Software (San Diego, CA).

4.4 Results

4.4.1 CS-induced pulmonary inflammation is reduced in TTP knock-in mice

To evaluate the acute CS-induced inflammatory responses in the lung, both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice were exposed to CS for four days (**Figure 4.1a**) and pulmonary inflammation was assessed in BAL fluid. CS exposure of *Zfp36*$^{+/+}$ mice significantly increased total leukocytes, neutrophils, macrophages and lymphocytes in BAL fluid compared to normal air-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.1b-e**). CS exposure of *Zfp36*$^{aa/aa}$ mice significantly increased total leukocytes and neutrophils, but not macrophages or lymphocytes in BAL fluid compared to normal air-exposed *Zfp36*$^{aa/aa}$ controls (**Figure 4.1b-e**). Interestingly, CS-exposed *Zfp36*$^{aa/aa}$ mice had reduced neutrophils and lymphocytes in BAL fluid compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.1c and e**).

Given that *Zfp36*$^{aa/aa}$ mice had reduced airway inflammatory responses after four days of CS exposure, we next sought to determine if active TTP could reduce inflammation in a chronic CS exposure that induces experimental COPD. CS exposure of both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice for eight weeks significantly increased total leukocytes, neutrophils, macrophages and lymphocytes in BAL fluid compared to normal air-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ controls (**Figure 4.2b-e**). Notably, CS-exposed *Zfp36*$^{aa/aa}$ mice had reduced total leukocytes and neutrophils in BAL fluid, but not macrophages or lymphocytes compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.2b-e**).

Next, inflammatory cell numbers in the parenchyma were assessed. Eight weeks of CS exposure of both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice significantly increased the numbers of inflammatory cells in the parenchyma compared to normal air-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ controls (**Figure 4.2f**). However, the numbers of parenchymal inflammatory

cells in CS-exposed *Zfp36*$^{aa/aa}$ was significantly reduced compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.2f**).

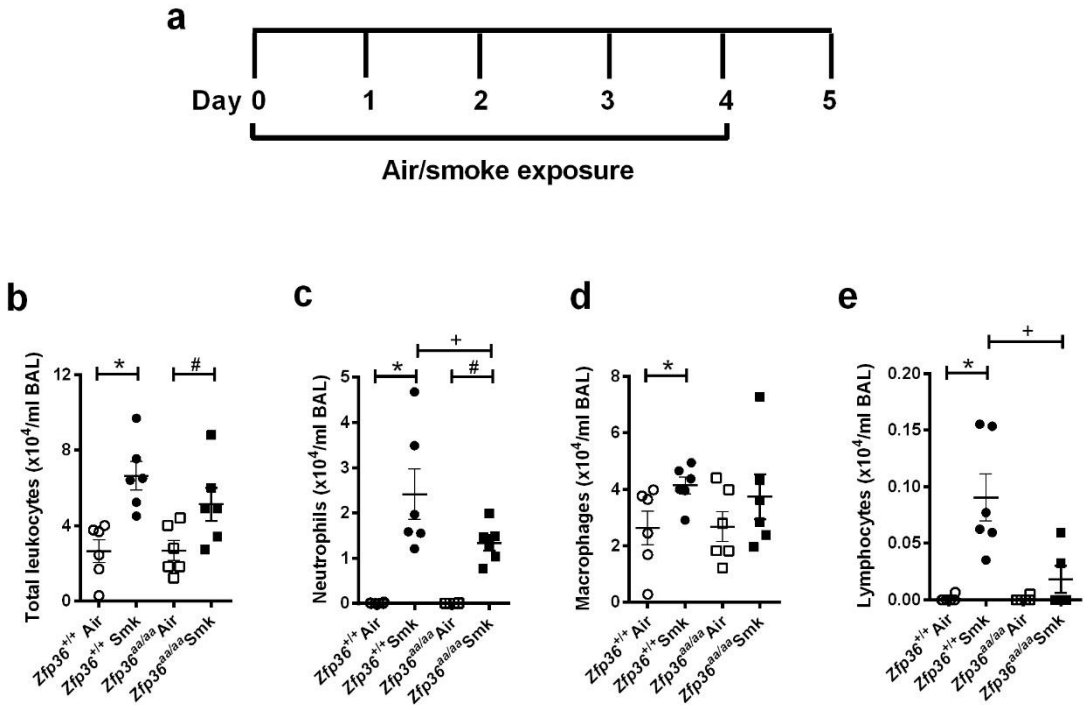


Figure 4.1: Acute cigarette smoke (CS)-induced pulmonary inflammation is reduced in tristetraprolin (TTP) knock-in mice. (a) Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were exposed to CS or normal air for four days. **(b)** Total leukocytes, **(c)** neutrophils, **(d)** macrophages, and **(e)** lymphocytes in bronchoalveolar lavage (BAL) fluid. Data (n=5-6) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Air, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Air, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Smk.

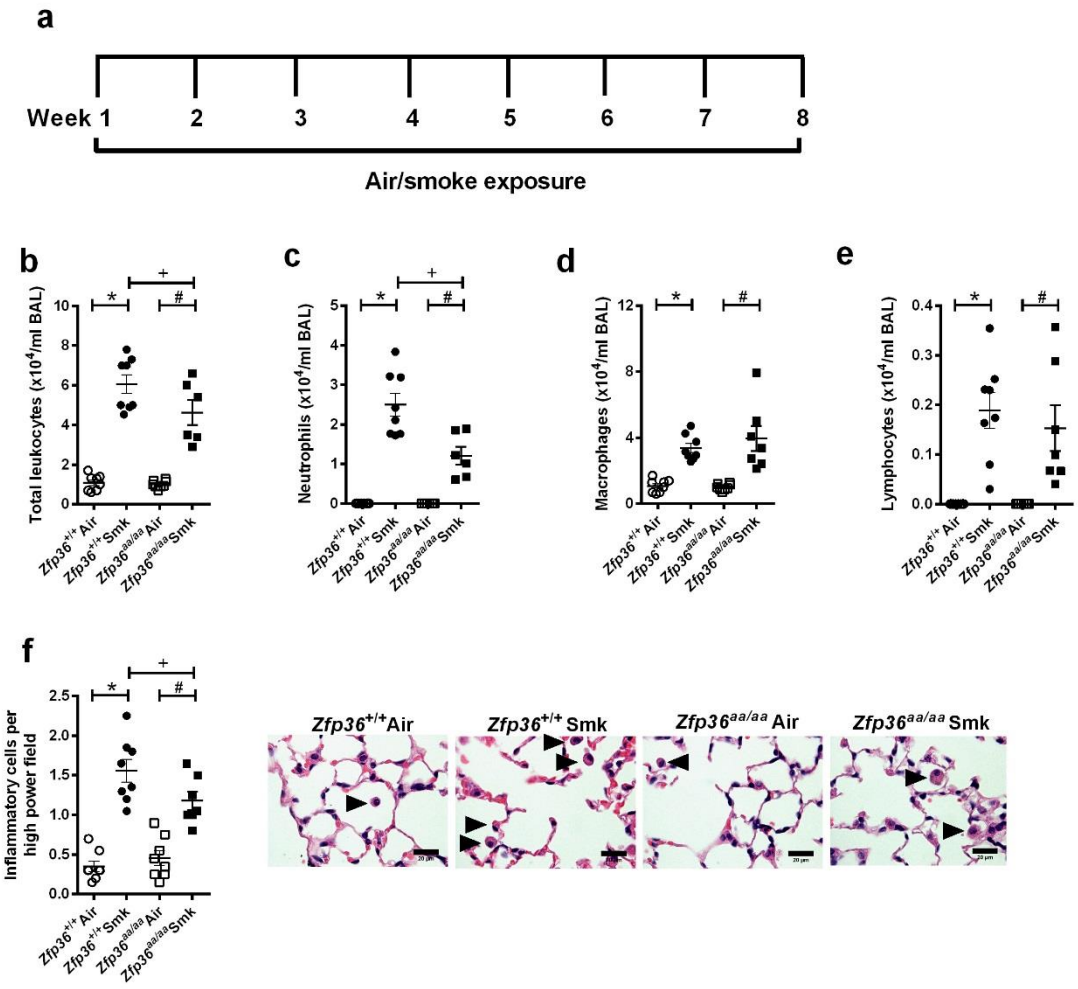


Figure 4.2: Chronic cigarette smoke (CS)-induced pulmonary inflammation is reduced in tristetraprolin (TTP) knock-in mice. (a) Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were exposed to CS or normal air for eight weeks. **(b)** Total leukocytes, **(c)** neutrophils, **(d)** macrophages, and **(e)** lymphocytes in bronchoalveolar lavage (BAL) fluid. **(f)** Numbers of parenchymal inflammatory cells (arrowheads) were determined in haematoxylin and eosin-stained lung sections. Scale bars= 20μm. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Air, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Air, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Smk.

4.4.2 CS-induced pro-inflammatory cytokine and chemokine mRNA expression is reduced in TTP knock-in mice

Given that CS-exposed *Zfp36*$^{aa/aa}$ mice had reduced pulmonary inflammation, mRNA expression of associated inflammatory cytokines and chemokines that are also TTP targets were assessed in lung homogenates. Acute CS exposure of both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice for four days increased the mRNA expression of IL-6, TNF-α, CXCL1 and CXCL2 compared to normal air-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ controls (**Figure 4.3a-d**). There was a significant reduction in the mRNA expression of the pro-inflammatory cytokines, IL-6 and TNF-α in CS-exposed *Zfp36*$^{aa/aa}$ mice compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.3a and b**). There was no change in CXCL1 mRNA expression between CS-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice, but there was a significant reduction in CXCL2 mRNA expression in CS-exposed *Zfp36*$^{aa/aa}$ mice compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.3c-d**).

Next, mRNA expression of inflammatory cytokines and chemokines in the lungs after chronic eight weeks of CS exposure was assessed. CS exposure of both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice increased the mRNA expression of IL-6, TNF-α, CXCL1 and CXCL2 compared to normal air-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ controls (**Figure 4.4a-d**). There were no significant differences in the mRNA expression of IL-6, TNF-α, or CXCL1 between CS-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice, but there was a significant reduction in the mRNA expression of CXCL2 in CS-exposed *Zfp36*$^{aa/aa}$ mice compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.4a-d**).

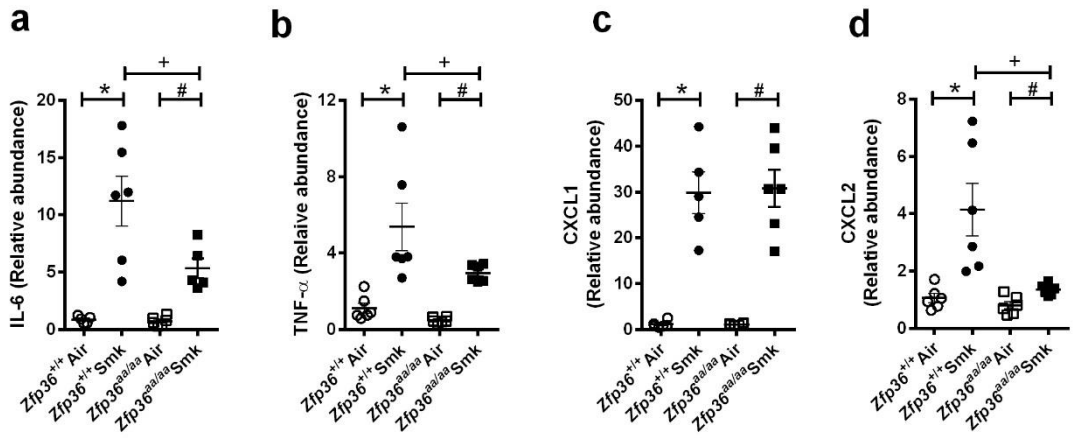


Figure 4.3: Acute cigarette smoke (CS)-induced pro-inflammatory cytokine and chemokine mRNA expression is reduced in tristetraprolin (TTP) knock-in mice. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were exposed to CS or normal air for four days. **(a)** Interleukin (IL)-6, **(b)** tumour necrosis factor alpha (TNF-α), **(c)** chemokine (C-X-C motif) ligand CXCL1 and **(d)** CXCL2 mRNA expression were determined in lung homogenates. Data (n=5-6) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Air, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Air, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Smk.

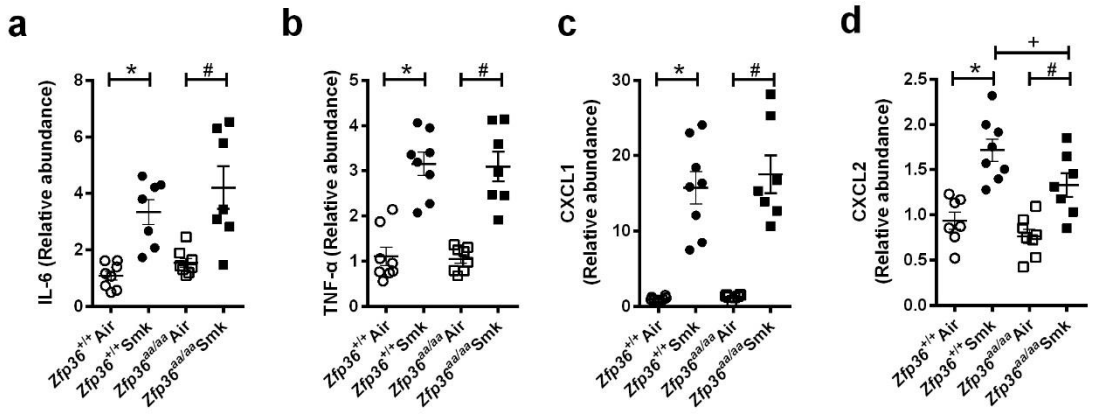


Figure 4.4: Chronic cigarette smoke (CS)-induced CXCL2 mRNA expression is reduced in tristetraprolin (TTP) knock-in mice. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were exposed to CS or normal air for eight weeks. **(a)** Interleukin (IL)-6, **(b)** tumour necrosis factor alpha (TNF-α), **(c)** chemokine (C-X-C motif) ligand (CXCL)1 and **(d)** CXCL2 mRNA expression were determined in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Air, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Air, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Smk.

4.4.3 CS-induced small airway remodelling is decreased in TTP knock-in mice

We have previously shown that mice develop small airway remodelling characterised by increases in collagen deposition around the small airways, small airway epithelial cell area and numbers of nuclei in the small airways in experimental COPD [113, 375]. As expected, CS exposure of *Zfp36*$^{+/+}$ mice increased collagen deposition around the airways compared to normal air-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.5a**). CS-exposed *Zfp36*$^{aa/aa}$ mice were protected against collagen deposition with no differences compared to normal air-exposed *Zfp36*$^{aa/aa}$ controls, but had significantly reduced collagen

deposition around the small airways compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.5a**).

CS exposure of *Zfp36*$^{+/+}$, but not *Zfp36*$^{aa/aa}$ increased the mRNA expression of the pro-fibrotic/remodelling protein fibronectin in the lungs compared to their respective controls. Interestingly, CS-exposed *Zfp36*$^{aa/aa}$ had significantly lower expression of fibronectin in the lungs compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.5b**).

Next, small airway epithelial cell area was determined. CS exposure significantly increased small airway epithelial cell area in *Zfp36*$^{+/+}$ mice compared to normal air-exposed *Zfp36*$^{+/+}$ controls, but not in *Zfp36*$^{aa/aa}$ compared to normal air-exposed *Zfp36*$^{aa/aa}$ controls. Again, CS-exposed *Zfp36*$^{aa/aa}$ mice had significantly reduced small airway epithelial cell area compared to CS-exposed *Zfp36*$^{+/+}$ controls. (**Figure 4.5c**).

