Exploring the role of early life respiratory infection in asthma

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ABSTRACT

The underlying cause of asthma is yet to be determined. Asthma is presently the most common chronic disease affecting children, and it is becoming clear that disease originates in early life and as a result of complex synergistic interactions of various environmental exposures. There has been increasing interest in the role of neonatal infections; with evidence emerging that asymptomatic pneumococcal colonisation is a strong predictor of future asthma, which can be exacerbated by viral infection. It is therefore paramount that appropriate models of disease are developed in order to elucidate these complex immunological interactions that have profound pathogenic potential during this window of lung development. Importantly, small airways dysfunction is present in the majority of asthmatics, and remains relatively undertreated by existing therapy, contributing considerably to the severity of disease. The lung slice technique is a powerful *in vitro* tool that can be used to explore airway and vascular pharmacology, offering a unique experimental link between cell-based assays and *in vivo* experimentation. Methodological studies compared airway reactivity in lung slices in varying experimental conditions, and application of the lung slice technique to characterize reactivity following acute respiratory infection *in vivo*, permitting application of this technique within the rest of this thesis. Utilising novel mouse models of asthma, parameters of bronchial reactivity, lung immunity and structural changes were assessed in adulthood following exposures in infancy, elucidating key mechanisms driving the deleterious effects caused by early life exposures. Using a mouse model of neonatal respiratory co-infection, the effect of early life co-infection with *Streptococcus pneumoniae* (SP) and influenza A virus (IAV) on mouse lung health, immunity and structure in adulthood was investigated. Co-infection caused a significant increase in central airway resistance that was not associated with conventional airway remodelling such as mucus overproduction, smooth muscle thickening or epithelial leakage. Airways hyperresponsiveness (AHR) was not maintained in lung slices *in vitro*. An increase in hysteresivity was
observed in both IAV and co-infected mice. Hence, it is possible that co-infection in infancy is causing distinct structural changes that contribute to ventilation heterogeneity, which persists into adulthood. A novel model of early life co-infection combined with house dust mite (HDM) aeroallergen challenge was developed and characterised. Neonatal co-infection in the background of HDM sensitisation resulted in chronic lung colonisation, exacerbated neutrophilic inflammation, increased mucin production and increased AHR. Hence, pneumococcal colonisation does not protect against allergic airways disease which is predominately driven by mechanisms that are independent of T helper 2 immunity. This study identified SAA, IL-17A and G-CSF as molecular markers for this phenotype, which parallels features of more severe asthma. Taken together, the strong neutrophilic/T helper 17/SAA signal, accompanied by AHR and mucus hyper-secretion generated in the present study in early life co-infection plus HDM group suggests this model may be capturing key features of severe, steroid insensitive asthma, providing novel insight into clinically relevant pathology. Ultimately, this model presents a unique opportunity to develop new treatment strategies to circumvent a highly relevant cause of childhood asthma.
DECLARATION

I, Meaghan FitzPatrick, certify that:

i. the thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the Preface,
ii. due acknowledgement has been made in the text to all other material used,
iii. the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Signature: ___________________________ Date: ____________
PUBLICATIONS ARISING FROM THIS THESIS

Parts of this thesis have been presented in a peer-reviewed format, as is listed below. Selected articles have been included as Appendices to this thesis.

PEER REVIEWED JOURNAL ARTICLES


CONFERENCE ABSTRACTS (SELECTED)

M FitzPatrick, S Royce, S Langenbach, P Reading, O Wijburg, GP Anderson, AG Stewart, JE Bourke & S Bozinovski (2016). Neonatal pneumococcal colonisation caused by Influenza A infection alters lung function in adult mice. Thoracic Society of Australia and New Zealand Annual Scientific Meeting, Perth, Australia
M FitzPatrick, S Royce, S Yatmaz, O Wijburg, P Reading, S Langenbach, AG Stewart, JE Bourke & S Bozinovski (2015). How does early life viral-induced pneumococcal infection impact the adult mouse lung? Victorian Infection & Immunity Network Young Investigator Symposium, Walter & Eliza Hall Institute, Melbourne, Australia

M FitzPatrick, J McQualter, S Yatmaz, O Wijburg, P Reading, S Langenbach, AG Stewart, JE Bourke & S Bozinovski (2015). How does early life viral-induced pneumococcal infection impact the adult mouse lung? Thoracic Society of Australia and New Zealand Annual Scientific Meeting, Gold Coast, Australia

M FitzPatrick, J McQualter, S Yatmaz, O Wijburg, P Reading, S Langenbach, AG Stewart, JE Bourke, & S Bozinovski (2014). How does early life viral-induced pneumococcal infection impact the adult mouse lung? Australasian Society for Experimental Pharmacologists and Toxicologists, Melbourne, Australia


M FitzPatrick, CE Wright & JE Bourke (2014). Endothelin-1-mediated contraction of intrapulmonary airways and arteries in rat lung slices. Thoracic Society of Australia and New Zealand Annual Scientific Meeting, Adelaide, Australia

M FitzPatrick, CE Wright, JE Bourke (2013). Visualisation of endothelin-1–mediated contraction of rat airways and arteries and mouse airways in situ using lung slices. Australasian Society for Experimental Pharmacologists and Toxicologists, Melbourne, Australia
M FitzPatrick, CE Wright, JE Bourke (2013). Characterization of endothelin-1-mediated contraction in intrapulmonary airways and arteries in rat lung slices. Pulmonary Hypertension Society of Australia and New Zealand Annual Scientific Meeting, Melbourne, Australia

M FitzPatrick, C Donovan, M Simoons, J Esposito, JE Bourke (2013). Rosiglitazone, but not salbutamol, opposes contraction to diverse agonists in small airways in mouse lung slices. Thoracic Society of Australia and New Zealand Annual Scientific Meeting, Darwin, Australia
**Preface - Contribution of Others**

In order to present a cohesive set of experiments, the following people contributed data sets to this thesis.

The image of inflated rat lungs in Figure 3.4 was provided by Ms Maggie Lam. Phase contrast images, methacholine (MCh) representative trace and grouped data for MCh and serotonin presented in Figure 3.6 were performed by Dr Chantal Donovan.

qRT-PCR on interleukin (IL)-5, IL-13, IL-33 presented in Figure 4.6 was performed by Mr Selcuk Yatmaz from previously generated cDNA.

qRT-PCR for *Streptococcus pneumoniae* in lung tissue presented in Figure 5.11 and qRT-PCR for expression of IL-17 in Figure 5.21 was performed by Dr Hao Wang. Quantification of α-smooth muscle actin-positive stain area in Figure 5.14 and blinded scoring of inflammation in haematoxylin & eosin sections in Figure 5.18 was performed by A/Prof Steven Bozinovski.
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COMMON ABBREVIATIONS

A list of common abbreviations used throughout this thesis is provided below.

5-HT 5-hydroxytryptamine; serotonin
αSMA α-smooth muscle actin
AAD allergic airways disease
Ab-PAS Alcian blue – Period Acid Schiff
AC adenylate cyclase
AECOPD acute exacerbation of chronic obstructive pulmonary disease
AHR airways hyperresponsiveness
ANOVA analysis of variance
ASM airway smooth muscle
BAL bronchoalveolar lavage
BALF bronchoalveolar lavage fluid
Ca\(^{2+}\) calcium ion
[Ca\(^{2+}\)]\(_i\) intracellular calcium
cAMP cyclic adenosine monophosphate
CFU colony forming units
COPD chronic obstructive pulmonary disease
CQ chloroquine
CXCL1/2 chemokine (C-X-C motif) ligand 1 or 2
DC dendritic cell
DMEM Dulbecco’s Modified Eagle Medium
DMF dimethyl formamide
DMSO dimethyl sulphoxide
EC-SOD extracellular superoxide dismutase
ET\(_{A/B}\) endothelin receptor A/B
Et-1 endothelin-1
FCS fetal calf serum
FOT forced oscillation technique
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>h</td>
<td>hour(s)</td>
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<tr>
<td>HA</td>
<td>histamine</td>
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<tr>
<td>HBA</td>
<td>horse blood agar</td>
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<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<tr>
<td>HDM</td>
<td>house dust mite</td>
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<tr>
<td>H&amp;E</td>
<td>haemoxylon &amp; eosin</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>IAV</td>
<td>influenza A virus</td>
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<tr>
<td>iBALT</td>
<td>inducible bronchus-associated lymphoid tissue</td>
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<tr>
<td>ICS</td>
<td>inhaled corticosteroid</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ILC2</td>
<td>innate lymphoid cell type 2</td>
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<tr>
<td>ISO</td>
<td>isoprenaline</td>
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<tr>
<td>K+</td>
<td>potassium ion</td>
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<tr>
<td>l</td>
<td>litre(s)</td>
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<tr>
<td>LRI</td>
<td>lower respiratory tract infection</td>
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<tr>
<td>m</td>
<td>milli-</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>MCh</td>
<td>methacholine</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cell</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
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<tr>
<td>MLC</td>
<td>myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
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<tr>
<td>MLCP</td>
<td>myosin light chain phosphotase</td>
</tr>
<tr>
<td>MT</td>
<td>Masson’s trichrome</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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</table>
Muc5AC/B mucin markers
N/A not applicable
NO nitric oxide
NS not significant
o/n overnight
OVA ovalbumin
PA pulmonary artery
PAH pulmonary arterial hypertension
PBS phosphate buffered saline
PCLS precision cut lung slice
PFU plaque forming units
PGI₂ prostacyclin
PKA protein kinase A
PLC phospholipase C
PPARγ peroxisome proliferator-activated receptor γ
qRT-PCR quantitative reverse transcription polymerase chain reaction
RGZ rosiglitazone
ROK Rho-A associated kinase
RSV respiratory syncytial virus
RT room temperature
RV rhinovirus
RVen right ventricle
s second(s)
SAA serum amyloid A
SAL salbutamol
SEM standard error of mean
SERCA sarco/endoplasmic reticulum Ca²⁺-ATPase
SOD superoxide dismutase
SOD KO superoxide dismutase knockout
SP Streptococcus pneumoniae
SR sarcoplasmic reticulum
Stfpc/d pulmonary-associated surfactant protein C or D
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>TNFβ</td>
<td>tumour necrosis factor β</td>
</tr>
<tr>
<td>$T_{\text{H}}1/2$</td>
<td>T helper cell type 1 or 2</td>
</tr>
<tr>
<td>Trp63</td>
<td>transformation related protein 63</td>
</tr>
<tr>
<td>$T_{\text{reg}}$</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>VSM</td>
<td>vascular smooth muscle</td>
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<tr>
<td>μ</td>
<td>micro</td>
</tr>
<tr>
<td>ZO-1</td>
<td>intracellular tight junction protein</td>
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</table>
CHAPTER 1

General Introduction
1.1 Overview

This thesis is divided into six chapters.

Chapter 1 is the General Introduction that discusses asthma broadly, and covers disease mechanisms, the involvement of small airways and treatment strategies, as well as severe disease, exacerbation and early life origins of asthma. Experimental models of asthma and selected measurements of airway reactivity are also discussed.

Chapter 2 is the General Methods used throughout this thesis.

Chapters 3 to 5 are results chapters, comprising comprehensive evaluation of lung slice methodology and experimental applications in rats and mice (Chapter 3). The impact of early life respiratory bacterial and viral co-infection on adult mouse lung health is explored (Chapter 4), and a novel model of neonatal co-infection and aeroallergen sensitisation is characterised (Chapter 5).

Chapter 6 is the General Discussion, which summarises the key findings of this thesis, and how this has provided novel insight into the field of asthma research, also discussing the inherent limitations of these studies and suggested future directions of this work.

Some components of this thesis have been published as original articles or as conference papers, as listed in the preceding section of this thesis. Additionally, one journal article originating from research presented in this thesis is included in the appendix.
1.2 Asthma

Asthma is a chronic inflammatory disease of the lung that can affect people of all ages whereby the airways contract ‘too easily or too much’ to a wide range of stimuli (Woolcock et al., 1998). Asthma affects 1 in 10 adults, however prevalence rates of asthma are historically high in Australia particularly, with recent estimates of doctor-diagnosed asthma in up to 21% of Australian adults (To et al., 2012). Asthma is the most common chronic disease in children, however prevalence is variably reported as 9 – 17 % (Centers for Disease Control & Prevention, 2011). There are an estimated 235 million sufferers of all ages worldwide and 250,000 asthma-related fatalities were recorded in 2005. At the 61st World Health Assembly, WHO Director-General Dr Margaret Chan stated in her opening address that asthma is “on the rise everywhere” (Hurd & Minelli, 2008).

An acute asthma attack is symptomatically characterised by breathlessness, wheeze, coughing and chest pain that can be triggered by exposure to inhaled allergens or irritants, during exercise, stress or cold weather. Asthmatic lungs are chronically inflamed and hyper-responsive, which collectively occur in conjunction with airway remodelling processes and structural changes in the lung. There are three major components to airway remodelling in asthma namely; i) airway smooth muscle hyper-proliferation leading to excessive contractile forces in response to constricting agents, ii) goblet cell hyperplasia and mucous hyper-secretion occurring in moderate/severe disease that can completely occlude small airways and iii) collagen deposition around airways leading to stiff fibrotic airways that are insensitive to bronchodilator therapy (Holgate et al., 2015). The relative contribution of each remodelling process in the overall pathophysiology of asthma remains unknown and varies greatly between patients (Holgate, 2010).
The underlying cause of airway remodelling in asthma remains poorly understood, however it is generally accepted that disease initiation relies on complex genetic and environmental interactions. Environmental factors such as exposure to pollution, allergens, chemicals or cigarette smoke have all been linked to asthma risk (Salam et al., 2004). Genetic studies remain elusive; there are 25 genes that have been associated with asthma across multiple populations, with >100 genes noted in smaller populations, suggestive that asthma is a remarkably heterogeneous condition (Ober & Hoffjan, 2006).

Clinically, measurements of lung function are used as a measure of asthma severity, most commonly FEV$_1$ readings (maximal amount of air forcefully exhaled in 1 s, converted to % of normal/predicted). Mild-moderate asthmatics experience asthma symptoms multiple times per week with intermittent exacerbations, and display lung function at FEV$_1$ <80% predicted. Severe asthmatics experience symptoms daily and often experience exacerbations, associated with FEV$_1$ <60% predicted (severe asthma is discussed in more detail in 1.2.5) (Enright et al., 1994). Currently, asthma is unpreventable and incurable, but lung function can be improved with asthma therapies, including bronchodilator agents, such as β$_2$-adrenoceptor agonists, and anti-inflammatory glucocorticosteroids.

### 1.2.1 Asthma phenotypes

It is now recognised that asthma is a remarkably heterogeneous condition, and has been divided into multiple disease phenotypes based on observable characteristics that describe the differing clinical and inflammatory profiles (Wenzel, 2012). Asthma was historically divided simply into extrinsic (atopic/allergic) and intrinsic (non-allergic) classifications (Rackemann, 1947). Atopy describes a genetic predisposition to a heightened immune response, and atopy was associated with extrinsic, allergic types of asthma. Intrinsic asthma developed later in life and was typically not associated with atopy. Critically, the concepts of immunity and the discoveries around the
crucial role of T helper (Th) immune pathways (discussed in 1.2.2) in allergic and inflammatory diseases led to asthma being widely defined as an allergic, eosinophilic, Th2-mediated, steroid-sensitive disease (Grünig et al., 1998; Leckie et al., 2000). Additionally, it was found that levels of Th2 cytokines were comparable between the extrinsic and intrinsic phenotypes, and that both were similarly responsive to corticosteroids, and so these classifications were no longer sufficient to describe this complex disease (Humbert et al., 1996).

Table 1.1 Asthma phenotypes in relation to characteristics.

Definitions of distinct asthma phenotypes are continually evolving however can be differentiated according to clinical features, pathology & biomarkers and response to therapy. Abbreviations: IgE, immunoglobulin-E; IL, interleukin; Th, T helper. Adapted from Wenzel 2012.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Clinical features</th>
<th>Pathology &amp; biomarkers</th>
<th>Response to therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-onset allergic</td>
<td>Allergic symptoms</td>
<td>Specific IgE, Th2 cytokines, thickened subepithelial layer</td>
<td>Corticosteroid-responsive; Th2 targeted</td>
</tr>
<tr>
<td>Late-onset eosinophilic</td>
<td>Sinusitis; less allergic</td>
<td>Corticosteroid-refractory eosinophilia; IL-5</td>
<td>Responsive to IL-5 therapy; steroid refractory</td>
</tr>
<tr>
<td>Obesity-related</td>
<td>Very symptomatic; adult onset</td>
<td>Lack of Th2 biomarkers; oxidative stress</td>
<td>Responsive to weight loss and antioxidants</td>
</tr>
<tr>
<td>Exercise-induced</td>
<td>Mild, intermittent with exercise</td>
<td>Mast-cell activation, Th2 cytokines</td>
<td>Responsive to β agonist therapy</td>
</tr>
<tr>
<td>Neutrophilic</td>
<td>Poor lung function, air trapping</td>
<td>Sputum neutrophilia, Th17 pathways</td>
<td>Possible responsive to macrolide antibiotics</td>
</tr>
</tbody>
</table>

The definition and characterisation of asthma phenotypes are continually evolving, and requires a consistent natural history of disease, consistent clinical and physiological characteristics, and identifiable biomarkers underlying pathobiology with predictable responses to general and specific therapies. (Error! Reference source not found.). Currently, asthma phenotypes typically encompass; early-onset allergic, non-allergic, late-onset
eosinophilic, exercise-induced, obesity-related, and neutrophilic asthma, but may also include subgroups of non-allergic or asthma with fixed airflow limitation (Global Initiative for Asthma, 2016). The age of onset of asthma appears to be a critical determinant of disease phenotype, as those under 12 at onset typically exhibiting allergic asthma (Miranda et al., 2004). Allergic asthma is associated with past history of other atopic disease, with sputum eosinophilia responding well to inhaled corticosteroid (ICS) therapy. Late-onset asthma is usually not associated with atopy, and is often steroid insensitive. Asthma owing to obesity has prominent respiratory symptoms with little evidence of airway eosinophilia. Additionally, asthma with fixed airflow limitation occurs in patients with long-standing disease with excessive airway wall remodelling. However, no present system of subgrouping can achieve all the requirements for a ‘true’ phenotype, and there are numerous co-morbidities and confounders that can alter asthma phenotypes such as smoking history, hormonal influences, occupational exposures and respiratory infections (Wenzel, 2012). The importance of Th2 type processes have been described across multiple phenotypes of asthma with varying severity, however non-Th2 asthmatics are likely to represent a large proportion of patients, who are more likely to be steroid insensitive (Wenzel, 2012).

More recently, clinical definitions of asthma in terms of disease endotypes have been proposed to overcome the heterogeneity of the clinical disease phenotypes. Endotypes, which describe distinct pathobiological mechanisms, involve patients being stratified on the basis of dominant molecular pathways underlying their disease, rather than clinical and observational traits without mechanistic implication (Anderson, 2008). The proposed endotype definitions of asthma differ from the currently described phenotypes, in that one asthma phenotype may contain multiple distinct endotypes and vice versa (Lötvall et al., 2011). Whilst their definitions and specific classifications remain limited, endotypes may provide a better
conceptual framework than phenotypes to guide asthma research and develop novel targeted treatment strategies (Agache, 2013; Corren, 2013).

1.2.2 \textit{T}_{\text{H}2} inflammation in asthma

The pathogenesis of asthma is largely attributed to chronic airway inflammation, which is involved in driving key features of disease including airway and vascular remodelling processes, and non-specific bronchial hyper-reactivity. Chronic lung inflammation is seen in allergic and non-allergic asthma.

Asthmatic inflammation is primarily coordinated by T-helper type 2 (\textit{T}_{\text{H}2}) cells, eosinophils, group 2 innate lymphoid cells (ILC2), basophils, mast and dendritic cells (Figure 1.1) (Wenzel, 2012). T cells are involved in the adaptive immune response, and play a key role particularly in atopic asthma. Naïve T cells can proliferate into \textit{T}_{\text{H}0} cells upon recognition and verification of an allergen presented by a dendritic cell, which then differentiate into \textit{T}_{\text{H}1} or \textit{T}_{\text{H}2} cells depending on the cytokine environment. \textit{T}_{\text{H}1} cells secrete interleukin (IL)-2, interferon (IFN)-\gamma and tumour necrosis factor (TNF)-\alpha. \textit{T}_{\text{H}2} cells release multiple cytokines, including IL-4, IL-5, IL-13 and granulocyte macrophage colony-stimulating factor (GM-CSF), which stimulate type 2 immunity. Release of these factors (IL-4, -5, -13) orchestrates a cellular inflammatory cascade that encompasses \textit{T}_{\text{H}2} cell survival, mucus secretion, fibrosis, smooth muscle effects, mast cell activation and growth, eosinophilia, serum antibody production and basophil recruitment. In particular, IL-4 and IL-13 drive humoral immune responses that trigger allergy, via isotype switching of B cells for immunoglobulin-E (IgE) production that in turn drives atopy (Wenzel, 2012).

There is strong evidence that \~50\% of asthmatics across varying severity of disease display aspects of the classical ‘\textit{T}_{\text{H}2}-high’ phenotype, with heightened airway hyperresponsiveness (AHR), higher serum IgE, blood and airway eosinophilia, and evidence of more extensive airway remodelling compared
to ‘T_{h}2-low’ asthmatics, which display airway T_{h}2 cytokine levels indistinguishable from healthy controls (Woodruff et al., 2009). Eosinophilic asthma is defined when >2% of all inflammatory cells in a sputum sample are eosinophils. These cells play an effector role by releasing pro-inflammatory or cytotoxic mediators, which can then contribute to vascular leakage (oedema), mucus hyper-secretion, smooth muscle contraction, epithelial shedding and AHR, also initiating the remodelling processes by release of cytokines and growth factors (Bousquet et al., 2000; Rothenberg 1998).

ILC2s were recently identified as an alternative source of inflammatory type 2 cytokines that may be implicated in asthma pathogenesis. ILC2s are classified as non-B, non-T cell and do not express dendritic or myeloid markers (Licona-Limon et al., 2013). Epithelial cell-derived cytokines including IL-25 and IL-33 are released during tissue damage, pathogen recognition or allergen exposure, and these cytokines in turn stimulate ILC2s. Importantly, this process occurs in the absence of the adaptive immune system, and ILC2s generate T_{h}2 cytokines including IL-13 which plays a key role in asthmatic remodelling and AHR (Kim et al., 2012). Additionally, ILC2s have been found to represent a large proportion of the IL-5 and IL-13-producing cells in experimental models of asthma (Wolterink et al., 2012).

T_{h}17 cells were recognised as a functionally distinct from T_{h}1 and T_{h}2 T cell populations, which are also involved in inflammation via secretion of IL-17 (Ouyang et al., 2008). IL-17 is directly implicated in the recruitment and survival of neutrophils, evident in more severe forms of asthma that are associated with steroid insensitivity (Nembrini et al., 2009). Although there is still debate as to whether a true neutrophilic asthma phenotype exists, it is evident that neutrophils accumulate with increasing severity of disease (Kamath et al., 2005).