We then determined if the increase in epithelial cell area was associated with an increase in the number of nuclei in the small airways, which is indicative of increased epithelial cell number. CS exposure increased nuclei numbers in the small airways of both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice. However, there was no difference in nuclei numbers in the small airways between CS-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice (**Figure 4.5d**).

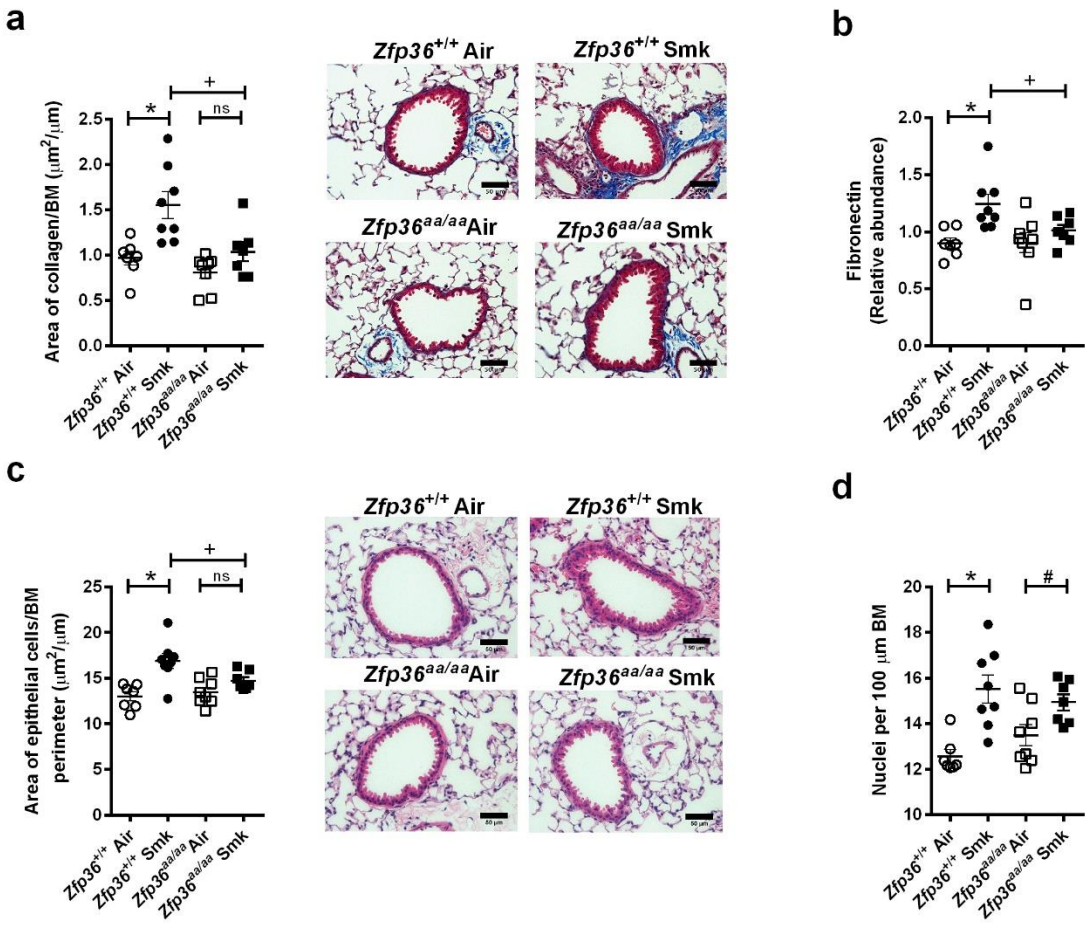


Figure 4.5: Chronic cigarette smoke (CS)-induced airway remodelling is reduced in tristetraprolin (TTP) knock-in mice. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were exposed to CS or normal air for eight weeks. **(a)** Area of collagen deposition (μm^2) per basement membrane (BM) perimeter (μm) in Masson's Trichrome stained lung sections. Scale bars= 50μm. **(b)** Fibronectin mRNA expression was determined in lung homogenates. **(c)** Small airway epithelial thickness in terms of epithelial cell area (μm^2) per BM perimeter (μm) in haematoxylin and eosin (H&E) stained lung sections. Scale bars= 50μm. **(d)** Number of epithelial cells in H&E stained lung sections was assessed by enumerating the number of nuclei per 100 μm of BM perimeter. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared

to *Zfp36*$^{+/+}$ Air, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Air, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Smk, ns represents not significant.

4.4.4 CS-induced emphysema-like alveolar enlargement and lung function impairment is reduced in TTP knock-in mice

We have previously shown that mice develop emphysema-like alveolar enlargement and impaired lung function after eight weeks of CS exposure [113, 375, 376]. In this study, CS exposure of *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice for eight weeks resulted in increased alveolar diameter and septal damage, using the mean linear intercept and destructive index methods respectively, compared to normal air-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ controls (**Figure 4.6a**). Notably, alveolar diameter and septal damage were significantly reduced in CS-exposed *Zfp36*$^{aa/aa}$ mice compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.6a**). CS exposure of both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice increased the mRNA expression MMP-12 in the lungs compared to their respective controls (**Figure 4.6b**). However, the expression of MMP-12 was significantly reduced in CS-exposed *Zfp36*$^{aa/aa}$ mice compared to CS-exposed *Zfp36*$^{+/+}$ controls (**Figure 4.6b**).

Next, we assessed the potential of active TTP to prevent CS-induced impairment of lung function. CS exposure of *Zfp36*$^{+/+}$ mice increased airway resistance and total lung capacity (**Figure 4.6c and d**) and reduced FEV_{100}/FVC ratio (**Figure 4.6e**) compared to normal air-exposed *Zfp36*$^{+/+}$ controls. Notably, *Zfp36*$^{aa/aa}$ were protected against changes in airway resistance, total lung capacity and FEV_{100}/FVC ratio, which were the same as in normal air-exposed *Zfp36*$^{aa/aa}$ controls (**Figure 4.6c-e**). Airway resistance and total lung capacity were significantly reduced in CS-exposed *Zfp36*$^{aa/aa}$ mice compared to CS-exposed *Zfp36*$^{+/+}$ controls, while FEV_{100}/FVC ratio was significantly increased in CS-exposed *Zfp36*$^{aa/aa}$ mice (**Figure 4.6c-e**). CS exposure of *Zfp36*$^{+/+}$ mice also increased

compliance, determined during a pressure-volume loop manoeuvre compared to normal-air exposed *Zfp36*$^{+/+}$ controls (**Figure 4.6f and g**). *Zfp36*$^{aa/aa}$ mice exposed to CS were protected against changes in pressure-volume loops and lung compliance, which were the same as in normal air-exposed *Zfp36*$^{aa/aa}$ controls (**Figure 4.6f and g**).

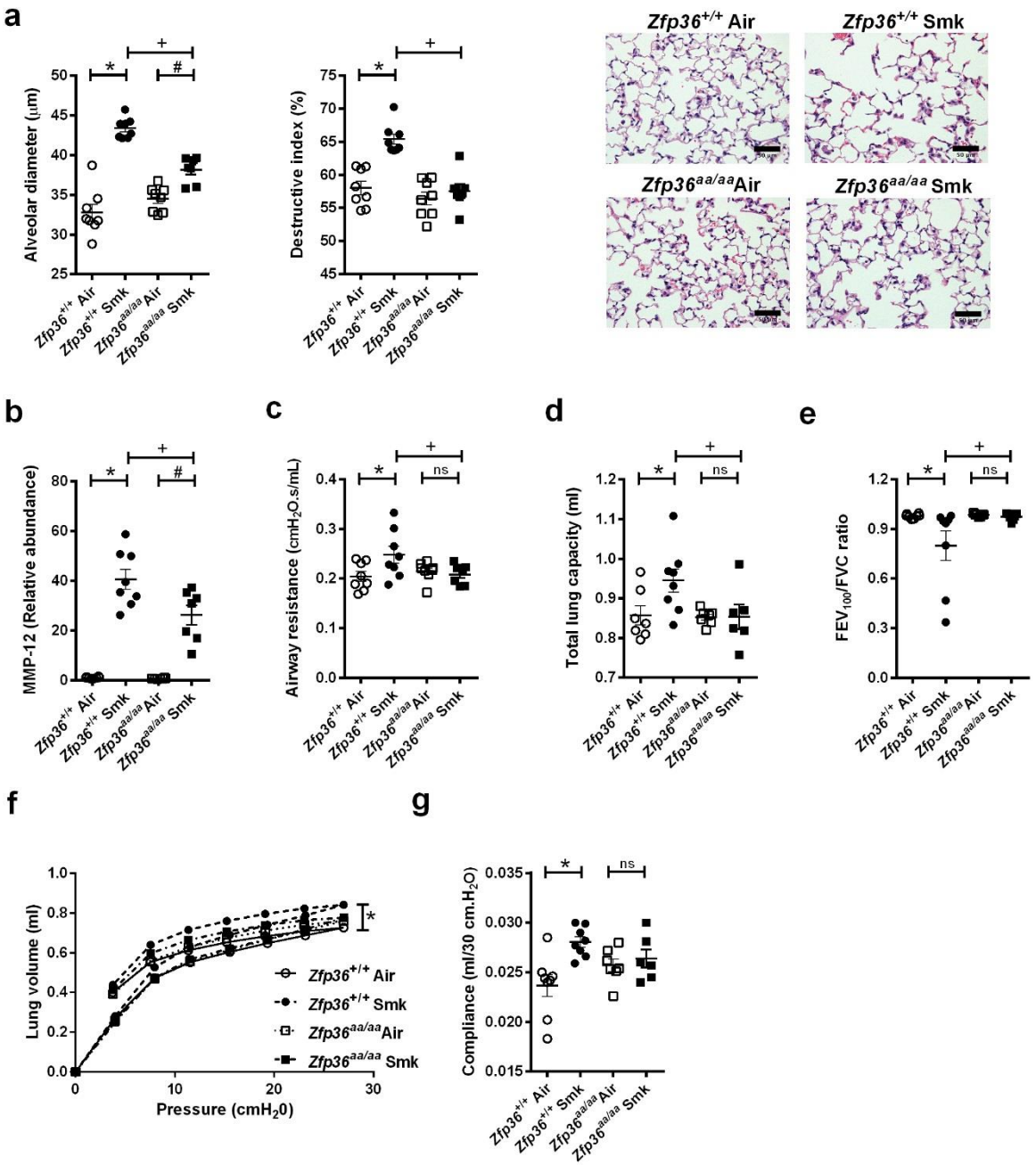


Figure 4.6: Chronic cigarette smoke (CS)-induced emphysema-like alveolar enlargement and lung function impairment are reduced in tristetraprolin (TTP) knock-in mice. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were exposed to CS or normal air for eight weeks. **(a)** Alveolar diameter (μm) was determined in haematoxylin and eosin-stained lung sections using the mean linear intercept and destructive index technique. Scale bars= 50μm. **(b)** Matrix metalloproteinase (MMP)-12 mRNA expression was determined in lung homogenates. Lung function was assessed in terms of **(c)** airway resistance, **(d)** total lung capacity, **(e)** forced expiratory volume in 100 seconds /forced vital capacity (FEV_{100}/FVC) ratio, **(f)** pressure–volume loops and **(g)** lung compliance at 30 cmH_20. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Air, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Air, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Smk, ns represents not significant.

4.5 Discussion

In this study, a previously unrecognised role for TTP in CS-induced pulmonary inflammation, airway remodelling, emphysema and impaired lung function was identified. Using mouse models of CS-induced experimental COPD and *Zfp36*$^{aa/aa}$ mice, we demonstrate that the presence of active TTP significantly reduced CS-induced pulmonary inflammation, characterised by decreased neutrophils in BAL fluid and numbers of inflammatory cells in the parenchyma. These reductions in inflammatory cells were associated with reduced expression of pro-inflammatory cytokines and chemokines that recruit neutrophils to the airway lumen and induce pulmonary inflammation in response to CS exposure. Mice expressing active TTP were protected against CS-induced small airway remodelling, with no excess collagen deposition or increases in small airway epithelial cell area. Importantly, the presence of active TTP in mice reduced emphysema-

like alveolar enlargement and improved lung function. Collectively, these findings highlight a novel therapeutic potential of increasing the activity of TTP in COPD.

CS exposure for four days enabled the evaluation of early smoke-induced inflammatory responses in the lung, whilst exposure for eight weeks causes the development of many of the hallmark features of COPD that are observed in humans [113]. These include pulmonary inflammation (chronic bronchitis), small airway remodelling, emphysema-like alveolar enlargement and impaired lung function [112]. The *Zfp36*$^{aa/aa}$ mouse was generated by replacing both serines 52 and 178 in the endogenous murine *Zfp36* locus with the non-phosphorylatable alanines. The resulting phenotype has a constitutively active TTP (unphosphorylated form) due to the inability of TTP to be phosphorylated by the p38 MAPK-MK2 signalling pathway [356]. This active TTP is able to induce mRNA decay of target cytokines. As TTP$^{-/-}$ mice develop severe inflammation and have a high mortality rate in the absence of exogenous inflammatory stimuli, using these mice to study inflammatory diseases is not appropriate [311]. Hence, *Zfp36*$^{aa/aa}$ mice provided a valuable and novel tool to investigate the role of active TTP in regulation of inflammatory responses in the lung and in the pathogenesis of experimental COPD.

CS exposure for both four days and eight weeks resulted in increased pulmonary inflammation characterised by exaggerated levels of total leukocytes, neutrophils, macrophages and lymphocytes in the airway lumen. These cells have previously been shown to be increased in both experimental models and in human COPD [98, 99, 113, 375]. CS-exposed *Zfp36*$^{aa/aa}$ mice had significantly reduced influx of neutrophils in the airway lumen after four days and eight weeks of CS exposure compared to CS-exposed *Zfp36*$^{+/+}$ controls. Neutrophils have been implicated in the development of alveolar destruction in COPD through the release of serine proteases [89, 438]. Increased levels of sputum neutrophils have also been associated with rapid declines in lung function and increases

in COPD disease severity [98, 99, 101]. CS-exposed *Zfp36*$^{aa/aa}$ mice also had reduced numbers of parenchymal-associated inflammatory cells in the lungs after eight weeks of CS exposure. These findings suggest that active TTP regulates inflammatory responses to CS. This likely results from active TTP inducing the degradation of mRNA of target cytokines, such as TNF-α and CXCL2 that are known to recruit inflammatory cells [311, 318]. Our findings are consistent with those of others that have previously shown that a lack of TTP leads to severe inflammation as a result of exaggerated TNF-α expression [311].

In support of our inflammatory cell data, CS exposure also increased the mRNA expression of key pro-inflammatory cytokines IL-6 and TNF-α and the chemokines CXCL1 and CXCL2, in both CS-exposed *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice. Levels of IL-6 have been shown to be increased in induced sputum of COPD patients and this was associated with declined in lung function [439, 440]. Increased expression of TNF-α has been observed in mice and humans following CS exposure [113, 441, 442]. This cytokine is known to induce the expression of the neutrophil chemoattractants CXCL1 and CXCL2 [443]. Notably, the mRNA expression of IL-6 and TNF-α were significantly lower in the lungs of CS-exposed *Zfp36*$^{aa/aa}$ mice compared to CS-exposed *Zfp36*$^{+/+}$ controls after four days, but not after eight weeks of exposure. This may be attributed to differences in acute versus chronic phases of the disease, where the induction of pro-inflammatory cytokines predominates in the acute phase, while this is less critical in the chronic phase where features of remodelling and emphysematous-like changes occur. There was a significant reduction in the mRNA expression of CXCL2, but not CXCL1 in the lungs of CS-exposed *Zfp36*$^{aa/aa}$ mice compared to CS-exposed *Zfp36*$^{+/+}$ controls after eight weeks of CS exposure. Both these chemokines are functional homologs of human IL-8 and have been shown to be increased in both mouse models and human COPD [113, 444, 445]. The decreased in CXCL2 expression is consistent with the reduced neutrophils observed in the BAL of

CS-exposed *Zfp36*$^{aa/aa}$ mice. As CXCL2 is a known target of TTP [318], our findings suggest that the presence of active TTP can induce the decay of CXCL2 mRNA in response to CS, and subsequently prevents the recruitment of neutrophils to the lung.