Regulatory T cells (T_{regs}) are involved in the homeostatic regulation of the immune responses. T_{regs} are able to suppress the damaging, pro-asthmatic
T\textsubscript{H}2, T\textsubscript{H}17 and B cells and regulate antigen presenting cell activity, however in the asthmatic state T\textsubscript{reg} display a significantly reduced capacity to exert this suppressive effect (Ling et al., 2004). For this reason, defective T\textsubscript{reg} may play an important role in the dysregulated immune responses involved in asthma initiation, progression and exacerbation (Thorburn & Hansbro, 2010).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{T\textsubscript{H}2 immunity in asthma.}
\end{figure}

The pathway is initiated by T\textsubscript{H}2 cell development and subsequent production of interleukin (IL)-4, IL-5 and IL-13. These cytokines stimulate eosinophilic inflammation, epithelial and airway smooth muscle changes, and development of allergy through production of immunoglobulin E (IgE), collectively driving asthmatic pathobiology. Additionally, the development of T\textsubscript{H}17 immunity drives neutrophilia evident in more severe asthma. Regulatory T cells (T\textsubscript{reg}) suppress damaging pro-asthmatic cellular activity
and may be defective in asthmatic patients. Innate lymphoid cells type 2 (ILC2s) are an alternative source of Th2 cytokines. Key: DC, dendritic cell; IFNγ, interferon γ; IgE, immunoglobulin E; IL, interleukin; ILC2, innate lymphoid cell type 2; TH, T helper cell; TNFβ, transforming growth factor β; Treg, regulatory T cell. Adapted from Wenzel, Nat Med 2012 (Wenzel, 2012).

1.2.3 Airway remodelling and airway hyperresponsiveness

Asthmatic airways undergo significant structural changes that are especially prominent in more severe/chronic disease (Figure 1.2). In asthma, the number of goblet cells is increased in proximal airways, and also evident in the peripheral airways where they are otherwise absent, collectively resulting in mucus overproduction and hyper-secretion. Goblet cell hyperplasia (increase in number of goblet cells due to cell proliferation) tracks with asthma severity; with more stored and secreted mucus in moderate asthma than is seen in mild disease (Ordoñez et al., 2001). Additionally, in severe cases, endobronchial mucus suffocation can occur resulting in fatality, where airways are completely occluded by viscous mucus plugging (Aikawa et al., 1992). The asthmatic lung is also hyper-vascularised, where there is proliferation of vessels and nerves (Holgate, 2008). Hyperplasia of airway smooth muscle (ASM) cells leads to a 3–4-fold increase in ASM bulk in asthmatic airways which is evident throughout both the proximal and distal lung (Hogg, 1993). Hypertrophy of ASM cells occurs in the large airways in both fatal and nonfatal asthma (James et al., 2012).

The epithelium represents an important physical barrier that is normally a highly regulated and relatively impermeable structure with tight junctional proteins maintaining cell-cell and cell-extracellular matrix interactions (Godfrey, 1997). The epithelium plays a fundamental role in asthma pathogenesis; it is particularly fragile, with evidence of epithelial damage and shedding in most asthmatics (Nelson et al., 2003). These changes contribute to greater epithelial permeability, allowing inhaled allergens, irritants, microbes and pollutants greater access to underlying tissue layers (Xiao et al., 2011), and also permits allergens enhanced antigen presentation to
dendritic cells (DCs). The epithelium itself can also worsen Th2 inflammation, as damaged barrier epithelial cells release danger-associated molecular patterns, amplifying the production of thymic stromal lymphopoietin, GM-CSF, IL-25, IL-33 which stimulate ILC2 proliferation and survival, and in turn their production of Th2 cytokines (Hammad & Lambrecht, 2015). The asthmatic epithelium is intrinsically abnormal, with evidence for an aberrant repair process following injury (Hackett & Knight, 2007), releasing epidermal growth factors that induce goblet cell metaplasia and contribute to mucus hypersecretion (Lambrecht & Hammad, 2012). Additionally, remodeling occurs at the sub-epithelial layer (or basement membrane) which is thickened by increased deposition of collagen and extracellular matrix proteins (Bai & Knight, 2005).

![Image](image.png)

**Figure 1.2 Airways in asthma undergo significant structural remodelling.**

Medium-sized airways from a tissue biopsy from normal patient (left) and a severe asthmatic patient (right) were stained with Movat’s pentachrome stain, showing mucus hyperplasia (blue) and increased smooth muscle volume (red). Compared to the normal airway, the severe asthmatic airway has undergone remodeling events collectively resulting in a significantly narrowed airway: mucus cell hyperplasia and excessive mucus deposition.
into the airway, thickened basement membrane, increased smooth muscle mass, and evidence of fibrosis. Key: Bv, blood vessel; Ep, epithelium; Bm, basement membrane; Sm, smooth muscle. Scale bar 100 μm. Image from (Wadsworth et al., 2011).

Whilst historically it was thought that inflammation was the underlying driver of asthma, both inflammation and airway remodelling are now proposed to occur in parallel, as both processes contribute to disease progression despite anti-inflammatory therapy. AHR and lung function are clinical measures of asthma severity, and have been linked to both airway inflammation and remodelling processes. Indeed, the interactions between the epithelium particularly and the immune system are likely to be major drivers of asthma initiation and pathology (Lloyd & Saglani, 2010). Whilst some T_{h}2 cytokines have potent remodelling properties, there is emerging evidence that remodelling is initiated in early stages of disease rather than as a consequence of inflammation. It has been shown that epithelial shedding and basement membrane thickening is evident in childhood asthma (Barbato et al., 2006), emphasizing the importance of the airway microenvironment in the disease (Nelson et al., 2003). Morphometry studies suggest that airway wall thickening in asthmatic airways could account for a large component of AHR in established disease (James et al., 1989; Wiggs et al., 1992a), whilst the acute inflammatory response following inhalation of allergen or exposure to virus is known to reduce lung function (Woolcock et al., 1984). Furthermore, non-eosinophilic asthmatic airways display comparable extent of remodelling (epithelial thickness, epithelial shedding and vascularisation) as that seen in eosinophilic disease (Baraldo et al., 2011), suggesting that neutrophils may also be contributing to remodeling processes.

1.2.4 Small airways dysfunction in asthma

Small airways are defined as non-cartilaginous airways with diameter < 2 mm in adults, extending from the 8th generation airways to the alveoli, accounting for 10% of the total lung volume. It has been known for some time that peripheral airway resistance is elevated in asthmatic patients
compared to healthy control, as assessed directly using wedged bronchoscopy (Wagner et al., 1998), however this ‘silent zone’ can bear extensive disease with little or no observable abnormality via conventional pulmonary function tests. Additionally, it has been shown that small airways exhibit more severe inflammation than large airways, with higher counts of activated eosinophils present in asthmatic airways with diameter < 2 mm (Hamid et al., 1997). Remodelling processes occur in the peripheral airways, and the increased ASM described in the small airways in both fatal and nonfatal asthma are also likely to contribute to asthma severity (Carroll et al., 1993; Wiggs et al., 1992b). Mucus plugging and closure of the small airways may limit aerosolised drug delivery, as the efficacy of β2-adrenoceptor agonists is relatively decreased in peripheral airways (Broadley, 2006; Finney et al., 1985).

With the use of advanced physiological and imaging techniques permitting direct investigation of the distal airways, the prevalence and extent of small airways disease in asthma can now be evaluated non-invasively (Usmani & Barnes, 2012). A current systematic review revealed that small airways disease has been identified in almost 60% of asthmatics, over multiple diverse populations, in patients with mild to severe asthma (Usmani et al., 2016). The small airways are clearly an important site of disease pathology, and their involvement may be a prerequisite for the development of asthma (Bjermer, 2014).

1.2.5 Severe Asthma

A subset of patients (5 – 10 %) suffer from severe asthma, accounting for ~ 450 deaths per year in Australia, consuming a disproportionate amount of healthcare costs associated with the disease [http://asthmaaustralia.org.au/Statistics.aspx]. Global estimates of severe, persistent disease range from 11 – 32 % depending on region (Rabe et al., 2004). Severe asthma is distinct from poorly controlled asthma where patients are non-compliant or undertreated with available therapies. Severe
asthma is often steroid insensitive and so is ultimately undertreated, with current therapeutic strategies unable to adequately manage disease in a number of patients (Barnes & Adcock, 1995; Broadley, 2006; Holgate & Polosa, 2006).

The inflammatory profile of the severe asthmatic lung can shift from persistent eosinophilia to neutrophilia, in the presence or absence of elevated eosinophils (Kamath et al., 2005). Importantly, neutrophilic airway inflammation is resistant to steroid therapy, and neutrophilia may actually be caused/exacerbated by steroid therapy as glucocorticoids inhibit neutrophil apoptosis (Cox, 1995). Clinically, the co-existence of neutrophils and eosinophils is more prominent in patients with worse asthma control, lower lung function and higher need for healthcare (Hastie et al., 2010). In addition, up to 50% of severe asthmatics display very little inflammation, so steroid insensitivity may occur at numerous levels, not just a lack of effect of steroids on inflammation (Wenzel, 2005). Severe disease is associated with fixed airflow obstruction as a distinctive characteristic, which cannot be relieved with bronchodilators (Abraham et al., 2003; ten Brinke et al., 2001). Remodelling is also more marked in severe disease, where a constant cycle of tissue injury and repair creates a chronic ‘wound status’ (Holgate et al., 2000). Production of mucus extends to the small airways, where its effects on lung function are likely to be substantial and small airway closure may occur. Additionally, lung elastic recoil is impaired by loss of alveolar-airway attachments (Holgate & Polosa, 2006).

1.2.6 Exacerbations

The chronic inflammation observed in asthma is punctuated by episodes of acute inflammatory events known as exacerbations. Acute exacerbations of asthma are a major cause of disease morbidity and mortality, and are defined as a worsening of symptoms lasting 3 days or more requiring additional treatment or healthcare intervention, with severe asthmatics exacerbating >2 times per year (Holgate & Polosa, 2006). Exacerbations impose a major
financial burden on health care providers, and have significant detrimental effect on patient quality of life. Respiratory infections, exposure to dust, animal dander or irritants can trigger acute exacerbations that are associated with peripheral airway dysfunction and manifests as debilitating breathlessness (Nicholson et al., 1993). Viruses play a key role in exacerbation of disease, and strains such as rhinovirus (RV), respiratory syncytial virus (RSV) or influenza can induce exacerbation particularly in severe disease, where they would otherwise have mild effect (Lambrecht & Hammad, 2015). This increased susceptibility to viruses may be due to impaired barrier function, epithelial fragility, or by defective antiviral immunity, and can continue to occur despite adequate control of the disease. Importantly, neutrophilic inflammation that can occur during viral exacerbation is poorly responsive to steroid treatment (Wark et al., 2001). The critical roles of respiratory viruses in asthma causation are discussed in 1.4.1.

1.3 Current treatments and novel strategies

There is presently no cure for asthma, and so therapy aims to control symptoms. The focus of asthma treatment is to reduce damaging inflammation using anti-inflammatory corticosteroids and to relieve symptomatic bronchoconstriction with β-adrenoceptor agonists (Barnes, 1989).

β-adrenoceptor agonists are used as reliever therapy in asthma management, where inhaled preparations can act directly on β2-adrenoceptors and relax ASM and elicit bronchodilation, without affecting underlying disease progression. Salbutamol (SAL) is a short-acting selective β2-adrenoceptor agonist used clinically. Isoprenaline (ISO) is a non-selective β-adrenoceptor agonist used experimentally as it may elicit non-selective cardiovascular actions via β1-adrenoceptors present on the heart (Broadley, 2006). These β-
adrenoceptor agonists are routinely used in assessment of altered dilator response in asthma studies in vitro (Chapter 3).

Glucocorticoids are the most potent anti-inflammatory controller treatment available for asthma, where they suppress immune responses and also inhibit the synthesis of inflammatory mediators, namely prostaglandins and leukotrienes. The beneficial anti-inflammatory effects of glucocorticoids in asthma therapy are largely mediated via their modulation of T\textsubscript{H}2 cytokines. Importantly, glucocorticoids can inhibit goblet cell hyperplasia and mucus hypersecretion driven by T\textsubscript{H}2 immunity, which is a major clinical feature of asthma (Kim et al., 2003). Their broad and non-specific actions can produce an unfavourable side effect profile, which is minimised in inhaled preparations that reduce systemic exposure. Patients with eosinophilia (or T\textsubscript{H}2-high asthma) generally respond well to glucocorticoid therapy although lack of compliance with medications can be a major cause of non-response to treatment in this phenotype of asthma. Cysteinyl leukotrienes are highly potent endogenous bronchoconstrictors that can also cause tissue oedema, eosinophil migration, airway secretion and ASM proliferation (O’Byrne et al., 2009). Cysteinyl leukotriene receptor antagonists, such as montelukast and zafirlukast, have been developed as alternative/add-on therapeutics however are less effective than corticosteroids, which remain the preferred therapy (Global Initiative for Asthma, 2016).