The continuous inflammatory response in COPD leads to the development of airway remodelling [93]. Excessive production of extracellular matrix proteins such as fibronectin by airway epithelial and smooth muscles cells in response to CS have been linked to the development of airway remodelling in COPD [446, 447]. In our study, while CS exposure increased collagen deposition around the airways and increased airway epithelial cell thickness in *Zfp36*$^{+/+}$ mice, these effects were not observed in CS-exposed *Zfp36*$^{aa/aa}$ mice, highlighting a role for active TTP in suppressing airway remodelling. Increased collagen deposition has been reported to be higher in emphysematous lung tissue and has been postulated to be associated with lung destruction in human emphysema [448-450]. Increased epithelial thickness has also been observed in COPD patients [451]. The extracellular matrix protein fibronectin has been shown to be higher in the airways of COPD patients and have been reported to contribute to airway remodelling in COPD and have an inverse correlation with lung function changes in COPD patients [446, 447]. The reduction in airway remodelling observed in *Zfp36*$^{aa/aa}$ mice could be associated with reduced levels of fibronectin in the lung.

We previously showed that chronic CS exposure leads to emphysema-like alveolar enlargement in experimental COPD [113, 141, 375, 376, 452]. In this study, *Zfp36*$^{aa/aa}$ mice were protected against CS-induced alveolar enlargement. This was associated with decreased mRNA expression of MMP-12 in the lungs. MMP-12 is a proteinase that is primarily produced by macrophages and plays a role in the destruction of alveolar walls and the development of emphysema [453-455]. Mice deficient in MMP-12 are protected against CS-induced emphysema [89]. Similarly, guinea pigs exposed to CS for 6 months

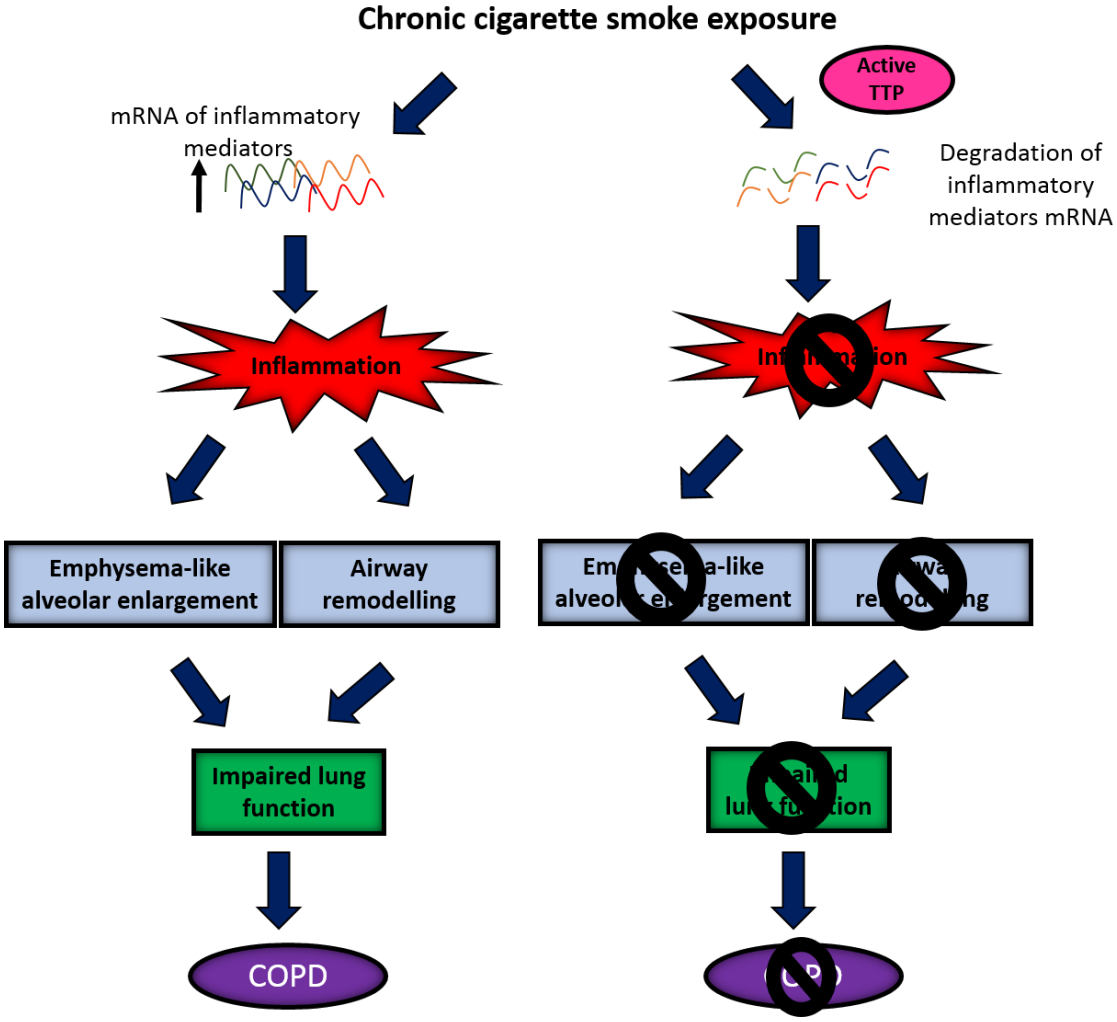
that were treated with the dual MMP-9/MMP-12 inhibitor were also protected against CS-induced emphysema. [456].

Impairment of lung function and breathing difficulties are the most important features of human COPD. CS-exposure of *Zfp36*$^{+/+}$ mice for eight weeks resulted in impaired lung function with increased airway resistance and total lung capacity, reduced FEV_{100}/FVC ratio and increased compliance, determined during a pressure-volume loop manoeuvre. Importantly, CS-exposed *Zfp36*$^{aa/aa}$ mice did not have impaired lung function. Protection against increased airway resistance in CS-exposed *Zfp36*$^{aa/aa}$ is consistent with the decrease in collagen deposition around the airways while no increases in total lung capacity or decreases in FEV_{100}/FVC ratio are consistent with reduced alveolar enlargement. Taken together, our findings suggest that active TTP protects against multiple deleterious effects of CS exposure on lung function. This could be due to the ability of active TTP to induce mRNA decay of pro-inflammatory cytokines and chemokines, which then reduced pulmonary inflammation in response to CS and subsequently leads to reduced features of airway remodelling and consequently improve lung function.

This study highlights the importance of having active TTP to control CS-induced inflammation and subsequent pulmonary remodelling and impaired lung function in mice. The inability of TTP in *Zfp36*$^{aa/aa}$ mice to be phosphorylated in response to CS means that TTP remains in its active form, and hence is able to promote degradation of pro-inflammatory cytokine and chemokine mRNA produced in response to CS. The precise mechanisms of how TTP suppresses CS-induced COPD remains to be explored and are likely to be complex. It has been shown that TTP can induce the mRNA decay of the pro-inflammatory cytokines and chemokines IL-6, TNF-α, CXCL1 and CXCL2 [311, 318, 319, 457]. The reduction in these inflammatory factors in *Zfp36*$^{aa/aa}$ mice likely prevents the

progression of the disease. TTP could also potentially be regulating the mRNA of other factors involved in the development of airway remodelling (fibronectin) and emphysema (MMP-12). Further studies will be required to investigate these possibilities. We have previously shown that macrophages play crucial roles in the development of experimental COPD as their depletion with clodronate-loaded liposomes suppressed CS-induced emphysema-like alveolar enlargement, airway remodelling and improved lung function [113]. A recent study highlighted that BMMs and peritoneal macrophages from *Zfp36*$^{aa/aa}$ mice had reduced expression of TNF-α and CXCL1 in response to LPS [356]. This finding suggests that the expression of TTP in macrophages, and possibly other cells is important in controlling pro-inflammatory cytokine expression and the subsequent development of COPD. Collectively, these data suggest that TTP is a negative regulator of inflammation and is able to confer protection from the development of CS-induced emphysema, airway remodelling and lung function impairment (**Supplementary figure 4.7**).

In summary, this study reveals for the first time that active TTP plays an important suppressive role in CS-induced experimental COPD. It is pivotal in suppressing hallmark features of COPD including pulmonary inflammation, emphysema-like alveolar enlargement, airway remodelling and lung function impairment. Taken together, these findings identify the activation of TTP as a potential therapeutic strategy for COPD.



Supplementary figure 4.7: Proposed mechanisms of how tristetraprolin (TTP) confers protection against the development of cigarette smoke (CS)-induced experimental COPD. CS exposure increases the messenger ribonucleic acid (mRNA) expression of inflammatory mediators resulting in pulmonary inflammation. Ongoing inflammation drives the development of emphysema-like alveolar enlargement and airway remodelling. These features collectively result in lung function decline. The presence of active TTP degrades the mRNA of inflammatory mediators, preventing the development of pulmonary inflammation, emphysema-like alveolar enlargement, airway remodelling and subsequently lung function decline. *TTP: tristetraprolin, mRNA: messenger ribonucleic acid, COPD: chronic obstructive pulmonary disease*

CHAPTER 5:

Enhancing tristetraprolin activity reduces the severity of influenza virus infection in mice

In this chapter, we explored the role of TTP in a mouse model of influenza virus infection. We showed in Chapter 4 that active TTP can suppress hallmark features of experimental COPD (chapter 4). We furthered our investigation to determine if enhancing the activity of TTP would have any beneficial effects in influenza virus infection. Our study demonstrates that active TTP reduced the severity of infection by enhancing protective antiviral responses, decreasing pro-inflammatory cytokine and chemokine production, and suppressing PI3K activity. Our study identifies a novel role for active TTP in reducing the severity of influenza virus infection. It highlights the potential of enhancing TTP activity as a therapeutic approach for the prevention and/or treatment of influenza.

This chapter is currently in preparation for submission as an original research article to *Infection and Immunity*.

The study in this chapter was conducted in collaboration with Professor Alaina Ammit from the University of Sydney and Professor Andrew Clark from the University of Birmingham, who provided us with the *Zfp36*$^{aa/aa}$ mice used in this study. I conducted majority of the work and was heavily involved in experimental design, data interpretation and wrote the manuscript.

5.1 Abstract

Influenza is a major cause of significant morbidity and mortality around the world. The immune factors that regulate clearance of influenza virus and resolution of the associated inflammation are incompletely understood. TTP is a destabilising RNA-binding protein, which in its active form can enhance mRNA decay, including factors involved in the immune response to influenza virus infection. Here, we investigated the role of TTP in this infection using *Zfp36*$^{aa/aa}$ mice that have constitutively active TTP. *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ C57BL/6 mice were infected with the mouse adapted H1N1 influenza virus A/PR/8/34 and were sacrificed at 1, 3, 5, 7 and 9 days post infection (dpi). Infected *Zfp36*$^{aa/aa}$ mice had significantly reduced viral load at 3, 5, 7 and 9 dpi, which was associated with less severe weight loss from 3 dpi, compared to infected *Zfp36*$^{+/+}$ controls. Infected *Zfp36*$^{aa/aa}$ mice also had reduced total leukocytes and neutrophils in BAL fluid, peribronchial, perivascular and parenchymal inflammation compared to infected *Zfp36*$^{+/+}$ controls. There were significant increases in IFN-β and IFN-λ mRNA expression in infected *Zfp36*$^{aa/aa}$ mice and reduced IL-6, TNF-α, CCL3, CCL5, CCL20, CXCL9 and CXCL10 protein levels in the lung compared to infected *Zfp36*$^{+/+}$ controls. *Zfp36*$^{aa/aa}$ mice also had decreased constitutive PI3K activity, which may reduce influenza virus entry and replication. This study identifies for the first time a novel role for active TTP in reducing the severity of influenza virus infection in mice by enhancing protective IFN responses, suppressing pro-inflammatory responses and reducing PI3K activity.

5.2 Introduction

Influenza is one of the most important respiratory viral infections in the world affecting 5-10% adults and 20-30% children and causing approximately 250,000 to 500,000 deaths annually [119]. In addition, influenza also causes substantial economic burden due to loss of productivity, medical costs and indirect costs of preventative measure such as vaccination programs [458]. Development of resistance due to constant genetic changes in the influenza viral genome limits the efficacy of vaccines and antiviral drugs.

Influenza virus infections typically occurs when the virus invades the respiratory tract through binding of viral HA to sialic acid on the surface of host cell [459]. This initiates a cascade of immune responses by the host immune system. Binding of viral RNA by the PRR, RIG-I, initiates the production of type 1 IFNs (IFN-α/β) and type III IFNs (IFN-λ 1/2/3) [460]. The production of these IFNs further induced the production of more than 300 IFN-stimulated genes that inhibit protein synthesis by cleaving viral RNA [164, 461]. Infection also results in the induction of an array of cytokines and chemokines including IL-6, TNF-α and CXCL10 that signals the immune system to defend the host against the infection [462-464]. The infection can also lead to an excessive production of cytokines and chemokines as a result of uncontrolled immune responses. This 'cytokine storm' has deleterious effects on host tissues and can lead to death [143, 144]. Influenza viruses utilise the PI3K/Akt pathway for viral entry and replication [137]. Influenza viruses produce NS1 protein that binds to the PI3K regulatory subunit, p85, and subsequently converts PIP_2 to PIP_3, which in turn phosphorylates Akt and activates the PI3K signalling pathway [138]. This consequently promotes viral entry and replication.

TTP is an anti-inflammatory protein encoded by the *Zfp36* gene in mice and humans. TTP is also known as TIS11, Nup475 and G0S24 [297]. It plays a pivotal role in

promoting mRNA decay of cytokines by binding to the AREs in the 3'-UTR of cytokine mRNAs [434]. The p38 MAPK pathway is an important regulator of TTP. Phosphorylation of TTP at serines 52 and 178 by MK2 inactivates TTP, leading to stabilisation of target mRNA.[348] The phosphorylated form of TTP is inactive, while the unphosphorylated form of TTP is active and is often targeted by the UPS for degradation [347]. The lack of TTP in mice leads to the development of a severe inflammatory phenotype as a result of increased mRNA stability of the pro-inflammatory cytokines, TNF-α and GM-CSF [313, 436]. The roles and effects of TTP in viral infections are poorly understood. As TTP regulates the mRNA stability of numerous inflammatory genes, enhancing the activity of TTP may be beneficial in influenza.

In this study, the role of active TTP in influenza was investigated using a mouse model of influenza virus infection and *Zfp36*$^{aa/aa}$ mice that express the active form of TTP due to the inability of TTP to be phosphorylated by the p38 MAPK-MK2 pathway. These mice are the most appropriate tool for investigating the role of TTP in influenza, as mice deficient in this factor develop severe spontaneous autoimmune inflammation making it difficult to assess its role in infection models [311]. Here, we demonstrate for the first time that active TTP protects mice from influenza virus infection by reducing viral load and pulmonary inflammation, improving antiviral IFN-responses, and decreasing pro-inflammatory cytokine and chemokine levels in the lung. This protection is mediated by a reduction in PI3K activity. This study demonstrates a previously unrecognised role for active TTP in promoting antiviral IFN responses and also suggest a possible link between active TTP and the PI3K signalling pathway.