More recently, an anti-IgE monoclonal antibody omalizumab has been approved for asthma patients with severe allergic disease. Omalizumab decreases allergic responses and eosinophilia in the airways, improves pulmonary function, reducing asthma exacerbations, and permits lower doses of steroid co-therapy (Busse et al., 2001; Soler et al., 2001). However, the cost of omalizumab is high making this treatment inaccessible to many asthmatics. There is also a need to develop biomarkers that are predictive of treatment response to anti-IgE therapy given the expensive nature of this medication. Mepolizumab, an anti-IL-5 monoclonal antibody, has been
approved for use in multiple continents in patients with severe eosinophilic asthma. Administered in conjunction with steroid, mepolizumab reduces asthma exacerbations, eosinophilic inflammation and reliance on steroid therapy (Ortega et al., 2014; Pavord et al., 2012). Benralizumab, an anti-IL-5 receptor α antibody is presently in development and may provide additional, more specific eosinophil depletion than mepolizumab therapy (FitzGerald et al., 2016). The development of novel biologics continues to provide innovative treatment strategies for subgroups of asthmatics, however to date these agents have demonstrated limited effect on airway function, and so may be utilised as adjunct therapy (Fahy, 2015).

Collectively, these therapies aim to provide disease control, which can be critically assessed via Global Initiative for Asthma (GINA) Guidelines [available at http://ginasthma.org]. Clinically, asthma is controlled when patients demonstrate >80% predicted FEV₁, with fewer than two episodes of daytime symptoms and associated use of bronchodilator medications per week, with no activity limitations or nocturnal awakening. If any of these criteria are not met, a patient’s asthma is considered partially controlled, and is considered uncontrolled if several criteria are not met. More recently, there has been a shift in focus towards curative strategies and disease prevention; hence a better understanding of the origins of asthma in early life is paramount (Section 1.4).

1.4 Origins of asthma in early life

Although the underlying cause of asthma is incompletely understood, it is generally accepted that the asthma trajectory is entrenched in early life, and disease develops as a consequence of specific genetic and environmental exposures interacting during lung development (Martinez, 2007). Whether asthmatics are born with abnormally ‘small airways’, or if lung function is reduced as a consequence of inflammatory insult in early life remains to be fully elucidated (Martinez, 2009). Most adult asthmatics manifested
persistent wheeze and airway obstruction before the age of 6 years, suggesting a ‘developmental window of susceptibility’ during early life where normal growth is disrupted (Gern et al., 1999). Cohort studies have reported that children who experience repeated wheezy episodes in infancy have persistent asthma-like symptoms up to 7 – 10 years of age (Noble et al., 1997; Sigurs et al., 2000; Stein et al., 1999). Atopic sensitisation and/or severe lower respiratory tract infections (LRI) in the first years of life may induce wheezy illness, and have been identified as risk factors for asthma causation.

Strachan previously proposed the “hygiene hypothesis” to explain the increasing incidence of asthma and allergic disease (Strachan, 1989). This theory postulated that limited exposure to certain microbes (bacteria, virus) in early life resulted in insufficient stimulation of Th1 cells, polarising the immune systems towards Th2 and predisposing to allergy. Indeed, exposure to diverse microbes present in a farming environment, such as stables and unpasteurised farm milk in early life conferred protection against atopy, hay fever and asthma (Riedler et al., 2001). However, in a large cohort study, this protection was observed independent of atopic status (Fuchs et al., 2012). The hygiene hypothesis has been scrutinized over the years which have followed, and it has been proposed that there is no definitive evidence to support this theory (Brooks et al., 2013). It is now emerging the protective effects attributable to the hygiene hypothesis may be orchestrated by altering the gut microbiota, which can educate immune cells to dampen their response to allergens (Holgate et al., 2015). This is an area of developing research that will progress with technological and bioinformatics advances.

A causal relationship was postulated between exposure to allergen, specifically house dust mite (HDM), in early life and subsequent development of asthma (Sporik et al., 1990). This idea was strengthened by an Australian study correlating childhood asthma risk directly with the amount of HDM exposure (Peat et al., 1996). In more recent studies, however, this causal link has been challenged, as larger prospective cohort studies have reported no
effect of exposures to HDM on the development of asthma specifically, but rather suggests that sensitisation to allergen and development of atopy is key (Torrent et al., 2007). Furthermore, the European Network For Understanding Mechanisms Of Severe Asthma (ENFUMOSA) study reports mild asthmatics were more atopic than severe asthmatics, suggesting that allergen exposure alone does not contribute directly to disease severity (Abraham et al., 2003).

1.4.1 Early life respiratory infection

There exists a close epidemiological relationship between early life respiratory infection and wheeze, and viral infections have long been associated with the subsequent development of asthma. Viral aetiologies have been identified in 90% of nasal lavages from children suffering from wheeze, most commonly RSV and RV (Jackson et al., 2008). Wheezing episodes are very common in infancy, and milder viral infections are a benign prognosis for asthma development in later life (Kneyber et al., 2000; Martinez et al., 1995).

More severe viral LRI can lead to bronchiolitis or febrile events, which parallels many features of childhood asthma including inflammation and airway obstruction (Gern & Busse, 2002). In a cohort of high-risk Australian children, where one parent had respiratory allergy, early life viral infection lead to a 10-fold increased asthma risk in childhood (Jackson et al., 2008). Moreover, the frequency of severe viral LRI in early life continued to contribute to asthma risk at 5 and 10 years of age (Holt et al., 2010; Kusel et al., 2007). The Tennessee Asthma Bronchiolitis Study (TABS) of over 95,000 children reported a dose-response relationship between the severity of viral bronchiolitis in infancy and the increased risk of childhood asthma (Carroll et al., 2009). Furthermore, infants that had been infected with RSV in the first year of life possessed more reactive airways and slight bronchoconstriction at rest that persisted into teenage years (Sigurs et al., 2010; Sigurs et al., 2005). Severe respiratory viral infection within this ‘critical window’ of early
life lung development may predispose children to persistent asthmatic symptoms in later life, however, it remains to be elucidated whether infection in early life is the cause of wheezy illness and disease, or is merely unmasking a respiratory vulnerability (Postma & Koppelman, 2014; Sly et al., 2010).

Although the majority of childhood cohort studies investigate asthma risk in terms of early life viral infection, there is emerging evidence that bacterial pathogens may play a role as they also can cause wheezy illnesses. Pneumococcal infection is preceded by asymptomatic, often transient, colonization of the upper airways. Bacterial colonisation in neonates has been identified as risk factor for recurrent wheeze in early life (Bisgaard et al., 2007). A recent study of the infant nasopharyngeal microbiome identified asymptomatic Streptococcus pneumoniae (SP) colonisation specifically in the first months of life as a strong predictor of asthma (Teo et al., 2015). This is highly significant, as SP colonisation rates can be up to 80% in children under 5 years old (Adegbola et al., 2001). Additionally, an episode of bacterial pneumonia in early life is associated with reduced lung function and asthma extending into adulthood (Chan et al., 2015). Whilst microbial colonization during infancy plays an instrumental role in the development of the immune system, these early-life events can also disrupt host-commensal interactions and critically, contribute to the development of inflammatory diseases including asthma (Gensollen et al., 2016).

1.4.2 Respiratory co-infection

More recently, there has been increasing interest surrounding the role of bacterial and viral co-infection in early life. Birth cohort studies have repeatedly identified the presence of both bacterial and viral microbes in airway aspirates during acute wheezy episodes in young children (Bisgaard et al., 2010; Carlsson et al., 2015). Severe bacterial pneumonia following influenza infection has been well described, but the associations of more
clinically relevant viral strains in young children, such as RSV and RV, are less clear (Brealey et al., 2015; O’Brien et al., 2000).

A recent study found an elevated SP bacterial burden in nasopharyngeal samples from children hospitalized with RSV LRI compared to healthy counterparts (Rosas-Salazar et al., 2016), and there was high bacterial load in the lower airways in up to 40% of infants (Thorburn et al., 2006). These bacterial super-infections increase morbidity and mortality in the paediatric population, with longer hospital stays, increased requirement for ventilator support, and more frequent admissions to intensive care units. The severity of RSV infection has been directly associated with bacterial carriage, suggesting co-infection drives more damaging airway inflammation and subsequent hospitalisation (de Steenhuijsen Piters et al., 2016).

There is growing evidence for enhanced disease severity with co-infection, however the precise mechanisms by which viral and bacterial pathogens act synergistically is likely to be highly complex and possibly bidirectional, and the longer-term clinical consequences are yet to be fully appreciated (Brealey et al., 2015). This novel area of research has great potential, as both bacteria and viruses are capable of modulating the immature immune system, and it is therefore plausible that early life co-infection may play a significant role in asthma induction.

1.4.3 Respiratory infection and aeroallergen sensitisation

Holt & Sly have postulated that co-exposure to respiratory infection and allergen in early life presents a uniquely potent “two-hit” risk factor for asthma (Holt & Sly, 2012). Specifically, early life viral infection can interact with atopy and predispose infants to asthma, if exposure occurs within a critical period during early developmental. However, not all children who experience allergen sensitisation and wheezy illnesses develop asthma, suggesting a complex synergy of these inflammatory insults (Jackson et al., 2012; Kusel et al., 2007).
1.5 Experimental models of asthma

As there are still many aspects of asthma pathology and causation that are incompletely understood, animal models of disease represent an important biological tool for potential discovery of novel therapeutics or curative strategies. Given that there are significant ethical and logistical limitations in directly assessing disease parameters in humans, it is essential that animal models replicate key aspects of human asthma, namely airway inflammation, remodelling and bronchial hyperresponsiveness (Sagar et al., 2015). Most pre-clinical models of asthma utilise small rodents, most commonly mice, due to their low cost, ease of handling, short reproductive cycle, and well-characterised genome (Zosky & Sly, 2007). Whilst there are clear benefits with approximating human disease features in mouse models, it must be considered that the anatomy of the mouse lung does differ markedly from human. Firstly, the total lung capacity in a mouse is ~1 ml, compared to 6000 ml in humans. Architecturally, humans and mice both possess 5 lung lobes in total, however mice only possess a single lobe in the left lung whilst human is divided into two. The airway pattern in mouse lungs is monopodial, with 13 – 17 respiratory generations, compared with the dichotomous branching with 17 – 21 generations in human lung (Irvin & Bates, 2003). Nonetheless, mice provide relative benefit in terms of disease modelling as they can be genetically modified to delete, suppress or enhance specific pathways, enabling direct assessment of their mechanistic importance in an in vivo setting. Potential therapeutic agents can then be evaluated for efficacy in disease context. Furthermore, given the heterogeneity in human asthma, well-characterised experimental mouse models can offer unique insight into specific molecular drivers pertinent to distinct asthma phenotypes to advance targeted therapeutics in this setting.

An ideal animal model of T\(_h\)2 type asthma should demonstrate key features of disease, such as AHR and remodelling of airways, and immunologically an ideal model should display the classical T\(_h\)2 profile (elevated IL-4, -5 and -
13), with high levels of antigen-specific IgE, and eosinophilia (Holt et al., 1999). Since laboratory animals do not naturally develop disease in the absence of allergen exposure (Szelenyi, 2000), airway dysfunction must be initiated via administration of allergen, infection or a combination. ‘Asthmatic’ mouse models usually involve sensitization to an allergen (such as ovalbumin (OVA) or HDM), then repeatedly challenging the airways with the same allergen in order to elicit an allergic response. Respiratory viral infections represent an important contributor to exacerbations of severe asthma. Whilst it is primarily RV causing clinical asthma exacerbations, most serotypes of RV do not replicate in mice. Therefore, studies often employ influenza A virus (IAV) or RSV, as strains of these viruses with varying virulence do replicate in mice if the inoculum is sufficiently large, and these pathogens are still clinically relevant with respect to human asthma exacerbation (Starkey et al., 2013a). Additionally, the timing of exposure to allergens and infections is of importance. It is now generally accepted that early life events, namely exposure to allergen and infections, can disturb the developing lung such that asthma is initiated (Section 1.4). The specific immunological and structural alterations that these insults induced is still incompletely understood, and so it is important to develop mouse models of asthma that enable investigation of this critical period of neonatal development, when the immune system is naive. Specific allergic and infectious agents used in mouse models of asthma are described further in Results Chapters 4 and 5.

Animal models of asthma have yielded important insights into human disease. Of particular note was the identification of the role of Th2 cytokine IL-5 in a mouse model of asthma, where treatment with anti-IL-5 monoclonal antibody reduced inflammation (Shardonofsky et al., 1999). This led to the testing of benralizumab, an anti-IL-5 receptor α monoclonal antibody, which was able to reduce peripheral blood eosinophils in mild asthmatics, and has subsequently completed Phase III clinical trials in severe eosinophilic asthmatics (Busse et al., 2010; FitzGerald et al., 2016). Mechanistic studies
carried out in mice have also highlighted the importance of additional Th2 cytokines, IL-4 and -13, in the asthmatic inflammatory response (Brusselle et al., 1995; Kumar et al., 2002).

### 1.5.1 Measuring airway dysfunction in animal models of asthma - *in vivo*

As AHR is a primary characteristic of asthma, assessment of lung function is of particular importance and relevance in animal models of disease. Respiratory mechanics of the whole lung can be investigated *in vivo* in small animals such as mice by use of varied techniques, such as unrestrained plethysmography and forced oscillation (a comprehensive comparison of available *in vivo* lung mechanics techniques is reviewed elsewhere (Sly et al., 2004)). However, in these measures a compromise is made between precision and non-invasiveness, so as to achieve the most reliable data whilst simultaneously allowing the animal to remain in the most ‘natural’ conditions.

Plethysmography (also known as enhanced pause; Penh) involves placing the animal in a small, closed box and measuring pressure changes during normal respiration. These pressure changes are derived from the compression and decompression of gas within the thorax of the mouse, but can also occur from the humidification and warming of inspired air, which is not related to lung mechanics. This pressure change is a dimensionless readout with no physical meaning, which cannot be used to compare mice, nor as a baseline value for subsequent bronchoconstrictor challenge. Whilst this method is relatively simple way of assessing respiratory pattern, does not require intubation or anaesthesia, and permits full recovery in the mouse following measurements, its validity in assessing respiratory mechanics has been heavily criticised (Irvin & Bates, 2003; Lundblad et al., 2002).

Conversely, the forced oscillation technique (FOT) is highly invasive, yet is considered the gold standard in lung function testing as it provides sensitive, reproducible measurement of lung mechanics in anaesthetised,
tracheotomised mice (Vanoirbeek et al., 2010). FOT measures the imput impedance ($Z_{rs}$); the relationship between oscillatory pressure and flow measured at the airway opening; reflective of the dissipative and elastic properties of the lung (Sly et al., 2004). The animal is ventilated with a complex flow wave produced by a computer-controlled piston, such as is delivered by the Flexivent (SCIREQ, Montreal, PQ, Canada). FOT permits more complex modelling of the lung, one approach utilises a constant phase model, which is able to partition $Z_{rs}$ into airway (resistance) and tissue (damping, elastance) components of lung mechanics (Hantos et al., 1992). However, assessment of lung mechanics via tracheostomy by FOT is a terminal procedure, and temporary apnoea is necessary during mechanics measurements. Nonetheless, FOT is able to detect changes in baseline lung function, as well as altered responses to bronchoconstrictor challenge. In addition to FOT systems like Flexivent, a forced manoeuvres system (Buxco-Force Pulmonary Maneuvers [Buxco Research Systems, Wilmington, NC]) has been developed, which is likewise conducted in anaesthetised, tracheotomised mice, however is unable to measure all variables captured by FOT (Vanoirbeek et al., 2010). As such, FOT is often the preferred method by which to investigate disease-like changes in lung function using animal models of asthma.

1.5.2 Measuring airway dysfunction in animal models of asthma – in vitro

Whilst whole-animal in vivo experimentation is highly informative, measurements in isolated tissues in vitro may help to identify physiological changes occurring in specific regions of the lung in disease models, from the trachea to the small airways. The specific lung function parameters measured by in vitro techniques are varied, but may include pulmonary conductance and compliance in isolated perfused intact lungs, oscillation mechanics in lung strips, and force responses of ASM to pharmacological agents or electric field stimulation (Duguet et al., 2000; Held & Uhlig, 2000). As these tissues are isolated, by nature, important factors that influence lung mechanics and
airway reactivity, such as pulmonary surfactants, neural input or inflammatory cells, may be absent from the preparation. Additionally, a fundamental difference exists in the method of agonist delivery in such preparations, as compared to in vivo techniques. In in vitro preparations, the agonist is applied directly to the muscle, enabling examination of airway muscle responses only, with little indication of epithelial influence on reactivity. Nonetheless, differences in airway responses between in vivo and in vitro preparations may be mechanistically informative (Sly et al., 2004).

However, the direct assessment of small airways cannot be readily achieved using in vivo lung function testing. Given that the peripheral airways have been identified as a site of severe obstruction contributing to airway dysfunction in asthma (Section 1.2.4), in vitro techniques are required to investigate this area of the lung.

Development of the precision-cut lung slice (PCLS) technique has permitted direct observation of small airway reactivity in situ, and currently is used to assess pulmonary pharmacology, toxicology and physiology (Liberati et al., 2010; Sanderson, 2011). Unlike other in vitro studies using lung strips or tracheal segments, lung slices preserve surrounding parenchymal structures that may influence reactivity. Up to 50 lung slices can be generated from a single animal, allowing for internal control during experimentation. Additionally, the lung slice technique can be extended to permit simultaneous investigation of small arterial reactivity, however to date the application of this technique has been limited (Sanderson, 2011). This technique can be applied to animals that have been sensitised to allergens in vivo (Chew et al., 2008; Donovan et al., 2013) and is described further in Chapter 3.
1.6 Aims of this thesis

The current understanding is that small airways are an important but neglected target in the management of severe asthma, with few experimental methods established to directly assess this. In addition, a major cause of deleterious small airway remodelling resulting in the development of more severe asthma is the combination of early life respiratory infections and aeroallergen exposure. Novel insights can be gained by integration of validated in vivo models with assessment of lung function in vivo and assessment of small airway reactivity ex vivo. Moreover, the immunological and cellular mechanisms that drive pathological processes remain unresolved, and are vital for the development of future therapeutic interventions.

The overall hypothesis is that early life pneumococcal colonisation of the airways and viral infection, augmented by allergic sensitisation, is a causal factor leading to development of asthma.

This thesis aimed to address the following key issues;

- develop and optimise the lung slice technique in small rodents with viable intrapulmonary airways and arteries in order to assess the impact of acute respiratory infection in the small airways (Chapter 3)
- investigate the role of neonatal viral-induced pneumococcal colonisation on adult mouse lung health and development (Chapter 4)
- investigate the mechanisms driving early-onset severe asthma in a mouse model of neonatal bacterial and viral co-infection in combination with aeroallergen sensitisation (Chapter 5)
CHAPTER 2

General Methods
The methods used consistently throughout this project are outlined in detail in this chapter. This includes preparation of infections for mouse treatments, \textit{in vivo} techniques and cellular analyses. Methods relating specifically to the individual studies are outlined in the methods section of the Results chapters.

2.1 Animals and ethics

All experimental procedures in mice presented in this thesis were approved by the Animal Ethics Committees of the University of Melbourne (approvals #1212485, #1111986, #1413288) and complied with the National Health and Medical Research Council (NHMRC) Australian Code of Practice. In this project, two strains of laboratory mice were used; inbred albino BALB/C and inbred C57BL/6 mice. All animals were obtained from the Animal Resources Centre, Western Australia, and housed in the Animal Facility, School of Biomedical Sciences, University of Melbourne. Animals were housed at 22 °C under normal 12:12 h light:dark cycle, and given free access to a normal diet and water.

2.1.1 Animal monitoring

Mice were monitored on a daily basis for signs of illness or distress such as loss of body weight (>10%, or lack of growth for mouse pups), loss of grooming, hunched appearance, social isolation, loss of appetite, panting. Advanced pregnant dams were housed separately and monitored for births with minimal disruption. Upon birth, dams were housed with their litters until age of weaning. Infant mice were weaned at 3 – 4 weeks of age and were housed 1 – 4 per cage. Weaned mice were weighed a minimum of twice weekly.

2.1.2 Euthanasia

At the end of the experimental protocol, mice were euthanized by intraperitoneal injection of anaesthetic (0.20 ml sodium pentobarbitone, 60
mg/ml) (Cenvet, Australia), administered via 26 G needle with 1 ml syringe attached.

2.2 Infections used in mouse models

2.2.1 Preparation of Streptococcus pneumoniae

*Streptococcus pneumoniae* EF3030 (SP; serotype 19F) was kindly provided by Dr Odilia Wijburg from the Department of Microbiology & Immunology, University of Melbourne. EF3030 is a clinical *S. pneumonia* isolate and typically colonises the nasopharynx without bacteremia (Briles et al., 1992). Pneumococci were statically grown in Todd-Hewitt broth supplemented with yeast extract (0.5%) at 37 °C to an optical density of 0.4 – 0.45 (at 600nm). Cultures were placed on wet ice for 5 min before freezing at −80 °C in glycerol (8% v/v) as per standard procedure (Diavatopoulos et al., 2010). Aliquots of SP with known bacterial counts were diluted in sterile PBS and stored on ice immediately prior to mouse infection protocols.

2.2.2 Preparation of influenza A virus

Influenza A virus HKx31 (H3N2; IAV) was kindly provided by Associate Professor Patrick Reading from the Department of Microbiology & Immunology, University of Melbourne. HKx31 is a laboratory-derived high-yielding reassortant of A/PR/8/34 (PR8; H1N1) and A/Aichi/2/68 (H3N2). These viruses were originally obtained from World Health Organisation Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia. HKx31 was grown in 10-day embryonated hen's eggs by standard procedures and then titrated on Madin-Darby canine kidney (MDCK) cells (Tate et al., 2009). Aliquots of HKx31 with known titre (in plaque forming units (PFU)) were stored at −80 °C and diluted in sterile PBS immediately prior to mouse infection protocols and stored on ice.
2.3 Measurement of airway reactivity in vivo – Flexivent

*In vivo* airway reactivity was measured in mice via low-frequency forced oscillation technique, using a small animal computer-controlled piston ventilator (Flexivent, SCIREQ® Montreal, QC, Canada). In all experiments, stock anaesthetic solution was prepared in sterile injectable saline (0.9 %, Pfizer) containing ketamine (10 mg/ml) and xylazine (2 mg/ml). Mice were anaesthetised with initial dose of ketamine (125 mg/kg) and xylazine (25 mg/kg), and appropriate sedation confirmed by absence of pedal reflex. A small incision was made using a scalpel blade to expose the trachea, and surrounding muscle bluntly dissected away, and a small incision made in the mid-trachea. A 1 cm length of polyethylene tubing (0.8 mm inner diameter; 1.2 mm outer diameter, Microtube Extrusions, North Rocks, AU) with beveled edge was inserted mid-trachea, secured in place with silk thread, and the upper trachea occluded with a knot of silk thread. The mouse was attached to the ventilator and allowed to equilibrate for >5 min to mechanical ventilation of ~300 breaths/min and 10 ml/kg. Airway responses at baseline were measured, and then mice received a 10 s challenge with nebulised saline or methacholine (MCh; 3, 10, 30, 100 mg/ml dissolved in sterile injectable saline) (Sigma Aldrich, AU). Airway mechanical parameters were recorded for a period of 8 s at 1 min following challenge, and a further 4 times at minute intervals (5 measurements in total for each dose). Mice were allowed to recover with ~5 min ventilation between each dose, and administered additional anaesthetic of ketamine (50 mg/kg) and xylazine (10 mg/kg) if required (i.e. if mouse recovering from anaesthesia, as noted from low coefficient of determination values or mouse breathing against ventilator during manoeuvres).
Table 2.1 Constant phase model parameters assessed using forced oscillation technique lung function testing.