5.3 Methods

5.3.1 Ethics statement

This study was performed in strict accordance with the recommendations in the Australian code of practise for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All protocols were approved by the Animal Ethics Committee of The University of Newcastle.

5.3.2 Influenza virus infection

Male, 6-9 weeks old, *Zfp36*$^{+/+}$ or *Zfp36*$^{aa/aa}$ C57BL/6 mice were anaesthetised with isoflurane and infected intransally (i.n.) with 33 plaque forming units (pfu) of the mouse-adapted H1N1 influenza virus A/PR/8/34 (WHO Collaborating Centre for Reference and Research of Influenza, Victoria, Australia) in 50µl of media vehicle (UltraMDCK, Lonza, NJ, USA). Controls were sham-inoculated with media. Mice were sacrificed at 1, 3, 5, 7 and 9 days post infection (dpi).

5.3.3 Viral titre

Madin-Darby Canine Kidney (MDCK) cells were grown until approximately 79% confluent. Cells were then washed with Dulbecco's Phosphate Buffered Saline (DPBS; Sigma Aldrich, MO, USA) three times and submerged in Leibovitz's L-15 (L-15) medium (Invitrogen, CA, USA) supplemented with 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid (HEPES; Invitrogen) and N-p-tosyl-L-phenylalanine chloromethyl ketone treated trypsin (trypsin-TPCK; Invitrogen). BAL fluid samples were serially diluted in L-15 medium supplemented with HEPES and added to the cells. Inoculum was removed after 60 minutes of incubation (37 ^{o}C with 5% CO_2). Next, a thin overlay of 1.8% agarose in L-15 medium containing trypsin-TPCK (Invitrogen) was placed on to the cell monolayers. Plaques were stained with 0.1% crystal violet and counted after 48 hours of incubation (37 ^{o}C with 5% CO_2) [141, 465, 466].

5.3.4 Airway inflammation

BAL was performed, cells were cytocentrifuged (300 x*g*, 10 minutes) stained with May Grunwald-Giemsa and differential leukocyte counts were determined according to morphological criteria from a total of 250 cells as previously described [372, 375, 376].

5.3.5 Histopathology

Lungs were formalin fixed, embedded and sectioned. Longitudinal sections were stained with H&E. Histopathology was scored in a blinded fashion according to a set of custom-designed criteria as described previously (**Table 5.1**) [374].

Table 5.1: Histopathological scoring system

Score 1: Airways inflammation score (/4)
0= Lack of inflammatory cells around airways- Absent
1= Some airways have small numbers of inflammatory cells- Mild
2= Some airways have significant inflammation- Moderate
3= Majority of airways have some inflammation- Marked
4= Majority of airways have significant inflammation- Severe
Score 2: Vascular inflammation score (/4)
0= Lack of inflammatory cells around blood vessels- Absent
1= Some blood vessels have small numbers of inflammatory cells- Mild
2= Some blood vessels have significant inflammation- Moderate
3= Majority of blood vessels have some inflammation- Marked
4= Majority of blood vessels have significant inflammation- Severe
Score 2: Parenchymal inflammation (at 10x magnification) (/5)
0= <1 % affected
1= 1-9 % affected
2= 10-29 % affected
3= 30-49 % affected
4= 50-69 % affected
5= >70 % affected
Total score= Score 1+ Score 2+ Score 3

5.3.6 mRNA expression

Whole lungs were collected and stored in RNA Stabilisation Reagent, RNA*later* (Qiagen, Chadstone Centre, Australia. RNA was extracted using guanidinium thiocyanate phenol chloroform (TRIzol) extraction. Extracted RNA was treated with DNAse I (Sigma, Castle Hill, Australia) and reverse-transcribed using Bioscript (Bioline, Alexandria, Australia) and random hexamer primers (Invitrogen, Mount Waverly, Australia). The relative abundance of cytokine cDNA for IFN-α4, IFN-β, IFN-γ, IFN-λ, IL-6, TNF-α, CXCL1 and CXCL2 was determined relative to the reference gene HPRT by qPCR using a ViiA 7 Real-Time PCR System (Life Technologies, Thermo Fisher Scientific). Custom-designed primers (IDT, Coralville, IA, USA) were used (**Table 5.2**) [375, 413].

Table 5.2: Custom-designed primers used in qPCR analysis

Primer	**Primer sequence (5' → 3')**
IFN-α4 forward	ACCAACAGATCCAGAAGGCTCAAG
IFN-α4 reverse	AGACTTCCTGGGTCAGAGGAGGTT
IFN-γ forward	TCTTGAAAGACAATCAGGCCATCA
IFN-γ reverse	GAATCAGCAGCGACTCCTTTTCC
IFN-β forward	CCCTATGGAGATGACGGAGA
IFN-β reverse	ACCCAGTGCTGGAGAAATTG
IFN-λ forward	CTTCAGGCCACAGCAGAGCCCAAG
IFN-λ reverse	ACACACTTGAGGTCCCGGAGGA
IL-6 forward	AGAAAACAATCTGAAACTTC CAGAGAT
IL-6 reverse	GAAGACCAGAGGAAATTTTCAATAGG
TNF-α forward	TCTGTCTACTGAACTTCGGGGTGA
TNF-α reverse	TTGTCTTTGAGATCCATGCCGTT
CXCL1 forward	GCTGGGATTCACCTCAAGAA
CXCL1 reverse	CTTGGGGACACCTTTTAGCA
CXCL2 forward	TGCTGCTGGCCACCAACCAC
CXCL2 reverse	AGTGTGACGCCCCCAGGACC
HPRT forward	AGGCCAGACTTTGTTGGATTTGAA
HPRT reverse	CAACTTGCGCTCATCTTAGGATTT

IFN: Interferon, IL: Interleukin, TNF-α: Tumour necrosis factor alpha, CXCL: Chemokime (C-X-C motif) ligand, HPRT: hypoxanthine–guanine phosphoribosyltransferase

5.3.7 Protein isolation

Proteins were extracted from whole lungs. Briefly, tissues were homogenised in 500µl of sterile Dulbecco's phosphate-buffered saline (Life Technologies, Mulgrave, Victoria, Australia) supplemented with PhoSTOP phosphatase and Complete ULTRA protease inhibitors cocktails (Roche Diagnostics, Manheim, Germany) using a Tissue Tearor stick homogeniser (BioSpec Products, Bartesville, OK) on ice. Homogenates were then centrifuged (8000x*g*, 10 minutes, 4 ^{o}C). Supernatants were collected and stored at -20 ^{o}C for assessment by ELISA [141].

5.3.8 Cytokine and chemokine concentrations in lung

Protein concentration in lung homogenates were determined for IL-6, TNF-α, IFN-γ, CCL3, CCL5, CCL17, CCL20, CXCL9 and CXCL10 using a LEGENDplex multi-analyte flow assay kit (Biolegend, San Diego, CA) according to manufacturer's instructions.

5.3.9 PIP_3 levels in the lung

The levels of PIP_3 in lung homogenates were determined using a PIP_3 Mass Elisa kit (Sapphire Bioscience, Australia) according to manufacturer's instructions.

5.3.10 Statistical analysis

Data are presented as means ± s.e.m. with 5-8 mice in each group and are representative of two independent experiments. Statistical significance for multiple comparisons was determined by one-way ANOVA with Bonferroni post-test, or non-parametric equivalent, where appropriate. The two-tailed Mann-Whitney test was used to determine differences between two groups. Analyses were performed using GraphPad Prism V.6 Software (San Diego, CA).

5.4 Results

5.4.1 Viral load and weight loss are reduced in TTP knock-in mice infected with influenza virus

To investigate the importance of active TTP in influenza virus infection, both wild-type (*Zfp36*$^{+/+}$) and *Zfp36*$^{aa/aa}$ mice were infected i.n with 33 pfu of mouse-adapted H1N1 influenza A/PR/8/34 strain and sacrificed at 1, 3, 5, 7 and 9 dpi (**Figure 5.1a**). Viral titre and weight loss were assessed.

Infected *Zfp36*$^{+/+}$ mice (A/PR/8/34) had a productive infection peaking at 3 dpi and largely resolved by 9 dpi (**Figure 5.1b**). In infected *Zfp36*$^{aa/aa}$ mice, increased viral titres were only detected at 3 and 5 dpi. There was a significant reduction in viral load in infected *Zfp36*$^{aa/aa}$ mice at all time points compared to infected *Zfp36*$^{+/+}$ controls. Notably, influenza virus was no longer detected in the lungs of infected *Zfp36*$^{aa/aa}$ mice at 7 dpi while virus was still detectable at low levels in infected *Zfp36*$^{+/+}$ mice even at 9 dpi.

Both infected *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ had increased weight loss compared to sham-inoculated controls (Sham) (**Figure 5.1b**). There were no significant difference in weight loss between infected *Zfp36*$^{+/+}$ and infected *Zfp36*$^{aa/aa}$ mice. However, infected *Zfp36*$^{aa/aa}$ had delayed weight loss and there was a trend to not losing as much weight as infected *Zfp36*$^{+/+}$ controls. Infected *Zfp36*$^{+/+}$ mice began losing weight from 3 dpi, while infected *Zfp36*$^{aa/aa}$ mice only lost weight from 5 dpi and weight loss was not as severe as in *Zfp36*$^{+/+}$ controls.

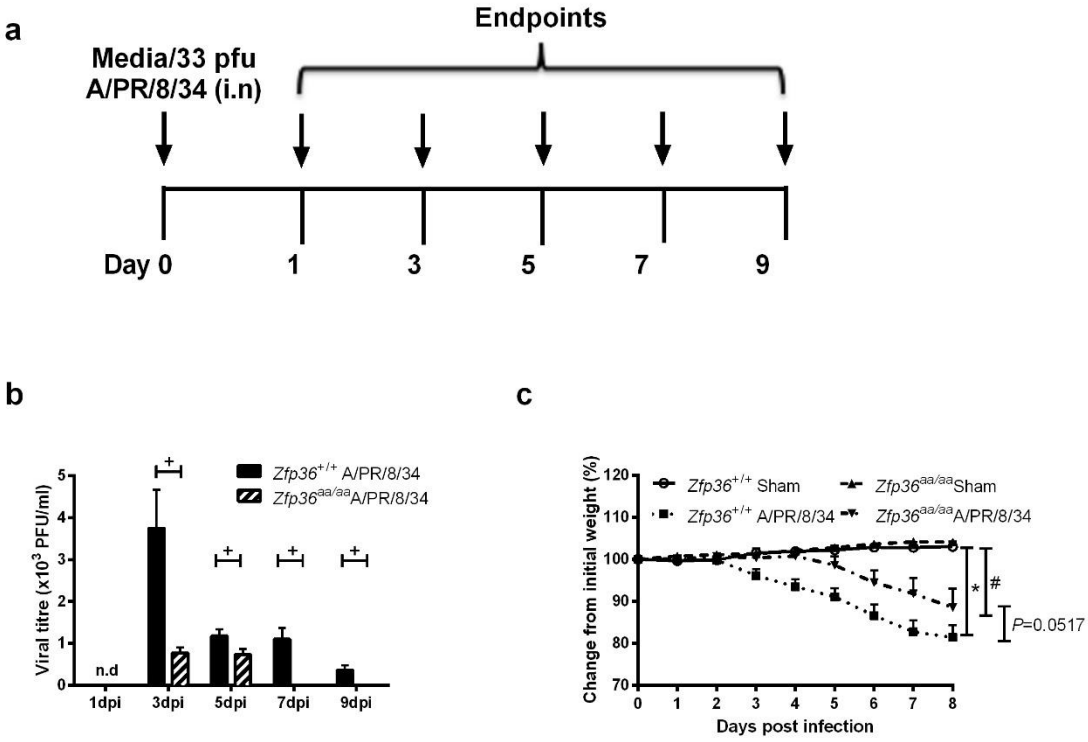


Figure 5.1: Viral load and weight loss are reduced in tristetraprolin (TTP) knock-in mice infected with influenza virus. (a) Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were inoculated with 33 plaque forming units of A/PR/8/34 influenza virus (A/PR/8/34) or media (Sham) and sacrificed at 1, 3, 5, 7 and 9 dpi. **(b)** Viral titres in bronchoalveolar lavage fluid. **(c)** Body weight. Data are presented as means ± s.e.m (n=5-8). * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Sham, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Sham, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ A/PR/8/34, n.d represents not detectable.

5.4.2 Airway inflammation is reduced in TTP knock-in mice infected with influenza virus

Next, the influx of inflammatory cells into the airways during influenza virus infection was assessed. Infection resulted in increases in the numbers of total leukocytes, neutrophils, lymphocytes and macrophages in BAL fluid in both infected *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice compared to their respective sham-inoculated controls at 3, 5, 7, and 9 dpi (**Figure 5.2a-d**). There were no differences in any of these cells at 1 dpi. Notably, infected *Zfp36*$^{aa/aa}$ mice had significantly reduced total leukocytes at 3, 7 and 9 dpi compared to infected *Zfp36*$^{+/+}$ controls and this was primarily due to a reduction in neutrophils (**Figure 5.2a and b**). Infected *Zfp36*$^{aa/aa}$ mice also had reduced lymphocytes at 3 and 9 dpi compared to infected *Zfp36*$^{+/+}$ controls (**Figure 5.2c**). There was no difference in macrophages between infected *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice at any time points assessed (**Figure 5.2d**).

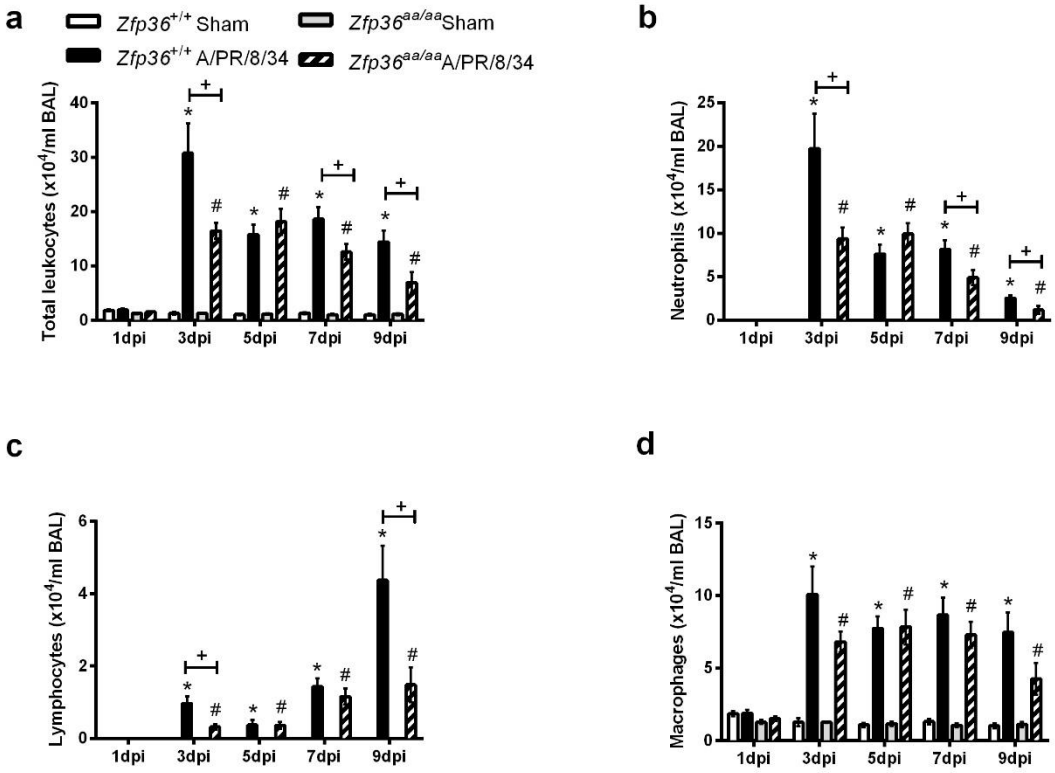


Figure 5.2: Airway inflammation is reduced in tristetraprolin (TTP) knock-in mice infected with influenza virus. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were inoculated with 33 plaque forming units A/PR/8/34 influenza virus (A/PR/8/34) or media (Sham). Inflammatory cells were assessed in bronchoalveolar lavage (BAL) fluid at 1, 3, 5, 7 and 9 days post infection. **(a)** Total leukocytes, **(b)** neutrophils, **(c)** lymphocytes and **(D)** macrophages. Data are presented as means ± s.e.m (n=5-8). * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Sham, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Sham, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ A/PR/8/34.