Description of the parameters of lung mechanics assessed using SCIREQ ® Flexivent apparatus [source: http://www.scireq.com/flexivent].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Name</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn</td>
<td>Newtonian resistance</td>
<td>Represents resistance of central or conducting airways</td>
</tr>
<tr>
<td>G</td>
<td>Tissue damping</td>
<td>Reflects energy dissipation in the alveoli during maneuver; closely related to tissue resistance</td>
</tr>
<tr>
<td>H</td>
<td>Tissue elastance</td>
<td>Reflects energy storage/conservation in the alveoli during maneuver; elastic recoil of the lung (tissue stiffness)</td>
</tr>
<tr>
<td>η (eta)</td>
<td>Hysteresivity</td>
<td>Ratio of $G/H$. A comparative change in both parameter reflects airway closure, whilst larger $G$ relative to $H$ suggests ventilation heterogeneity</td>
</tr>
</tbody>
</table>

The constant-phase model was used to measure respiratory system impedance ($Z_s$), partitioned into airway and parenchymal components to allow calculation of Newtonian resistance (Rn, equivalent to airway resistance (Raw) due to high compliance of the chest wall), tissue resistance (G) and tissue elastance (H) (Table 2.1) (Tate et al., 2009). Tissue hysteresivity ($\eta$) was calculated as the ratio $G/H$ (Fredberg & Stamenovic, 1989). Parameters were recorded only when coefficient of determination was >0.89. Rn values were averaged at baseline, and the highest Rn value (and accompanying G, H and $\eta$ values) was chosen for saline and each MCh dose.
2.4 Measurement of airway reactivity in vitro – lung slices

2.4.1 Lung slice – standard preparation

Figure 2.1 Preparation of mouse lung slice with viable airways and arteries.

Photographs show various stages of preparation of mouse lung slices with inflated airways and arteries in the laboratory.

All mice were culled on experimental day 0. Skin was dissected away from the chest and ribcage area, and the trachea exposed. The trachea was cannulated with an IV catheter containing two ports (20 G, Intima, Becton Dickinson); the chest cavity opened. A warm solution of ultra pure low-melting point agarose (GIBCO/Invitrogen, Australia) was injected via the tracheal cannula (~1.4 ml, 2% in 1X HBSS/HEPES at 44 °C) to inflate lungs, followed by ~1.5 ml air, to move agarose through airways into the alveolar space. The agarose was solidified in 1X HBSS/HEPES at 4 °C for 20 min. The heart and lungs were removed en bloc and the left lobe isolated, and adhered with superglue (cyanoacrylate) to the mounting piece of a vibratome (VT 1000S, Leica microsystems). Sequential slices (150 μm) were cut starting from the lobe periphery whilst tissue was bathed in HBSS/HEPES at 4 °C.
Lung slices were transferred into a 24-well plate containing Dulbecco’s modified eagle medium (DMEM; GIBCO/Invitrogen, Australia), supplemented with 1% penicillin-streptomycin solution (Sigma-Aldrich, Australia), 2 slices per well, and incubated at 37°C and 5% CO₂ for 24 or 28 h prior to use in experiments on day 1 and 2, respectively. 1 X HBSS/HEPES was used in all lung slice perfusion experiments unless otherwise stated.

2.4.2 Lung slice – mounting and microscopy

Lung slices were observed using phase contrast microscopy on an inverted microscope (Diaphot 300; Nikon, Japan), utilising 10 X objective lens, zoom adaptor, reducing lens and camera (CCD camera model TM-62EX; Pulnix/Adept Turnkey, Australia). Single slices were mounted in custom-built perfusion chambers of approximately 100 μL volume. A single airway (diameter 100 – 200 μm) from each slice was chosen for experimentation, based upon confirmation of ciliary activity in an intact layer of epithelial cells.

2.4.3 Lung slice – image capture

Digital images (744 x 572 pixels) were recorded in time lapse (0.5 Hz) using image acquisition software (Video Savant; IO Industries, Inc., Canada), converted into TIFF files, and subsequently analysed using NIH/Scion software (Scion Corporation; download www.scioncorp.com). The airway lumen area was distinguished from surrounding tissue by choosing an appropriate grey scale threshold to permit lumen area to be calculated with respect to time via pixel summation.

2.4.4 Lung slice – standard perfusion experiments and analysis

A gravity-fed perfusion system with eight separate channels was used to deliver drug solutions to the perfusion chamber at a rate of ~0.5 ml per min. All eight channels, each containing separate solutions, were connected to a manifold with a single outflow needle (Warner Instruments Inc., USA) to deliver drugs at timed intervals, regulated by a manually controlled valve.
(LFS Lee Company, USA). All experiments were conducted at room temperature, unless otherwise stated. Specific perfusion experimental protocols are detailed in individual chapters. In all experiments, data were averaged over the last min of the perfusion period, and fitted using non-linear regression (three parameter) to derive EC$_{50}$ and/or maximum values.

2.5 **Collection of bronchoalveolar lavage and lung tissue**

Immediately following euthanasia, bronchoalveolar lavage (BAL) was collected from each mouse via tracheotomy (21G catheter, SP3 Duran polyethylene tubing) proximal to the larynx. Using a 1 ml syringe, lungs were lavaged with 4 volumes of cold PBS (1 X 0.4ml, 3 X 0.3ml, total 1.3 ml). Approximately 1 ml of BAL was recovered from each mouse.

The left lobe was isolated and placed into a plastic cassette and stored in 10% neutral buffered formalin (Australian Biostain, Australia) for later histological analysis. Remaining lobes were placed in a 2 ml Eppendorf tube, snap frozen in liquid nitrogen prior to storage at -80 °C for later molecular analysis.

2.5.1 **Total and differential cell counts in bronchoalveolar lavage**

Total viable cell count was determined using the fluorescent viability stain ethidium bromide-acridine orange (Molecular Probes®, Invitrogen). A small aliquot (10 – 20 µl) of BAL was mixed with an equal volume of viability stain and counted on a standard Neubauer hemocytometer under fluorescent light on a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan). Via this method, viable cells incorporate acridine orange and appear bright green in colour.

To determine differential cell types within BAL samples, cytospins of cells were prepared by centrifuging 200 µl of sample at 400 rpm for 10 min. Cytospins were allowed to air-dry before staining with DiffQuik reagents (Dade Behring, USA); fixative (triarylmethane dye & methanol, 5 mins),
followed by xanthene dye (red; 10 min), thiazine dye (blue; 2 min) and finally excess dyes were gently washed away with running H₂O. Once dry, glass coverslips were adhered to the cytopins with entellan (Merck, Australia) and differential cell counts assessed by brightfield microscopy. Macrophages, neutrophils, eosinophils and lymphocytes were identified and counted according to standard morphological criteria , (lymphocyte; small, dark purple, macrophage; dark purple nucleus with pale cytoplasm, neutrophil; irregular shaped nuclear body with bluish cytoplasm, eosinophil; red cytoplasm) with a total of 500 cells counted per slide under oil immersion at 40X magnification (Olympus BX52, Olympus Optical, Japan).

### 2.5.2 Collection of bronchoalveolar lavage cells and supernatant

For assessment of proteins within the bronchoalveolar lavage fluid (BALF), BAL samples were centrifuged at 400 X G for 5 min to pellet the remaining cells. The fluid above the cells (BALF) was carefully collected and transferred to 1.5 ml Eppendorf tubes, and snap frozen in liquid nitrogen for storage at -80 °C for later analysis. The remaining cell pellet was lysed in 350 µl RLT buffer by drawing up and down the solution 10 times with a 21 G needle with 1 ml sterile syringe attached. The lysed cell pellet was stored at -80 °C for later analysis.

### 2.5.3 Measurement of total BALF protein

Total BALF protein was determined using standard Protein Assay Kit (Pierce™ bicinchoninic acid (BCA) Protein Assay Kit, Thermo Scientific). BALF samples were thawed on ice and assayed in duplicate as per manufacturer’s instructions. 10 µl of BALF sample was plated into a 96 well plate to which 200 µl of BCA reagent mixture was added. BCA reagent mixture was prepared from 50:1 mixture of BCA Reagent A and BCA Reagent B. A standard curve of albumin reference samples was also prepared and placed into the same plate. The plate was protected from light and incubated at 37 °C and absorbance read at t = 30 min using a plate reader set to 560 nm.
The protein concentration in each sample (mg or µg per ml) was deduced using bovine serum albumin standard curve performed in the same microplate.

2.6 Homogenisation of mouse lung and nasopharyngeal tissues

Mice were euthanized by injection of anaesthetic i.p. (0.20 ml sodium pentobarbitone, 60 mg/ml, Cenvet, Australia). Whole lungs and nasopharyngeal tissue were dissected out using sterile tools from treated mice at 20, 30 and 40 days of age. Each tissue sample was placed into a pre-weighed 5ml sterile round-bottom tube containing 1 ml complete maintenance media (composition as detailed below) and kept on ice. Tissues were homogenised inside a biohazard cabinet. The homogenizer probe (T25 digital Ultra-Turrax, IKA Malaysia) was sterilised by rinsing sequentially in 4 tubes containing 80% ethanol, 80% ethanol, sterile PBS, sterile PBS. Each tissue sample was homogenised until sufficiently ground (8–10 s) and placed back on ice; the probe washed with water to remove remaining tissue, and then rinsed sequentially, between each sample.

2.6.1 Determining pneumococcal load by viable count of homogenised mouse tissues

Bacterial burden was also assessed in the nasopharynx and whole lung tissue as was done for viral titre experiments. 3 x 15 µl droplets of homogenised tissue sample were plated into Horse Blood Agar (HBA) plates (Medical Preparation Unit, University of Melbourne, Australia). Each plate was designed to incorporate 45 µl of 4 concentrations of sample: neat, 1:10, 1:100 and 1:1000 (diluted in complete maintenance media). HBA plates were allowed to dry before being inverted and placed in an incubator at 37 ºC for 18-24 h. Total colony-forming units (CFU) for the highest countable dilution was recorded for each sample.
2.7 Fixing of mouse lung tissue for immunohistochemical analysis

Lung tissue stored in 10 % neutral buffered formalin (NBF; Amber Scientific, WA, Australia) for 24 hours was transferred into 80 % ethanol prior to paraffin embedding, and 4 µm sections of lung were serially cut from the periphery of the left lobe. Three lung sections were mounted on each glass slide. Haematoxylin & eosin (H&E), Alcian blue–periodic acid-Schiff (Ab-PAS) and Masson’s Trichrome (MT) stains on these sections were performed by School of Biomedical Sciences Histology Facility, University of Melbourne or Monash University Histology Department. Morphometric analyses were carried out on stained sections as detailed within individual results chapters.

2.7.1 α-smooth muscle actin immunohistochemical staining

α-smooth muscle actin (αSMA) immunohistochemical stain (Dako, North Sydney, AU) procedure was optimised in the laboratory using Dako EnVision ® + Dual Link System-HRP (DAB+) kit. Lung sections were deparaffinised and rehydrated using histolene and ethanol solutions before slides placed in a pre-heated coplin jar containing Tris-EDTA buffer antigen retrieval solution (10 mM Tris buffer-methylamine, 1 mM Ethylenediaminetetraacetic acid (EDTA) disodium salt, 0.05% Tween 20; pH 9), and incubated for 20 min at 95 °C. Slides were allowed to cool to room temperature (RT) for 20 min. A perspex incubation chamber with fitted lid was lined with wet paper towels. Individual lung sections on each slide were circled with Dako wax pen and placed in the incubation chamber. A bovine serum albumin (BSA) blocking step involved 30 min incubation at RT with BSA solution (BSA 5%, horse serum 10%, Triton-X 0.5% in sterile PBS). BSA solution was tapped off and then drops of Endogenous Enzyme Block (Dako EnVision ® kit) were applied to cover each section and incubated for 30 min at RT. Monoclonal mouse anti-human αSMA antibody (Clone 1A4; Dako) was diluted 1:300 in sterile PBS and mouse IgE negative control antibody (Dako) diluted 1:100. ~50 µl primary antibody, isotype control or sterile PBS was added to cover each one
of the three lung sections on each slide and slides incubated overnight at RT, protected from light.

Slides were washed 2 x 5 min intervals in 1 X wash buffer (prepared from 1:10 dilution in dH$_2$O of a 10 X stock solution containing Tris buffer – methylamine 13.9g, Trizma hydrochloride 60.6g, Tween20 5ml in 1 L distilled water (dH$_2$O)). Excess wash buffer was tapped off slides and drops of labelled polymer-HRP (Dako EnVision ® kit) added to cover sections on each slide, and slides incubated at RT for 1 hr. The washing step was repeated, excess wash buffer tapped off slides and DAB solution (Dako EnVision ® kit) added for 2 min before reaction stopped with dH$_2$O. Slides were lightly counterstained with Mayer’s Haematoxylin (Sigma Aldrich, AU) for 30 sec, rinsed in running tap water for 1 min, incubated in Schott’s tap water (3.5g MgSO$_4$ and 20g NaHCO$_3$ in 1 L dH$_2$O) and then slides dehydrated with ethanol and histolene solutions before being coverslipped using Entellen mounting medium (Merck).

2.8 Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for gene expression analysis of lung tissue

2.8.1 RNA extraction

RNA was purified from mouse lung tissue using RNeasy kit (Qiagen, worldwide) as per manufacturer’s instructions. Freshly dissected lung tissue was snap frozen in liquid nitrogen and stored at -80 °C until use. Using a mortar and pestle, tissue was ground into a fine powder under liquid nitrogen. Approximately 20 mg tissue was then transferred into an Eppendorf tube containing 600 µl RLT lysis buffer supplemented with β-mercaptoethanol (1:100, Sigma Aldrich, Australia) or a tube containing 1 ml Trizol (Life Technologies).
For β-mercaptoethanol RNA extraction procedures (used in Chapter 4) tissue was lysed 10 times using a 1 ml pipette tip, and then centrifuged at 15,000 rpm (or full speed) for 3 min.

For Trizol RNA extraction (used in Chapter 5), one stainless steel bead (Qiagen) was added to each tube, before placing each tube in a TissueLyser (Qiagen) for 10 min at 50 Hz to release mRNA and DNA from tissue samples. Tubes were briefly centrifuged and the homogenate transferred into a new 1.5 ml microcentrifuge tube. 200 µl chloroform (Sigma-Aldrich) was added to each homogenate and the sample mixed thoroughly with vortex (15 sec). Each sample was then centrifuged at 12000 X G for 15 min at 4 ºC. The upper, aqueous phase was transferred into RNeasy mini spin column and the remainder of the extraction was used for DNA purification as detailed in Chapter 5.

The lysate from either β-mercaptoethanol extraction or Trizol extraction was collected and transferred into the RNeasy spin column tube and centrifuged at 13,000 rpm for 30 sec, the column discarded, and the flow through mixed with equal volume 70% ethanol. 700 µl of sample was transferred to a new RNeasy spin column tube and centrifuged for 15 s at 10,000 x g and flow through discarded. The sample was then washed once with 700 µl RW1 buffer, then twice with 500 µl RPE buffer, with centrifuging between each wash (15 s at 10,000 x g). RNA was eluted from the column with RNase-free water (50 µl) and centrifuged for 1 min at 8000 X G.

RNA concentration, quality and purity were assessed on 1 µl sample of RNA using NanoDrop™ spectrophotometer (Thermo Scientific, Delaware, USA). All samples were confirmed to contain RNA concentration > 100 µg/ml and a ratio of absorbance readings at 260 and 280 nm within the range 2.0 – 2.15. RNA that was not immediately used for cDNA synthesis was stored at -20 ºC.
2.8.2 cDNA synthesis

cDNA was purified using a High Capacity RNA-to-cDNA kit (Life Technologies, Thermo Fisher, Australia). RNA was diluted to 100ng/µl in Rnase-free water and 9 µl transferred into micro eppendorf tubes. 2 X Reverse Transcription buffer (10 µl) and Reverse Transcription enzyme (1 µl) were added to form total reaction volume of 20 µl. The reaction mixture was then incubated in a thermocycler at 37 °C for 60 min, and then the reaction stopped with 5 min incubation at 95 °C. cDNA was stored at -20 °C until use.

2.8.3 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Taqman ® PCR primers (Life Technologies) were used to perform qRT-PCR. Samples were assayed with reaction volume of 7.5 µl in a 384 well optical well plate, in duplicate. Components of this reaction volume and specific primers are listed in individual results chapters. The plate was sealed with optical cover and briefly centrifuged to allow contents in each well to pool to the bottom of each well and eliminate air bubbles from skewing readings. The plate was then placed into the QuantStudio™ 7 Flex Real-Time machine. QuantStudio™ Real-Time PCR software was set up to run the following qRT-PCR experiment: uracil-N glycosylase activation at 50 °C for 2 min, pre-denaturation at 95 °C for 20 sec followed by 40 cycles of denaturation at 95 °C for 1 sec and annealing at 60 °C for 20 sec. Threshold cycle values (Ct) were normalized to an internal control (glyceraldehyde phosphate dehydrogenase, GAPDH). The ΔΔCt values (cycle difference of a gene of interest between the samples relative to a control sample, after normalising the loading variability) were obtained for each sample and data expressed as the normalised, relative fold change of the expression of the gene of interest in a sample relative to a control sample (2^–ΔΔCt).

2.9 Statistical analysis

Data are presented as the mean ± standard error of mean (SEM) for n animals throughout this entire thesis unless otherwise specified. All data were
statistically analysed using GraphPad Prism 5.0 (GraphPad, San Diego, CA). Where detailed within individual results chapters, one-way analyses of variance (ANOVA) with Bonferroni or Dunnett’s post-hoc tests were used. In other cases, unpaired Student’s t-tests were used to analyse data. In all cases, p <0.05 was considered to be statistically significant.
CHAPTER 3

Optimisation of mouse and rat lung slice experimental protocols for characterization of airway and vascular reactivity
3.1 Introduction

3.1.1 Exploring pulmonary pharmacology with lung slices

The lung slice technique is a powerful in vitro tool that can be used to explore airway and vascular pharmacology, offering a unique experimental link between cell-based assays and in vivo experimentation, (Liberati et al., 2010; Sanderson, 2011). Lung slices were first described in 1987. These slices (1 – 2 mm) were prepared from hamsters and rats using hand-held blades and were often centrally necrotic. The advent of the Krumdieck precision tissue slicer permitted thinner lung slices to be made to overcome this limitation (Fisher & Placke, 1987; Stefaniak et al., 1992). Optimisation of culture conditions enabled lung slice parenchymal structure and cellular viability to be maintained for periods beyond 60 days in very thin lung slices (Siminski et al., 1992). Historically, lung slices were primarily used in areas of toxicology and immunology. A study of small airway reactivity was first performed in 1996 in rat lung slices, where changes in airway lumen area in response to methacholine (MCh) were assessed via videomicroscopy (Martin et al., 1996). While many studies have now used lung slices for a variety of applications (Liberati et al., 2010; Sanderson, 2011), the aim of this methodological chapter was to develop optimal protocols around the use of mouse and rat lung slices for assessment of contraction and relaxation. Moreover, we sought to establish methodology in the laboratory for the preparation of rat lung slice with viable and reactive airways and arteries.

3.1.2 Preparation of viable lung slices containing airways and arteries

Lung slices have been prepared in numerous species including mouse, rat, guinea pig and human. Whilst the methodology of lung slice preparation has been extensively reviewed (Liberati et al., 2010; Sanderson, 2011), it has not yet been standardised across the literature (Table 3.1).

Generally, following euthanasia, the lungs are inflated with warmed low melting-point agarose solution delivered via the trachea, the volume used
depending on the species (Table 3.1). In guinea pigs, isoprenaline (ISO) is usually added to the agarose in order to oppose strong airway contraction that occurs in this species post-mortem (Ressmeyer et al., 2006). A bolus of air follows, to push the agarose into the alveolar space, beyond the airways to maintain their patency. With practice, this is a consistently successful procedure.

Various procedures have been applied to maintain patent pulmonary arteries in lung slices. Some groups (Held et al., 1999) suggest that bleeding the animal or perfusing the pulmonary circulation is sufficient to leave pulmonary arteries free of blood and therefore ‘inflated’, whereas others instil gelatin to physically fill the arteries, with the gelatin remaining in place until it dissolves when slices are cultured (Perez & Sanderson, 2005a). However, the reliable preparation of viable pulmonary arteries in lung slices continues to present challenges. One suggestion is that this is due to the relatively reduced tethering of arteries to the surrounding parenchyma compared to airways, so that arteries in lung slices often appear collapsed and poorly connected to the surrounding tissues (Sanderson, 2011).

Following single or dual inflation procedures, lungs are chilled either in situ or after removal. This allows the agarose/gelatin to gel and subsequently enables slices to be prepared from single lobes mounted in an automated tissue slicer. The thickness of slices varies between laboratory groups, but is usually 150 – 300 µm (Table 3.1).

Following cutting, lung slices are incubated in a small volume of maintenance media. If gelatin has been used to fill the arteries, it will dissolve under these conditions (Sanderson et al., 2010). Unlike other cell culture systems, stimulation of growth is not required. As such, serum is not added, as it contains growth factors and 5-HT that may induce airway and vascular tone (Abdullah et al., 1994). The use of various types of media and incubation conditions have been reviewed by Liberati et al, however have not been
systematically correlated with slice viability or physiological responsiveness (Liberati et al., 2010).

3.1.3 Experimental conditions for assessing reactivity in lung slices

To date, very little has been published discussing optimal experimental conditions to examine dynamic reactivity in airways and arteries in lung slices. Pharmacological agents used in reactivity experiments can be prepared in a volume of balanced salt solution such as HBSS (Bergner & Sanderson, 2002); alternatively in media such as DMEM (Held et al., 1999). Lung slice reactivity experimentation can extend to >1 hour, and so it is important to maintain slice viability and physiological pH during experimentation. A usual indicator of airway viability is the presence of beating cilia on epithelial cells as well as functional contractile responses. Experiments investigating in vitro reactivity of isolated larger airways, such as tracheal rings, are commonly conducted using Krebs-Henseleit (Krebs) physiological buffers, which contain bicarbonate to maintain pH. In lung slice experimentation, balanced salt solutions such as HBSS are supplemented with HEPES, a zwitterion that is highly effective at maintaining pH level within a physiological range (Held et al., 1999). The use of Krebs buffer has yet to be investigated in lung slice preparations, and is addressed in this chapter.

Since airway and artery lumen areas are imaged during these experiments, it is necessary to set up lung slices so that they remain in a fixed position as drug solutions are systemically changed throughout the course of the experiment. Some laboratories treat slices under static conditions, securing the slice using a weighted ring within a chamber containing buffer or media, which is not oxygenated (Rosner et al., 2013). Contractile or dilator agents are then added to a constant volume in the chamber, and airway images collected every minute to measure responses (Martin et al., 1996; Rosner et al., 2013). Others employ gravity-fed perfusion systems to constantly deliver drug solutions to a custom-made lung slice perfusion chamber containing
minimal volume, and image airway responses at shorter, 2 s intervals (FitzPatrick et al., 2014; Perez & Sanderson, 2005b).

The temperature used for lung slice reactivity experiments varies within the literature, with some groups assessing in vitro reactivity at ambient temperature (FitzPatrick et al., 2014), whilst others use heating equipment to maintain experimental temperature at 37 °C (Bai & Sanderson, 2008). The influence of temperature on small airway and artery reactivity in lung slices has not been systematically assessed. One of the aims of this chapter was therefore to compare the influence of different experimental temperatures and different buffers on contractile and dilator responses.
Table 3.1 Selected comparison of preparation of lung slices with preserved airways and arteries.