5.4.3 Pulmonary tissue inflammation is reduced in TTP knock-in mice infected with influenza virus

Next, tissue inflammation in the lungs was scored according to a set of custom designed criteria [374]. Infection resulted in increased total histopathology score in both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice at all time points assessed compared to their respective sham-inoculated controls (**Figure 5.3a**). This was characterised by increased airway, vascular and parenchymal inflammatory scores (**Figure 5.3b-d**). Inflammation in the lungs of infected *Zfp36*$^{aa/aa}$ mice was significantly increased at 7 dpi compared to infected *Zfp36*$^{+/+}$ controls. However, inflammation in infected *Zfp36*$^{aa/aa}$ mice began to clear at 9 dpi, and at this point, inflammation was significantly lower compared to infected *Zfp36*$^{+/+}$ controls. In contrast, inflammation in infected *Zfp36*$^{+/+}$ mice continued to increase at 9 dpi.

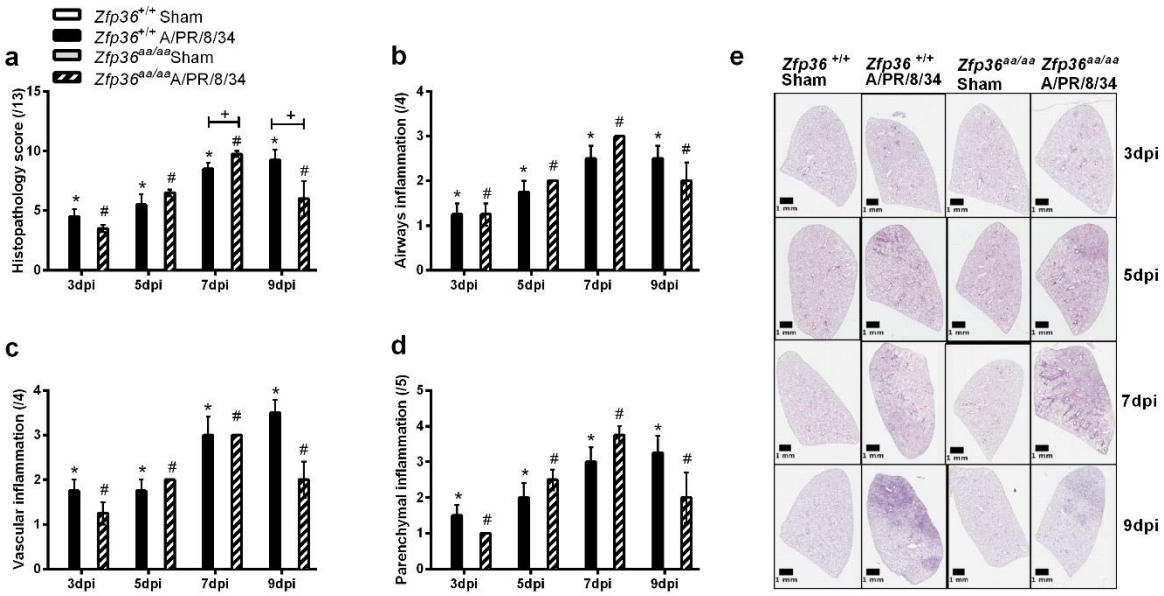


Figure 5.3: Pulmonary tissue inflammation is reduced in tristetraprolin (TTP) knock-in mice infected with influenza virus. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were inoculated with 33 plaque forming units of A/PR/8/34 influenza virus (A/PR/8/34) or media (Sham). Histopathological scores in haematoxylin and eosin stained lung sections were assessed at 3, 5, 7 and 9 days post infection. **(a)** Total, **(b)** airway, **(c)** vascular, and **(d)** parenchymal inflammatory score. **(e)** Representative images of whole lung sections. Scale bars= 1mm. Data are presented as means ± s.e.m (n=4). * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Sham, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Sham, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ A/PR/8/34.

5.4.4 IFN-β and IFN-λ responses are increased in TTP knock-in mice infected with influenza virus

The levels of antiviral IFNs in response to influenza virus infection were assessed. Infection increased the mRNA expression of IFN-α4, IFN-β, IFN-γ and IFN-λ in both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice compared to their respective sham-inoculated controls at 3, 5, 7 and 9 dpi (**Figure 5.4a-d**). There were no differences in the levels of IFN-α4 or IFN-γ between infected *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice (**Figure 5.4a and c**). Notably, the levels of IFN-β and IFN-λ in infected *Zfp36*$^{aa/aa}$ mice were significantly higher at 5 and 7 dpi compared to infected *Zfp36*$^{+/+}$ controls (**Figure 5.4b and d**).

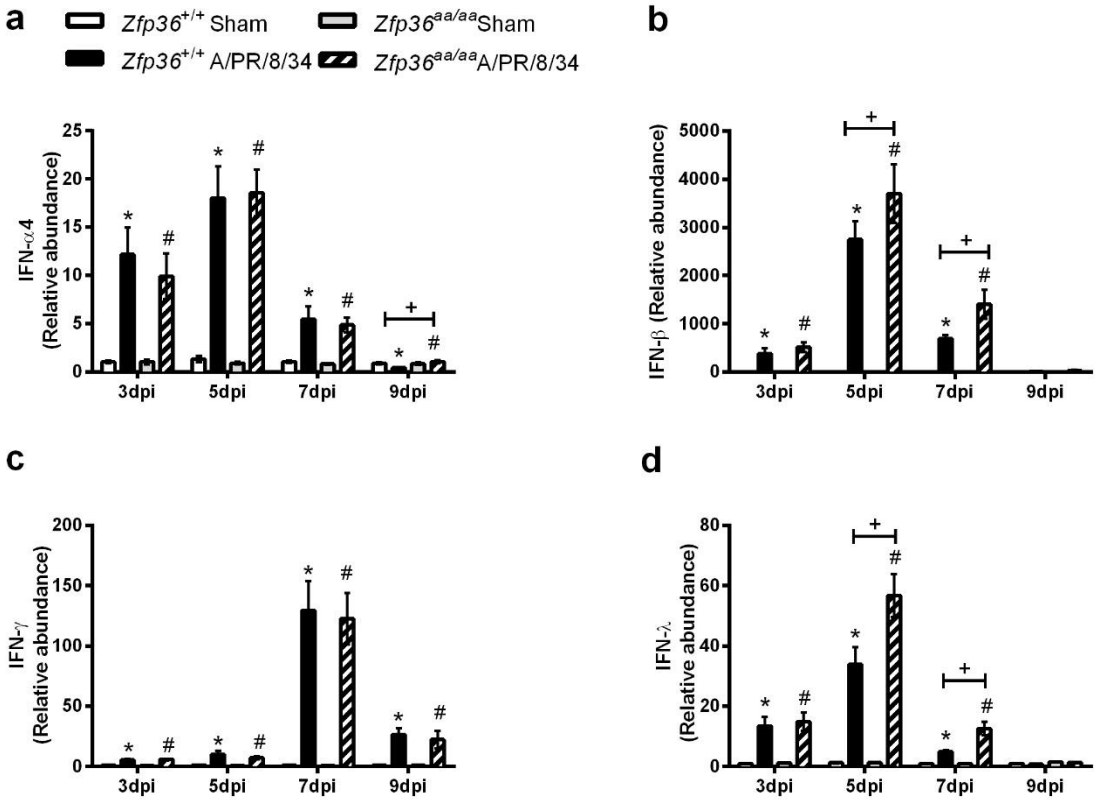


Figure 5.4: IFN-β and IFN-λ responses are increased in tristetraprolin (TTP) knock-in mice infected with influenza virus. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were inoculated with 33 plaque forming units of A/PR/8/34 influenza virus (A/PR/8/34) or media (Sham). Antiviral factors were assessed at 3, 5, 7 and 9 days post infection. **(a)** Interferon (IFN)-α4, **(b)** IFN-β **(c)** IFN-γ and **(d)** IFN-λ mRNA expression in lung homogenates. Data are presented as means ± s.e.m (n=5-8). * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Sham, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Sham, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ A/PR/8/34.

5.4.5 Pro-inflammatory cytokines and chemokines mRNA expression are altered in TTP knock-in mice infected with influenza virus

Given that infected *Zfp36*$^{aa/aa}$ had decreased pulmonary and tissue inflammation, mRNA expression of associated inflammatory cytokines and chemokines that are TTP targets were assessed in lung homogenates. Infection increased the mRNA expression of IL-6, TNF-α, CXCL1 and CXCL2 in both *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice compared to their respective sham-inoculated controls at 3, 5, 7 and 9 dpi (**Figure 5.5a-d**). There was a significant increase in the mRNA expression of the pro-inflammatory cytokine, IL-6 at 7 dpi in infected *Zfp36*$^{aa/aa}$ mice compared to infected *Zfp36*$^{+/+}$ controls (**Figure 5.5a**). Notably, infected *Zfp36*$^{aa/aa}$ mice had significantly lower mRNA expression of TNF-α and CXCL2 at 5 dpi and CXCL1 at 3 and 5 dpi compared to infected *Zfp36*$^{+/+}$ controls (**Figure 5.5b-d**).

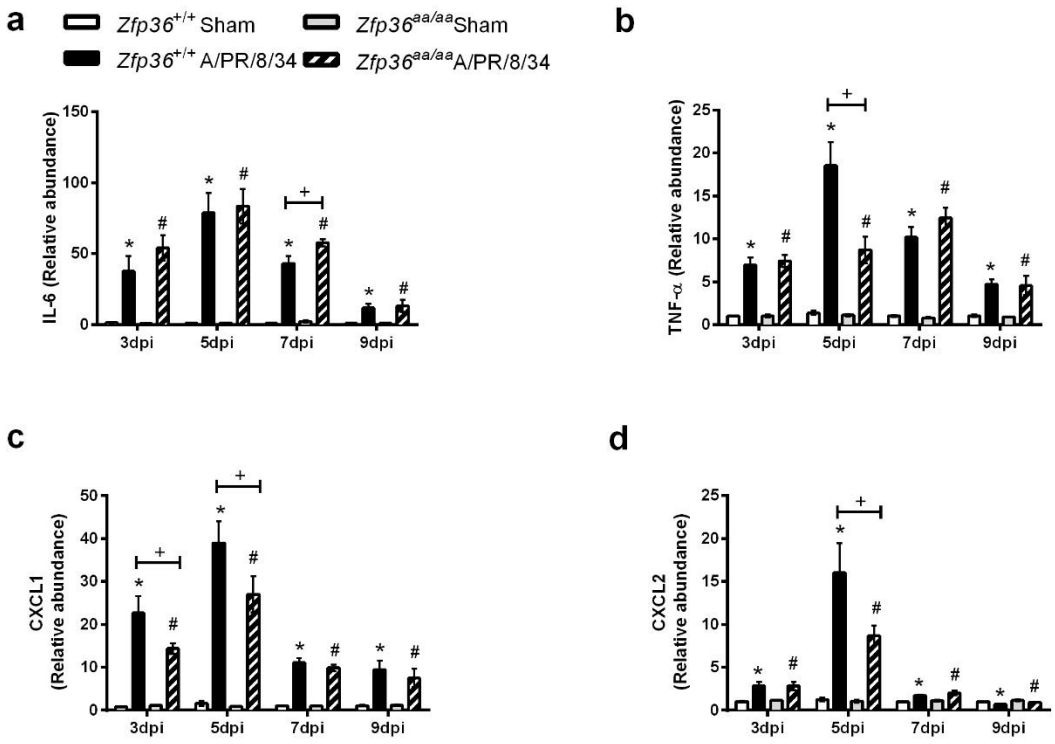


Figure 5.5: Pro-inflammatory cytokines and chemokines mRNA expression are altered in tristetraprolin (TTP) knock-in mice infected with influenza virus. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were inoculated with 33 plaque forming units of A/PR/8/34 influenza virus (A/PR/8/34) or media (Sham). Cytokine and chemokine mRNA expression in lung homogenates were assessed at 3, 5, 7 and 9 days post infection. **(a)** IL-6, **(b)** TNF-α, **(c)** CXCL1 and **(d)** CXCL2 mRNA expression in lung homogenates. Data are presented as means ± s.e.m (n=5-8). * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Sham, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Sham, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ A/PR/8/34.

5.4.6 Protein levels of pro-inflammatory cytokines and chemokines are reduced in TTP knock-in mice infected with influenza virus

Next, infection-induced cytokine and chemokine protein responses were assessed using a multiplex assay at 3, 7 and 9 dpi. Infection increased the levels of IL-6 (3 and 7 dpi), TNF-α (7 and 9 dpi), CCL3 (3 and 7 dpi), CCL5 (7 and 9 dpi), CCL20 (7 dpi), CXCL9 (7 and 9 dpi) and CXCL10 (3 and 7 dpi) in infected *Zfp36*$^{+/+}$ mice compared to sham-inoculated controls (**Figure 5.6a-g**). Infection also increased the levels of IL-6 (3 and 7 dpi), TNF-α (7 dpi), CCL3 (7 dpi), CCL5 (7 and 9 dpi), CCL20 (7 and 9 dpi), CXCL9 (7 dpi) and CXCL10 (3 and 7 dpi) in infected *Zfp36*$^{aa/aa}$ mice compared to sham-inoculated controls (**Figure 5.6a-g**). However, the protein levels were generally reduced in infected *Zfp36*$^{aa/aa}$ compared to infected *Zfp36*$^{+/+}$ controls with lower levels of IL-6 (3 dpi), TNF-α (7 and 9 dpi), CCL3, CCL5, CCL20, CXCL9 and CXCL10 (all at 7 dpi). Most reductions occurred at the peak of inflammation (7 dpi) but IL-6 was also decreased early (3 dpi) and TNF-α was also decreased during resolution (9 dpi).

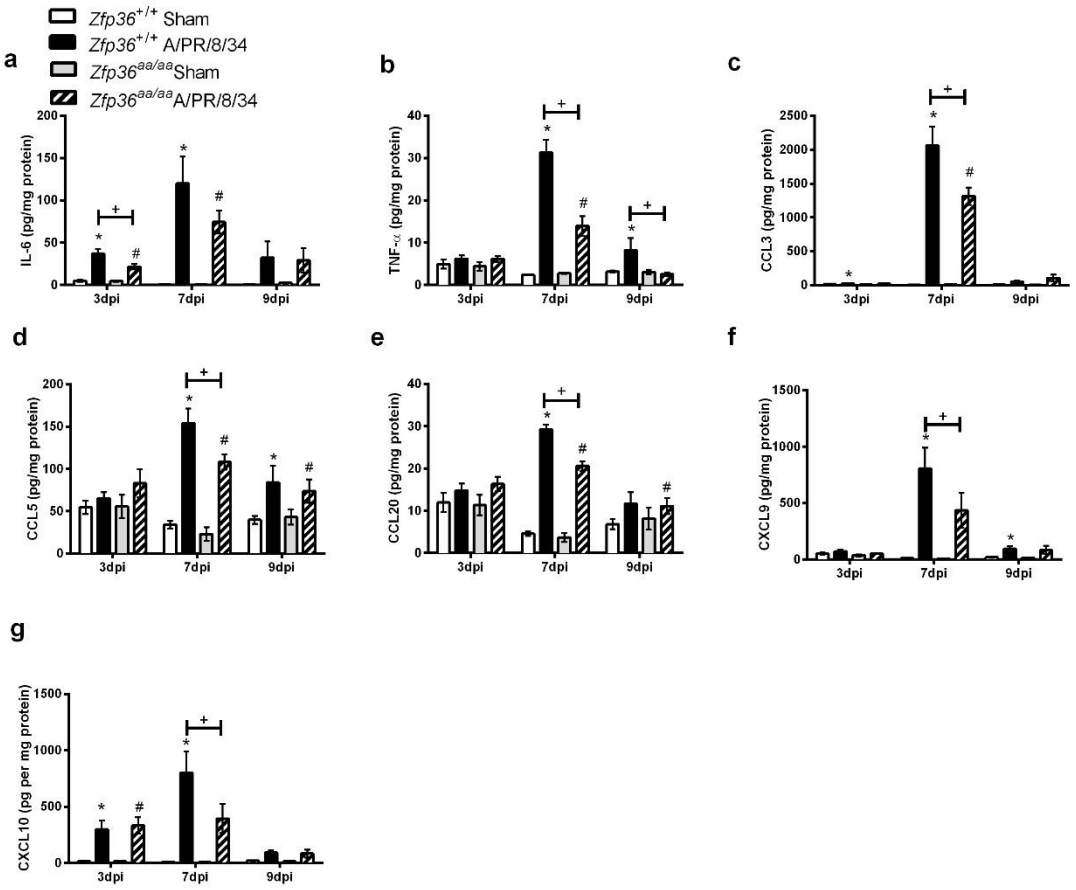


Figure 5.6: Protein levels of pro-inflammatory cytokines and chemokines are reduced in tristetraprolin (TTP) knock-in mice infected with influenza virus. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were inoculated with 33 plaque forming units of A/PR/8/34 influenza virus (A/PR/8/34) or media (Sham). Cytokine and chemokine levels in lung homogenates were assessed at 3, 7 and 9 days post infection. **(a)** Interleukin (IL)-6, **(b)** Tumour necrosis factor alpha (TNF-α), **(c)** Chemokine (C-C motif) ligand (CCL)3, **(d)** CCL5, **(e)** CCL20, **(f)** Chemokine (C-X-C motif) ligand (CXCL)9 and **(g)** CXCL10 protein levels in lung homogenates. Data are presented as means ± s.e.m (n=5-8). * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Sham, # represents $P \leq 0.05$ compared to *Zfp36*$^{aa/aa}$ Sham, + represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ A/PR/8/34.

5.4.7 PIP_3 levels are reduced in TTP knock-in mice

To determine the effect of active TTP on PI3K activity, the levels of PIP_3 were assessed as a surrogate marker. The levels of PIP_3 were significantly reduced in the lungs of sham-inoculated *Zfp36*$^{aa/aa}$ mice compared to sham-inoculated *Zfp36*$^{+/+}$ controls (**Figure 5.7a**). There was a trend towards a decrease in the levels of PIP_3 in the lungs of infected *Zfp36*$^{aa/aa}$ mice compared to infected *Zfp36*$^{+/+}$ controls (**Figure 5.7b**).

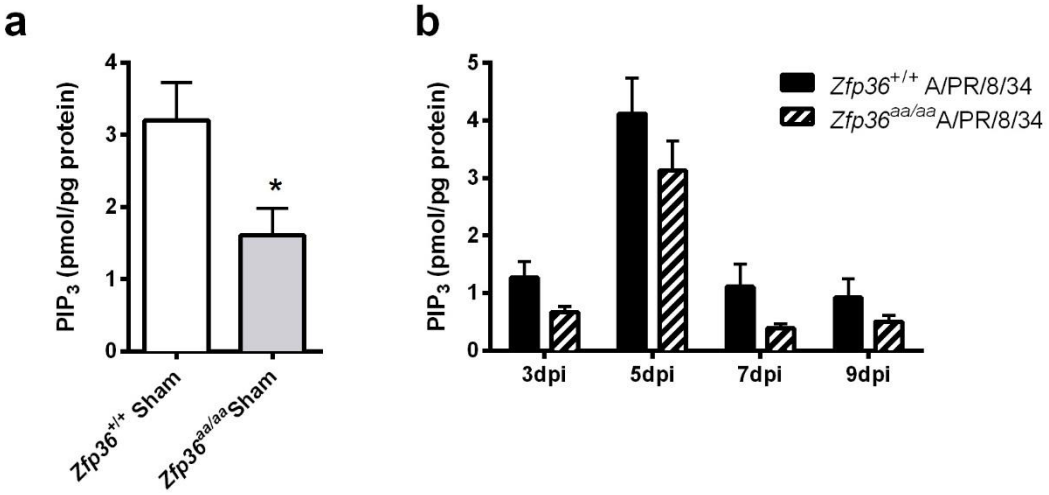


Figure 5.7: PIP_3 levels are reduced in tristetraprolin (TTP) knock-in mice. Wild-type (*Zfp36*$^{+/+}$) or TTP knock-in (*Zfp36*$^{aa/aa}$) C57BL/6 mice were inoculated with 33 plaque forming units of A/PR/8/34 influenza virus (A/PR/8/34) or media (Sham). Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) levels in lung homogenates were assessed at 3, 5, 7 and 9 days post infection (dpi). **(a)** PIP_3 levels in control mice. Data were combined from various time points. **(b)** PIP_3 levels in lung homogenates. Data are presented as means ± s.e.m (n=4-6). * represents $P \leq 0.05$ compared to *Zfp36*$^{+/+}$ Sham.

5.5 Discussion

In this study, a previously unrecognised role for active TTP in suppressing influenza virus infection and associated inflammation was identified. Using a mouse model of influenza virus infection and *Zfp36*$^{aa/aa}$ mice, we demonstrate for the first time that active TTP reduces influenza virus load and pulmonary inflammation, characterised by decreased neutrophils in BAL fluid and tissue inflammation. These effects were associated with improved antiviral responses and decreased expression of pro-inflammatory mediators (IL-6, TNF-α, CCL3, CCL5, CCL20, CXCL1, CXCL2, CXCL9 and CXCL10). Notably, mice expressing active TTP also had significantly lower PIP_3 levels in the lung, which is indicative of decreased constitutive PI3K activity. This may be responsible for reduced influenza viral entry and replication and improved IFN responses. Collectively, these findings highlight the importance of active TTP in influenza virus infection and its potential as a novel therapeutic target for influenza infection.

Influenza virus infection was characterised by increases in viral load that peaked at 3 dpi. Importantly, viral load in infected *Zfp36*$^{aa/aa}$ mice only increased at 3 and 5 dpi and was cleared by 7 dpi. This was in stark contrast to substantially greater viral load in infected *Zfp36*$^{+/+}$ controls at 3 and 5 dpi and infection was still present at 7 and 9 dpi in these mice. The reduction in viral load was associated with less severe weight loss in infected *Zfp36*$^{aa/aa}$ mice compared to infected *Zfp36*$^{+/+}$ controls. The ability of infected *Zfp36*$^{aa/aa}$ mice to clear virus more quickly and with less physiological effects compared to infected *Zfp36*$^{+/+}$ controls suggests that TTP may be regulating inflammation, viral entry and replication.

Influenza virus infection also led to increased airway inflammation that involved the influx of neutrophils, lymphocytes and macrophages, with neutrophils being the

dominant cell type recruited into the airway lumen. Neutrophils are the initial immune leukocyte population that are recruited to the airways and lungs during the early stages of influenza virus infection [467-469]. Notably, neutrophil influx was markedly decreased in infected *Zfp36*$^{aa/aa}$ mice, indicating the presence of less severe infection and inflammation. Neutrophils have also been proposed to serve as antigen presenting cells to CD8^{+} T cells during influenza virus infection, hence initiating the adaptive response [470]. The large increase in lymphocytes at 9 dpi in infected *Zfp36*$^{+/+}$, but not in *Zfp36*$^{aa/aa}$ mice is indicative of the initiation of adaptive immune responses in *Zfp36*$^{+/+}$ mice that likely contribute to more severe inflammation. This was also reflected in the lung tissue, where consolidation of the lung due to accumulation of inflammatory cells was observed in the lungs of infected *Zfp36*$^{+/+}$ mice at 9 dpi, but by this stage, the inflammation in the lungs starts to resolve in infected *Zfp36*$^{aa/aa}$ mice.

The reduced severity of influenza virus infection in *Zfp36*$^{aa/aa}$ mice could be attributed to the enhanced antiviral IFN responses observed in these mice. A substantial increase in IFN-β and IFN-λ were detected in the lungs of infected *Zfp36*$^{aa/aa}$ mice at 5 and 7 dpi compared to infected *Zfp36*$^{+/+}$ controls. Both IFN-β and IFN-λ are pivotal antiviral cytokines that play a crucial role in host defence against influenza virus infection [471, 472]. Mice deficient in IFN-β exhibited reduced survival rates and increased viral load when challenged with influenza A virus [471]. Similarly, mice lacking the IFN-λ receptor were reported to be more susceptible to influenza virus infection [473]. Infection also led to the induction of IFN-α4 and IFN-γ responses in the lungs, but these did not differ between infected *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice, suggesting that they may have minimal roles in the regulation of influenza virus infection by active TTP. Interestingly, it has been shown that IFNs curb inflammatory responses through the induction of TTP [333]. Upon stimulation with LPS, the mRNA and protein levels of TTP in BMMs from IFN-β deficient mice

reduced to baseline, highlighting that IFN-β is required for the expression of TTP [333]. The expression of LPS-induced IL-6, TNF-α, CCL2 and CCL3 in macrophages were reduced by IFN-induced TTP protein, suggesting that IFNs are able to modulate inflammatory responses through TTP [333]. In the current study, the presence of active TTP in *Zfp36*$^{aa/aa}$ mice could potentially induce a negative feedback loop that controls the deleterious inflammatory signals produced by influenza virus. Our findings also suggest that the presence of active TTP induces a heightened antiviral IFN response that is associated with reduced viral load.

In support of our inflammatory cell data, infection increased the mRNA expression of the cytokines IL-6, TNF-α, the chemokines CXCL1 and CXCL2 in both infected *Zfp36*$^{+/+}$ and *Zfp36*$^{aa/aa}$ mice. The levels of production of the mRNA of these factors were altered in *Zfp36*$^{aa/aa}$ mice. Increased levels of IL-6 and TNF-α have been reported in nasal lavage fluids in adults experimentally infected with influenza A virus and correlated with increased viral titre and symptom scores [474, 475]. Interestingly, infected *Zfp36*$^{aa/aa}$ mice had reduced expression of IL-6 at 7 dpi and TNF-α at 5 dpi compared to infected *Zfp36*$^{+/+}$ controls, which may be associated with reduced viral load and less severe weight loss. The functional homologs of human IL-8, CXCL1 and CXCL2, have also been reported to be increased in human airway epithelial cells and in mice infected with influenza A virus and play a role in recruiting neutrophils to the lung [476, 477]. The decreased neutrophils observed in infected *Zfp36*$^{aa/aa}$ compared to infected *Zfp36*$^{+/+}$ controls may be associated with reduced CXCL1 and CXCL2 expression in the lungs. As IL-6, TNF-α, CXCL1 and CXCL2 are known TTP targets, our findings suggest that the presence of active TTP in *Zfp36*$^{aa/aa}$ mice is able to induce the decay of the mRNA of these cytokines and chemokines, and hence prevent the recruitment of inflammatory cells to the lung in response to influenza virus [311, 318, 319].

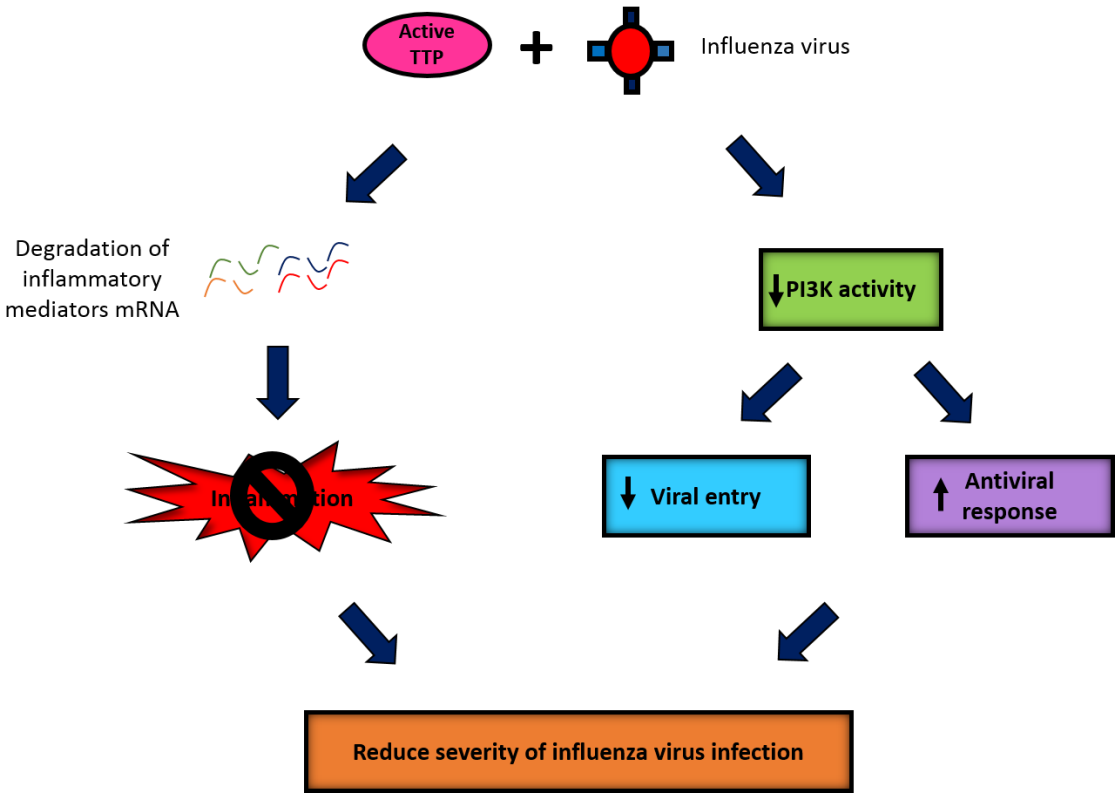
Exaggerated early cytokine production is associated with increased recruitment of inflammatory cells to the lung. This phenomenon known as the 'cytokine storm' was reported to be the major cause of morbidity during the 1918 Spanish flu and the 2009 swine flu outbreak [468, 478]. Infected *Zfp36*$^{aa/aa}$ mice had reduced levels of most of the cytokines and chemokines assessed and this effect was greatest at 7 dpi. Reduced levels of the pro-inflammatory cytokine IL-6 led to reduced influx of inflammatory leukocytes into the lung [479, 480]. Decreased levels of TNF-α and CCL3, which are also neutrophil chemoattractants, were associated with the reduced recruitment of neutrophils into the lung [481, 482]. The production of CCL5 by epithelial cells has been reported to play a pivotal role in the development of the 'cytokine storm' [483]. Production of CCL5 by A549 cells infected with influenza A virus enhanced the production of IL-6 and IL-8 [483]. Reduced levels of CCL5 in infected *Zfp36*$^{aa/aa}$ mice could lead to the decreased levels of IL-6 and subsequent inflammatory responses. CCL20 is upregulated in epithelial cells in response to viral proteins and is known to mediate DC activation and the recruitment of monocytes into the lung [484]. CCL20 has also been reported to be important in recruiting leukocytes into the lung at the early stages of respiratory syncytial virus infection [485]. Elevated levels of CXCL9 and CXCL10 have been observed in the plasma of H5N1 infected individuals [468]. These chemokines are commonly induced by IFN-γ and are chemoattractants for activated T lymphocytes [486-488]. Reduced levels of these chemokines in infected *Zfp36*$^{aa/aa}$ mice also means that there is reduced recruitment of inflammatory leukocytes and cells into the lung. Our findings highlight that active TTP could regulate the expression and production of cytokines and chemokines during influenza virus infection, likely by degrading the mRNA of these mediators or factors that regulate them, subsequently leading to decreased protein production.

To begin to identify the possible mechanisms underpinning the decreased susceptibility to influenza virus infection in *Zfp36*$^{aa/aa}$ mice, we explored the role of the PI3K/Akt signalling pathway. This pathway plays a pivotal role in promoting viral entry and replication, as well as controlling the induction of antiviral IFNs such as IFN-β [137, 138, 489]. In our study, we observed a significant reduction in the levels of PIP_3 in the lungs of *Zfp36*$^{aa/aa}$ mice, suggesting a decrease in constitutive PI3K activity. This observation was associated with reduced viral load, enhanced antiviral responses and suppressed cytokine and chemokine responses that likely leads to reduced recruitment of inflammatory cells into the lungs. These findings also provide a novel mechanistic link between reduced PI3K activity and active TTP. Delineating the interaction between TTP and PI3K/Akt signalling pathway requires further study.

Active TTP plays an anti-inflammatory role in the context of influenza virus infection. This suggest that the phosphorylation status of TTP plays a crucial role in determining its anti-inflammatory role. Altering the equilibrium between inactive (phosphorylated) and active (unphosphorylated) states of TTP could be beneficial in influenza virus infection and possibly other infections. However, as phosphorylation of TTP occurs on serines 52 and 178, which are subjected to strong selective pressure due to their conservation throughout vertebrates, alteration in the phosphorylation of TTP could increase the susceptibility to various pathogens due to impaired adaptive responses [490]. Mice deficient in *Mk2*, a factor that is involved in the phosphorylation of TTP, showed increased susceptibility to *L.monocytogenes* infection [491]. Our findings on the other hand indicate that altering the phosphorylation status of TTP may have minimal impact on the adaptive response towards influenza virus infection. This is supported by the evidence that *Zfp36*$^{aa/aa}$ mice were able to clear the virus much quicker compared to infected *Zfp36*$^{+/+}$ controls. Similarly, in the context of bacterial infection, Ross *et al*., showed that

Zfp36$^{aa/aa}$ mice were able to clear *Salmonella typhimurium* infection and also generated a protective immune response to vaccination against this bacterium [356].

In summary, this study reveals for the first time that active TTP plays an important role in reducing the severity of influenza virus infection. Active TTP reduced viral load, which in turn led to reduced pulmonary and tissue inflammation. Active TTP also enhanced the antiviral responses towards the infection leading to decreased viral load and pro-inflammatory cytokines and chemokines. TTP may be regulating influenza virus infection by decreasing PI3K activity, providing a possible link between active TTP and the PI3K signalling pathway (**Supplementary figure 5.8**). Collectively, these findings highlight the potential of enhancing TTP activity as a therapeutic target for influenza.



Supplementary figure 5.8: Proposed mechanisms of how tristetraprolin (TTP) reduces the severity of influenza virus infection. The presence of active TTP during an influenza virus infection degrades the messenger ribonucleic acid (mRNA) of inflammatory mediators, preventing the development of pulmonary inflammation. Active TTP also decreases phosphoinositide 3-kinase (PI3K) activity leading to decreased viral entry and enhanced antiviral responses. Collectively, these features lead to less severe influenza virus infection. *TTP: tristetraprolin, mRNA: messenger ribonucleic acid, PI3K: phosphoinositide 3-kinase.*

CHAPTER 6: General Discussion and Conclusion

In this chapter, the novel observations from my investigations of the role of RelB in the spontaneous development of AAI and the therapeutic potential of RelB-sufficient DCs for AAI will be discussed. I will also discuss how manipulation of PP2A and the UPS using various immunomodulatory drugs could have beneficial effects in AAD. Finally, I will discuss the novel observations from my investigation of the role of the anti-inflammatory molecule, TTP, in mouse models of both COPD and influenza virus infection as well as the therapeutic potential of manipulating the equilibrium between unphosphorylated and phosphorylated forms of TTP.

6.1 Significance of research

Respiratory diseases such as asthma, COPD and influenza are major causes of mortality and morbidity worldwide [72, 119, 365]. A lack of understanding of the mechanisms underpinning the development and progression of these diseases has led to treatments with only limited efficacy. Thus, there is an urgent need to better understand the mechanisms of pathogenesis and to develop new and effective therapies.

We have made important observations that demonstrate how RelB deficiency promotes the development of spontaneous AAI. This was achieved using $RelB^{-/-}$ mice, in which AAI occurred independently of allergen exposure. Treatment of $RelB^{-/-}$ mice with RelB-sufficient DCs ameliorated features of spontaneous AAI, which included lung inflammation, Th2-associated cytokines, chemokines and airway remodelling. This study indicates that RelB is an important molecule underlying AAI and that RelB-sufficient DCs are required to restore control through immune regulation of inflammation.

Using well-established murine models of acute OVA- and chronic HDM-induced AAD, we demonstrate that enhancing PP2A activity and inhibiting proteasome activity have beneficial effects on hallmark features of AAD. Treatment with immunomodulatory drugs that enhance PP2A activity (e.g. $AAL_{(S)}$) and/or inhibit proteasome activity (e.g. BORT) led to the suppression of eosinophils and Th2-associated cytokine levels and reduced airway remodelling and AHR. These studies identified the importance of PP2A and the UPS in the development of AAD and suggest that targeting these two systems either by activation or inhibition, respectively could be beneficial in AAD.

Finally, we demonstrate a novel and important role of TTP in COPD and influenza. The presence of active TTP reduced the severity of experimental COPD by reducing the influx of neutrophils, airway remodelling and emphysema-like alveolar enlargement that improved lung function. Active TTP also attenuated the severity of

influenza virus infection by reducing viral load, enhancing antiviral responses and suppressing pro-inflammatory cytokines and chemokines. These studies highlight the anti-inflammatory role of TTP in COPD and influenza and suggest that manipulating the equilibrium between phosphorylated and unphosphorylated states of TTP could promote anti-inflammatory functions in both these diseases.

6.2 RelB deficiency promotes the spontaneous development of AAI

Our data demonstrated that RelB-deficiency promotes the development of spontaneous AAI. RelB$^{-/-}$ mice had increases in pulmonary inflammation, Th2-associated cytokines and chemokines and airway remodelling. These features developed spontaneously and were independent of any allergen exposure, suggesting that RelB deficiency may be a crucial pathway involved in the development of AAI. Our data from mice indicate that a loss of function mutation or deletion of RelB could predispose an individual to an asthma-like AAI.

RelB is an important member of the NF-κB family that play pivotal roles in the development of lymphoid organ and DC function [194]. RelB has been associated with the development of several immune-mediated diseases such as atopic dermatitis, multi-organ inflammation and autoimmunity [199, 200, 213, 492]. RelB$^{-/-}$ mice develop atopic dermatitis with infiltration of CD4^{+} T cells and eosinophils into the skin and increased serum IgE [213]. The development of skin lesions in these mice is T cell dependent [213]. We observed similar findings in our study with the focus on the lungs. Our study supports the concept that human atopic dermatitis is often associated with respiratory problems [493]. In terms of contact sensitivity, RelB is required for normal delayed-type hypersensitivity. Besides the skin and lungs, other organs in RelB$^{-/-}$ mice such as the liver, stomach, skeletal muscles, salivary glands, ovaries and uterus have extensive infiltration of inflammatory cells [200]. The lack of mature DCs in the thymic medulla of RelB$^{-/-}$ mice means there is an impaired

ability to delete autoreactive thymocytes and splenocytes that then generate an autoreactive response [199, 492, 494, 495]. Consequently, this leads to the development of autoimmunity. It is possible that the response we observed in the lungs of $RelB^{-/-}$ mice is an autoimmune response as a result of the lack of mature DCs. The presence of autoantibodies in allergic diseases indicate the possible occurrence of autoimmunity that can trigger inflammation of the airways [496, 497].

The severe inflammation observed in the lungs and other organs of $RelB^{-/-}$ mice also suggests the possible involvement of secondary bacterial or viral infection. While this avenue was not explored in our work, several studies have demonstrated impaired immune responses against different infections. $RelB^{-/-}$ mice were highly susceptible and succumb to *L.monocytogenes* infection. This was postulated to be due to reduced phagocytic activity of neutrophils, thus impairing the clearance of the bacteria [200]. RelB deficiency also led to impaired clearance of lymphocytic choriomeningitis virus due to the inability to induce the expansion of $CD8^+$ T cells [200]. Similarly, another study reported impaired clearance of vaccinia virus encountered *via* the skin [498]. More recently, $RelB^{-/-}$ mice were shown to be highly susceptible to parasitic infection by *Toxoplasma gondii* and this was suggested to be due to the impaired ability to produce IFN-γ and reduced NK cell activity [403]. These findings highlight the inability of $RelB^{-/-}$ mice to induce appropriate T cell responses as a result of impaired antigen presenting function and the importance of RelB in regulating immune responses against viral and bacterial pathogens.

Surprisingly, we identified lung epithelial changes in $RelB^{+/-}$ mice despite the lack of inflammation. This suggests that even a defect or alteration in one allele could lead to mild to moderate structural changes in the lung. Recently, mutations in the *relB* gene that resulted in the lack of RelB protein expression was discovered in patients with immune deficiency and inflammatory diseases including respiratory tract infections and reactive

airway disease [401]. A genome-wide association study identified 10 new risk loci for atopic dermatitis, which is closely linked to asthma [364]. Many of these loci play pivotal roles in the regulation of T cell function, host defences and antigen presentation and these are all significantly altered in RelB$^{-/-}$ mice. Collectively, these findings in both mice and humans suggest that RelB is crucial in regulating the innate and adaptive immune responses. Our study contributes to the body of knowledge by showing the importance of RelB in the lungs and in that RelB-deficiency leads to the development of spontaneous AAI.

6.2.1 RelB-sufficient DCs ameliorate features of AAI

RelB is critical for the antigen presenting function of DCs and their maturation [195]. DCs from RelB$^{-/-}$ mice have impaired APC function, indicating a defect in the adaptive immune response [216]. The lack of splenic and thymic DCs in RelB$^{-/-}$ means that these mice are not able to delete autoreactive thymocytes and T cells, resulting in accumulation of self-reactive T cells and hence the profound inflammatory phenotype in these mice [216]. Our collaborators have previously shown that adoptive transfer of RelB-sufficient DCs to RelB$^{-/-}$ mice achieved long-term suppression of autoimmune disease and organ (liver, spleen and pancreas) inflammation in RelB$^{-/-}$ mice [208]. The suppressed inflammation is largely due to the presence of FoxP3^{+} Tregs in the periphery that are able to control proliferation and cytokine production by autoreactive memory T cells when signalled by RelB-sufficient DCs, which is dependent on IDO. In our study, we aimed to determine if adoptive transfer of RelB-sufficient DCs would have similar effects in controlling spontaneous inflammation in the lung. We demonstrate that RelB-sufficient DCs suppressed the hallmark features of AAI, including inflammation, Th2-associated cytokine and chemokine responses and airway remodelling. Collectively, these data indicate that RelB is required to regulate pulmonary homeostasis and also highlights the potential of RelB-sufficient DCs in controlling AAI.

To our knowledge, no study has shown decreased RelB expression or a loss of function in asthmatics. Sheridan and colleagues reported decreased RelB expression in lung fibroblasts from smokers with and without COPD.[499] Animal models of AAD and our human data (data not shown) show an increase in RelB expression.[248] This is expected as NF-κB activity is generally increased in response to inflammatory stimuli. Perhaps the increase in RelB is a compensatory mechanism to regulate inflammation as it has been suggested that upregulation of RelB may have beneficial effects in controlling inflammation [201, 202, 204]. Targeted overexpression of RelB using recombinant adenovirus encoding RelB inhibits CS-induced inflammation[202]. RelB in association with AhR is able to dampen CS extract-induced prostaglandin E2 production in mouse lung fibroblasts.[204] Furthermore, transfection of RelB cDNA into RelB-deficient fibroblasts reduced LPS-induced chemokine overexpression[201]. All these findings highlight the role of RelB in controlling inflammation.

It is important to note that RelB expression in different cell types under similar conditions may have different effects on immune responses. While RelB controls the production of chemokines from LPS-stimulated fibroblasts, it does not have the same effects on macrophages.[201] The lack of RelB in DCs prevents the maturation of DCs, which means DCs are unable to mount immune responses to control disease, therefore mice are chronically in a state of acute inflammation. In mouse models and in humans, whole lungs (mouse) and sputum (human-data not shown) were used to assess RelB expression [248]. Perhaps, if RelB expression were to be assessed in specific cell types, such as fibroblasts or DCs, differences in RelB expression may be observed.

6.3 Enhancing PP2A activity and inhibiting proteasome activity as therapeutic avenues for AAD

Only limited studies have investigated the role of PP2A and the UPS in asthma. In our study, we show that enhancing PP2A activity and inhibiting proteasome activity, either alone or particularly in combination, have beneficial effects in acute and chronic experimental models of AAD.

6.3.1 Enhancing PP2A activity as a potential therapy for asthma

PP2A is one of the most abundant serine/threonine phosphatases in mammals and is expressed ubiquitously [219, 222]. PP2A has been implicated in the development of cancer due to its function in maintaining normal cell division [222]. Over the years, several studies have highlighted the role of PP2A in asthma. Reduced PP2A activity has been observed in asthmatics and patients with severe asthma [248, 249]. Enhancing PP2A activity with the PP2A activator, $AAL_{(S)}$, suppressed AHR in HDM-induced AAD and rhinovirus-induced exacerbations of AAD [248, 249]. Our work adds to the current body of knowledge by comparing the effects of two different PP2A activators in AAD. We demonstrate for the first time that $AAL_{(S)}$ is superior to FTY720 in suppressing hallmark features of AAD such as tissue eosinophils and inflammation, Th2-associated cytokines, MSC numbers and AHR. Importantly, $AAL_{(S)}$ also suppressed collagen deposition around the airways in a chronic experimental model of AAD. These observations could be attributed to the specificity of $AAL_{(S)}$ in activating PP2A and its ability to avoid lymphocyte trafficking as it is devoid of SIPR1 agonism unlike FTY720 [259, 266, 500].

6.3.2 Inhibiting proteasome activity as a potential therapy for asthma

Homeostasis in mammalian systems is maintained through the degradation of proteins by the UPS. [274, 412]. Dysregulation of the UPS has been implicated in cancer, and more recently in asthma, suggesting that altering the activity of the UPS may have

beneficial effects [280, 289, 422]. The studies on the use of proteasome inhibitors in asthma are very limited. One study demonstrated reduced infiltration of eosinophils into the lung in a rat model of OVA-induced pulmonary eosinophilia using the proteasome inhibitor PS-519 [292]. Another study demonstrated the use of BORT in a chronic OVA mouse model, where administration of a high dose reduced OVA-specific IgE, but not airway inflammation or AHR [424]. These studies did not determine the effects of the inhibitors on other features of AAD such as MSC numbers, Th2 cytokines or remodelling [292, 424]. In our study, we demonstrate for the first time that treatment of allergic mice with the proteasome inhibitor, BORT, suppressed some features of AAD including eosinophil infiltration into the lungs, levels of IL-5 and IL-13 and AHR. Importantly, treatment also suppressed collagen deposition around the airways in a chronic experimental model of AAD. However, treatment did not reduce levels of IL-33, TSLP, serum IgE or MSC numbers. The suppression of only certain features of AAD could be partly due to the lack of specificity of BORT and the dose used in our study. Others have demonstrated that the suppression of dextran sodium sulfate-induced colitis in mice was dependent on the dose of BORT used [425]. An intermediate dose of BORT (0.2 mg/kg) was used in our study, while a higher dose (0.75 mg/kg) was used in the previous study that may explain why there was only a suppression in OVA-specific IgE and not inflammation and AHR, in contrast to our findings [424]. Nevertheless, our findings suggest that proteasome inhibition may be valuable as a potential therapeutic target for asthma.

6.3.3 Enhancing PP2A activity and inhibiting proteasome activity have synergistic protective effects on AAD

As both PP2A and the UPS have been implicated in the development of asthma, we hypothesised that enhancing PP2A and inhibiting the proteasome activity at the same time would have some synergistic effects on AAD. We are the first to show that treatment

of allergic mice with the PP2A activator, $AAL_{(S)}$, and inhibiting proteasome activity with BORT at the same time were able to reduce hallmark features of acute AAD and also airway remodelling in a chronic HDM model. While combined treatments suppressed many features of AAD similar to that of treatment with $AAL_{(S)}$ alone, combined treatment also suppressed BAL eosinophils, which was not affected with $AAL_{(S)}$ treatment alone. This suggest that combined treatments have a synergistic effect and is able to suppress additional hallmark features of AAD. Our findings provide valuable information for the development of a more efficacious treatment strategy targeting these two systems for asthma.

6.4 Enhancing TTP activity reduces the severity of CS-induced COPD and influenza virus infection

We demonstrated (chapter 3 and 4) for the first time that active TTP reduces the severity of experimental CS-induced COPD and influenza. In our model of experimental COPD, the presence of active TTP resulted in reduced pulmonary inflammation, airway remodelling, emphysema-like alveolar enlargement and improved lung function. Similarly, in our model of influenza virus infection, active TTP reduced viral load, enhanced antiviral responses and suppressed inflammation. Our studies highlight the importance of active TTP and suggest that activating TTP would be beneficial as a potential therapeutic approach in these respiratory diseases.

TTP is an anti-inflammatory molecule that regulates the mRNA decay of cytokines [297]. It is a RNA binding protein that plays a pivotal role in the post-transcriptional regulation of numerous genes, indicating that alterations in active TTP expression and activity could control the development and progression of multiple inflammatory diseases. The potential anti-inflammatory role of TTP has been primarily studied in rheumatoid arthritis, when it was first discovered that TNF-α, an important

cytokine in rheumatoid arthritis, was a TTP target in studies using TTP$^{-/-}$ mice [311]. However, the role of TTP in respiratory diseases remains poorly understood. We are the first to demonstrate that the presence of active TTP reduces the severity of experimental COPD and influenza using *Zfp36*$^{aa/aa}$ mice. We also explored the role of this molecule in AAD using our well established acute OVA and chronic HDM-induced AAD models as well as in a bleomycin-induced fibrosis model. However, we did not observe any protective effects in these models (data not shown in this thesis). We speculate that this may be due to the specific cell types that are involved in the different diseases. Neutrophils and macrophages are the predominant inflammatory leukocytes in COPD and the early stages of influenza virus infection, while eosinophils and lymphocytes are predominant in AAD and fibrosis models, respectively. Our findings suggest that enhancing TTP activity has more protective effects on models with neutrophil and macrophage dominant responses. In support of this, a recent study reported that enhancing stability of TTP mRNA protects mice against collagen antibody-induced arthritis and imiquimod-induced dermatitis, both models predominantly involve neutrophils as the major leukocytes [501]. It is likely that enhancing TTP activity is more effective in these models as several of the known TTP targets such as CXCL1, CXCL2 and TNF-α are all involved in neutrophil chemotaxis [502, 503]. Future work should explore the importance of enhancing TTP activity in models of neutrophilic asthma. This will further our understanding of the anti-inflammatory role of TTP in disease contexts.

Our studies may have also identified new targets for TTP. We observed reduced levels of several factors such as MMP-12 and fibronectin, which have not been previously identified as TTP targets. Further investigations will be required to determine if these factors are indeed TTP targets or whether these decreases are the result of indirect effects.

6.4.1 Pharmacological regulation of TTP expression

Altered phosphorylation status of TTP determines its activity. It is important to note that it is the phosphorylation status of TTP, and not its expression that determines the anti-inflammatory outcome in an inflammatory environment. Increased expression of TTP has been observed in rheumatoid synovia and atherosclerotic plaques [504, 505]. Whilst there was increased TTP expression, there was still overproduction of cytokines leading to progression of disease.

The TTP levels measure in those studies could be postulated to be the inactive form. The presence of the inactive form of TTP stabilises cytokine mRNA and consequently lead to inflammation.

Therefore, altering the dynamic equilibrium between unphosphorylated (active) and phosphorylated (inactive) forms of TTP may be beneficial in multiple inflammatory diseases.

The phosphorylation status of TTP was not determined in our study due to the lack of a suitable antibody. However, we hypothesise that in disease contexts (e.g. COPD and influenza viral infection), the phosphorylated (inactive) form of TTP predominates, leading to the stabilisation of pro-inflammatory cytokine mRNA and hence expression of these cytokines. The converse is observed in the *Zfp36*$^{aa/aa}$ mice, where the phosphorylation of TTP on serines 52 and 178 is halted and therefore TTP cannot be phosphorylated and hence remains in the active form. This results in the degradation of pro-inflammatory cytokine mRNA and thus reduced inflammation and consequently disease features.

There are several potential ways of targeting the equilibrium and shifting the balance towards the unphosphorylated/active form of TTP, although these strategies are

not specific for just TTP. Firstly, inhibiting the p38 MAPK pathway should theoretically prevent the phosphorylation of TTP and shift the equilibrium towards the active state. Conflicting evidence exists in the literature in regard to p38 inhibition and TTP activity. Mahtani *et al*., demonstrated that inhibition of p38 activity by SB203580 in LPS-stimulated RAW264.7 prevents the induction of TTP mRNA and protein [506]. However, Carballo *et al*., demonstrated that the p38 inhibitors SB203580 and SB220025 decreased the levels of LPS-stimulated TNFα in BMM by activating TTP [342]. The authors were able to show that the inhibitors reduced the levels of phosphorylated TTP by half, suggesting that minimal levels of unphosphorylated TTP may exist in the system, hence the destabilisation of TNF-α in BMMs. This supports the notion that even a small shift towards the active form of TTP may have strong anti-inflammatory effects. This was further confirmed by our collaborators who showed low expression of inflammatory mediators in TTP heterozygous mice ($Zfp36^{+/aa}$) [356]. Nevertheless, despite the evidence for potential beneficial effects, the use of these inhibitors has had minimal effects in clinical settings [507, 508].

The second approach is by increasing the activity of PP2A, which dephosphorylates TTP and therefore downregulates the expression of pro-inflammatory cytokines. Recently, it was demonstrated that the PP2A activators FTY720 and $AAL_{(S)}$ suppressed the levels of TNFα-induced IL-8 and IL-6 in A549 lung epithelial cells [256]. This was postulated to be due to the ability of the PP2A activators to shift the equilibrium towards the active state of TTP.

The third approach would be to suppress UPS activity. As the active form of TTP is unstable and prone to degradation by the UPS [347], preventing the degradation of unphosphorylated TTP would favour the degradation of pro-inflammatory cytokine mRNA. Treatment of RAW 264.7 with MG-132, an inhibitor of the 20S/26S proteasome

increased TTP protein levels by preventing its degradation [346]. Enhancing PP2A activity and preventing the degradation of TTP provide alternate means to targeting p38 MAPK, which would shift the equilibrium to favour the active form of TTP. However, as PP2A is an abundant protein phosphatase with diverse functions, enhancing PP2A activity could have off-target effects. Similarly, inhibiting proteasome activity with proteasome inhibitors tends to be non-specific and could also have off-target effects. Therefore, the key would be in developing suitable drugs that could specifically alter the equilibrium between unphosphorylated and phosphorylated TTP.

6.5 Future directions

6.5.1 Further investigations of the role of RelB in the spontaneous development of obstructive lung disease

We have made important observations that RelB deficiency promotes the spontaneous development of AAI independent of any allergen exposure. We have also shown that RelB-sufficient DCs can ameliorate the features of AAI, highlighting the importance of RelB in DCs (chapter 2). Our collaborators have previously demonstrated RelB deficiency in DCs from type 1 diabetes patients [509]. Determining RelB DNA binding activity in our future work would give us an indication if there is RelB deficiency in DCs from asthmatic patients. Our study did not determine how RelB deficiency caused the development of spontaneous features of AAI. It is likely that there is an alteration in the microbiome of these mice that ultimately lead to aberrant immune responses that may underlie AAI. Microbiome analysis of the different organs from these mice would provide an indication of the involvement of microorganisms in the spontaneous development of airway inflammation. Determining RelB expression in human lung tissue from asthmatic patients would also provide information on the potential causal role of RelB in allergic

asthma. There are no array data available from DCs from asthmatic patients to interrogate for these factors. In addition, gene editing technology such as CRISPR/Cas 9 could be utilised to delete RelB gene in human samples *in vitro* and determining if the lack of RelB leads to the development of allergic asthma.

We made novel observations that lung structure and function were altered in heterozygous $RelB^{+/-}$ mice despite the lack of inflammation, suggesting that alterations in one allele could lead to changes in lung structure and function. Further studies will investigate how changes in one allele could predispose an individual to obstructive lung disease. One potential experiment would be to expose $RelB^{+/-}$ mice to our models of AAD or CS-induced COPD to determine if alterations in one allele could lead to the development of more severe asthma and COPD.

6.5.2 Further investigations of the mechanisms of how enhancing PP2A activity and inhibiting proteasome activity suppresses AAD

We showed that enhancing PP2A activity with $AAL_{(S)}$ and inhibiting proteasome activity with BORT suppresses hallmark features of AAD. Our study did not determine the mechanism through which these immunomodulatory drugs suppressed features of AAD. Both PP2A and UPS have been shown to control the activity of NF-κB. Inhibition of PP2A with calyculin A increased NF-κB activity while proteasome inhibition with inhibitors such as MG-132 and BORT reduced NF-κB activity [253, 431, 432]. Thus, it is likely that enhancing PP2A activity with $AAL_{(S)}$ and inhibiting proteasome activity with BORT could reduce NF-κB activity, and decrease features of AAD [253, 431]. Determining the activity of NF-κB in our models of AAD as well as with the different treatments would give us an indication if this pathway is involved in the suppression of disease features with those drugs.

6.5.3 Determine if enhancing PP2A activity and inhibiting proteasome activity is efficacious in steroid resistance asthma models

The lack of effective therapies in severe asthma patients that do not typically respond to steroids means there is an urgent need for alternative therapeutic approaches [510]. Given that we have shown improved outcomes in our acute and chronic models of AAD by enhancing PP2A activity and inhibiting proteasome activity, future studies will look into determining the effect of these drugs in our models of steroid resistant asthma [372]. Future studies should also look into the effect of these drugs on exacerbations [411].

6.5.4 Further investigations of the role of TTP and the mechanisms of protection in experimental COPD and influenza viral infection models

We made the novel observations that the presence of active TTP improved disease features in both experimental COPD and influenza. The phosphorylation status of TTP determines its functional activity. It is active in the unphosphorylated form, while it is inactive in the phosphorylated form [310, 348]. Our studies did not determine the phosphorylation status of TTP in our disease models due to the lack of specific mouse antibodies. Our collaborators have now optimised methods for measuring the phosphorylation levels of TTP and work is under way to apply these to our models. We are also currently looking into determining the levels of TTP in human COPD patients and *in vitro* studies using pBECS infected with influenza virus to identify if there are any alterations in TTP levels. This will further strengthen our *in vivo* work and highlight the importance of TTP as a potential therapeutic target.

To further explore the mechanisms of how TTP is involved in the protection against the development of disease features, we will measure the mRNA stability of the different cytokines and chemokines in our models of experimental COPD and influenza. This will confirm that TTP can induce the decay of mRNA cytokines and chemokines

involved in those models. At the same time, we could potentially identify novel targets of TTP, which have not been previously reported, for example using microarray analysis of mRNA expression.

We have yet to determine the cellular sources of TTP in our study. Previous studies have reported increased production of TNF-α upon stimulation with LPS from BMMs and peritoneal macrophages from $TTP^{-/-}$ mice, confirming that TTP is expressed by macrophages [312, 436]. To determine the cells that express TTP and where these cells are localised, we will assess cellular expression by flow cytometry and determine tissue localisation by immunohistochemistry or immunofluorescence.

The balance between phosphorylated and unphosphorylated forms of TTP is important in determining its activity. This equilibrium could be manipulated by targeting the different pathways that regulates the phosphorylation of TTP. As PP2A is responsible for dephosphorylating TTP, and hence the ability to degrade mRNA cytokines, enhancing the activity of PP2A would shift the equilibrium towards the active form of TTP [310]. We will explore the role of enhancing PP2A activity using PP2A activators such as $AAL_{(S)}$ in our models of experimental COPD and influenza. Mice will be treated with $AAL_{(S)}$ during the course of disease and also after the establishment of disease features to see if there are any effects on disease features and if so are they directly linked to TTP. As the active form of TTP is not stable and subjected to degradation by the UPS, we will also treat mice with the proteasome inhibitor, BORT, to prevent the degradation of TTP. In theory, mice that are treated with both $AAL_{(S)}$ and BORT would have the active form of TTP that is stable. This would in turn lead to suppression of disease features observed similar to that in $Zfp36^{aa/aa}$ mice.

6.5.5 Assess the role of TTP in other diseases

We have shown the potential involvement of the PI3K signalling pathway as a potential mechanism by which TTP controls influenza viral infection in our study. Increased PI3K activity has been demonstrated in COPD exacerbation as a result of influenza virus infection [141]. An addition to further our study and identify the significance of PI3K/Akt pathway would be to subject the *Zfp36*$^{aa/aa}$ mice to our COPD exacerbation model, where mice will be exposed to cigarette smoke for 8 weeks and infected with influenza virus at the end of smoke exposure. This would further determine the effects of active TTP in exacerbations and at the same time determine the links between TTP and the PI3K/Akt pathway.

Given that we observed reduced disease severity in experimental COPD and influenza as a result of enhancing TTP activity, we will further explore the role of TTP in bacterial infections. We will subject the *Zfp36*$^{aa/aa}$ mice to our established *Streptococcus pneumoniae* and *Heamophilus influenza* respiratory and *Chlamydia muridarum* reproductive tract infection models and determine disease outcomes.

6.6 Conclusion

In conclusion, our studies have identified several molecules and pathways that could be targeted as potential therapies for respiratory diseases. Our studies demonstrate that RelB deficiency promotes the development of spontaneous AAI and airway remodelling, suggesting that loss of RelB function may lead to allergic-like airway inflammation and obstructive lung disease. We discovered that RelB-sufficient DCs can ameliorate disease outcomes. Our studies also demonstrate the importance of PP2A and UPS in AAD and that enhancing PP2A activity and inhibiting proteasome activity using immunomodulatory drugs is able to suppress features of AAD. Finally, we made the novel

observation that active TTP reduces the severity of experimental COPD and influenza, highlighting the potential of active TTP as a therapeutic target in these diseases. Collectively, our studies have extended our understanding of the pathogenesis and potential therapeutic approaches for asthma, COPD and influenza. The major limitation of the current studies is the lack of validation of the importance of the different molecular entities in human samples. While we show promising findings experimentally, we have yet to explore this in human tissues. This will be the next critical step to take these promising findings to the clinical disease in question.

7.0 References

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