Abbreviations: PA; pulmonary artery, RVen; right ventricle, ISO; isoprenaline, pen/strep; penicillin/streptomycin. (Held et al., 1999; Martin et al., 1996; Milara et al., 2012; Moreno et al., 2006; Paddenberg et al., 2006; Perez & Sanderson, 2005a; Pfaff et al., 2005; Ressmeyer et al., 2006; Rieg et al., 2011; Wright & Churg, 2008)

<table>
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<tr>
<th>Species</th>
<th>Airways</th>
<th>Arteries</th>
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<td>agarose 0.75% exsanguinate via vena cava</td>
<td>agarose 2%</td>
</tr>
<tr>
<td></td>
<td>agarose 1.5% perfuse vasculature with HEPES + heparin, pen/strep, sodium nitroprusside via RVen</td>
<td>agarose 1.5% perfuse vasculature with HEPES + heparin, pen/strep</td>
</tr>
<tr>
<td>Rat</td>
<td>agarose 0.75% heparin via RVen perfuse vasculature with Hank’s buffer</td>
<td>agarose 2% perfuse vasculature with Hank’s buffer via RVen</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>agarose 0.75% + ISO 1 µM exsanguinate via vena cava</td>
<td>agarose 2% perfuse vasculature with heparinized Hank’s via RVen</td>
</tr>
<tr>
<td>Human</td>
<td>agarose 1.5% + ISO 1 µM exsanguinate via vena cava</td>
<td>agarose 3%</td>
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<th>Reactivity</th>
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<td>Human</td>
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3.1.4 Examining pulmonary vascular pharmacology with lung slices

The lung slice technique has been most commonly used to assess reactivity of intrapulmonary airways because of their role in asthma and other chronic lung diseases affecting the airways (1.5.2). However, recent advances in the technique (described in 3.1.2) have also permitted preservation of intact intrapulmonary arteries in slices, with the potential to study vascular diseases of the lung. The pulmonary vasculature is a low resistance vascular bed with high surface area to facilitate gas exchange. Pulmonary arteries (PA) carry blood from the heart to the lung, with mean pressure 9 – 18 mmHg. In diseases such as PAH, mean PA pressure rises to >25 mmHg at rest, and the structure and function of these arteries are therefore compromised. PAH can be modelled experimentally in rodents using stimuli such as hypoxia (low O₂), or following intraperitoneal injection of the liver toxin monocrotaline. Rat is the preferred species in these models, as they are more sensitive to developing PAH-like changes, including pronounced vascular remodelling and elevated PA pressure as compared to mice (Stenmark et al., 2009).

Historically, studies of pulmonary arterial (PA) reactivity relied on in vitro organ bath or myograph apparatus, measuring changes in force in isolated tissues. However, this does not permit investigation of distal PAs, which are a major site of resistance in lung vascular diseases such as pulmonary arterial hypertension (PAH) (Simonneau et al., 2004). The lung slice technique has been extended to enable direct observation of the peripheral pulmonary arteries and veins (Ressmeyer et al., 2006), and provides a novel approach to visualise changes in vascular contraction and relaxation in disease models of PAH.

3.1.4.1 Comparative pharmacology of airways and arteries in lung slices

The simultaneous, comparative investigation of airway and arterial reactivity in lung slices has been established in mouse (Perez & Sanderson, 2005a), rat
Comparative airway and vascular pharmacology in response to serotonin, potassium chloride and endothelin-1 has been reported in mouse and guinea pig lung slices (Perez & Sanderson, 2005a; Perez & Sanderson, 2005b; Perez-Zoghbi & Sanderson, 2007; Ressmeyer et al., 2006). Collectively, these studies investigated the mechanisms underlying small airway and arterial contraction, as well as the contributions of Ca\(^{2+}\) oscillation frequency to these contractile responses in mouse and guinea pig lung slices. Another study demonstrated that while airways are sensitive to acetylcholine-induced contractions, endothelium-dependent relaxation to acetylcholine is evident in pre-contracted arteries in rat lung slices (Moreno et al., 2006). Importantly, this study examined rat lung slices under static conditions, where endothelium-derived factors could accumulate. Beyond these few reports, assessment of the comparative pharmacology of airways and arteries in lung slices, particularly from rats, is limited. In this chapter, protocols for preparation of rat lung slices with viable airways and arteries were developed, with the view to apply this to in vivo animal models of vascular disease.

### 3.1.5 Regulation of airway and vascular smooth muscle tone

The active effector of bronchial tone and reactivity is airway smooth muscle (ASM), located in the airway walls, controlled primarily via cholinergic innervation from the parasympathetic nervous system. Acetylcholine (ACh) is the main neurotransmitter released at pre- and post-ganglionic junctions in the ASM, and upon binding to muscarinic (bronchoconstriction mediated by subtype M\(_3\)) receptor, causes an influx of calcium (Ca\(^{2+}\)) into the cytosol from the sarcoplasmic reticulum (SR) (Figure 3.1) (Bergner & Sanderson, 2002). Bronchoconstriction occurs when intracellular calcium ([Ca\(^{2+}\)]\(_i\)) levels are increased, via the breakdown of PIP\(_2\) by phospholipase C, resulting in the production of inositol-1,4,5-trisphosphate (IP\(_3\)). IP\(_3\) binds to the IP\(_3\)-receptor
(IP₃R) to release Ca²⁺ from the SR stores. Ca²⁺-induced Ca²⁺-release from the SR further increases [Ca²⁺]. This results in the activation of calmodulin (CaM), followed by the activation of myosin light chain kinase (MLCK) that phosphorylates myosin light chain (MLC) resulting in ASM contraction. Methacholine (MCh) is a pharmacological agent that is also a non-selective muscarinic receptor agonist that induces potent contraction of human airway smooth muscle, mediated via the M₃ muscarinic receptor (Figure 3.1) (Roffel et al., 1990). Clinically, it has previously been used in an aerosolized form in broncho-provocation testing in asthmatic patients (Berend et al., 2008). A number of other endogenous agents can stimulate this pathway, including endothelin-1 and mast cell derived mediators (histamine in humans, 5-hydroxytryptamine (5-HT) in rodents), which are discussed in this chapter.

Opposing this airway contraction, adrenergic stimulation relaxes ASM (Figure 3.1). While β₂-adrenoceptors are highly expressed in human airways, adrenergic innervation is sparse so circulating adrenaline drives relaxation. Bronchodilation can be stimulated using β₂-adrenoceptor agonists, which bind and decrease levels of [Ca²⁺], by inhibiting IP₃-induced Ca²⁺-release from the SR, and PKA-mediated activation of myosin light chain phosphatase (Kotlikoff & Kamm, 1996). This allows for the dephosphorylation of MLC to reverse contraction and relax ASM (Figure 3.1).

Vascular smooth muscle (VSM) contraction also occurs when intracellular calcium ([Ca²⁺]) levels are increased, causing the conversion of MLC to MLC-P. However, in contrast to ASM, the major driver of contraction in VSM is Ca²⁺ entry into the cell rather than SR Ca²⁺ release. Several agonists (Figure 3.2, Figure 3.3) can contract both ASM and VSM. Whilst ACh elicits bronchoconstriction in ASM, activation of the muscarinic M₃ receptor on vascular endothelial cells leads to increased synthesis of nitric oxide, a secondary mediator which in turn relaxes VSM (Ignarro et al., 1987).
Figure 3.1 Mechanisms of airway smooth muscle contraction and relaxation.

Bronchoconstriction occurs when activated myosin light chain kinase (MLCK) phosphorylates myosin light chain (MLC) resulting in airway smooth muscle contraction. Conversely, bronchodilation occurs when myosin light chain phosphatase is activated, allowing the dephosphorylation of MLC to reverse contraction and relax ASM. Key: 5-HT; 5-hydroxytryptamine (serotonin), 5-HTR; 5-HT receptor, AC; adenylate cyclase, ATP; adenosine triphosphate, Ca²⁺; calcium ion, (c)AMP; (cyclic) adenosine monophosphate, Et-1; endothelin-1, ETₐ/ₜ; endothelin receptor A/B, IP₃(R); inositol triphosphate (receptor), M₃; muscarinic receptor, MCh; methacholine, MLC; myosin light chain, MLCK; myosin light chain kinase, MLC-P; phosphorylated myosin light chain, MLCP; myosin light chain kinase phosphatase, PLC; phospholipase C, PKA; protein kinase A, SERCA; sarcoplasmic or endoplasmic reticulum, SR; sarcoplasmic reticulum, ROK; RhoA kinase, RhoA; Ras homolog gene family member A.
3.1.6 Manipulation of airway and vascular smooth muscle tone

In addition to cholinergic agonists and β-adrenoceptor agonists, other pharmacological agents or stimuli can be employed to investigate airway and vascular smooth muscle reactivity. These agents are useful tools in assessing differential responses of airway and vascular smooth muscle and as such can yield important insight into disease processes. Pharmacological agents relevant to this thesis are discussed in the following sections.

3.1.6.1 5-Hydroxytryptamine (Serotonin)

5-Hydroxytryptamine (serotonin, 5-HT) is a potent vasoconstrictor in humans and rodents and bronchoconstrictor in rodents. It is primarily released in the gut, and stored in platelets via the serotonin transporter (5-HTT or SERT), so normal plasma concentrations of 5-HT are low (< 1 nM). 5-HTT is highly expressed in the lung, and lung levels are further increased in pulmonary vascular diseases (Eddahibi et al., 2001). In addition, 5-HTT is thought to play a key role in mediating the mitogenic effects of 5-HT in disease, leading to vascular proliferation (Marcos et al., 2004).

The actions of 5-HT are mediated not only by 5-HTT, but also the 14 structurally distinct 5-HT receptors, which are divided into 7 families. The mechanism of 5-HT-mediated smooth muscle contraction is comparable to that initiated by MCh, whereby both Ca\(^{2+}\) sensitivity and Ca\(^{2+}\) oscillations are employed (Appendix 1). Whilst 5-HT is a potent constrictor of smooth muscle cells, endothelium-dependent vascular relaxation has been observed in response to 5-HT in coronary, mesenteric and renal arteries in porcine and canine tissues (Cocks & Angus, 1983), and at high concentrations, bronchodilation was observed in isolated human bronchi (Goldie et al., 1982).

Whilst the 5-HT\(_{2A}\) receptors mediate contraction in airway smooth muscle and the systemic vascular beds, 5HT\(_{1B}\) receptors mediate vasoconstriction in
the human pulmonary circulation (MacLean et al., 1996a; Morecroft et al., 1999) (Figure 3.2). Importantly, 5-HT potently contracts airways of some species, such as rodents, however it does not contract human airways. 5-HT is therefore a useful alternative contractile agonist to assess both airway and vascular pharmacology in rodent models of disease.

![Figure 3.2 Mechanism of serotonin-mediated airway and vascular smooth muscle contraction.](image)

Serotonin (5-HT) binds to 5-HT$_{1B}$ receptors on pulmonary vascular tissue and 5-HT$_{2A}$ receptors on airway tissue to induce contraction. Abbreviations: AC; adenylate cyclase, cAMP; cyclic adenosine monophosphate, $G_{i}/G_{q}$, g proteins

### 3.1.6.2 Endothelin-1

Endothelin-1 (Et-1) is a 21 amino acid peptide, synthesized primarily in endothelial cells by a complex process that involves conversion of prepro-Et-1 to big Et-1, and finally to Et-1 via endothelin-converting enzyme. Et-1 has powerful broncho- and vasoconstrictor effects in both humans and rodents, and is also an inflammatory mediator and smooth muscle mitogen. The various actions of Et-1 are mediated by its two G-protein coupled receptors. The endothelin (ET)$_{A}$ receptors is the more abundant subtype in the human lung, primarily located on smooth muscle cells but also found in epithelial cells (Figure 3.3) (Markewitz et al., 1995). ET$_{B}$ receptors are found on both smooth muscle cells and endothelial cells (Fukuroda et al., 1994; Seo et al., 1994). Stimulation of ET$_{A}$ receptors elicits long-lasting, potent broncho- or vasoconstriction, whilst binding to ET$_{B}$ receptors on the endothelium
stimulates Et-1 clearance, and release of relaxing factors nitric oxide and prostacyclin, leading to vasodilation (Hirata et al., 1993; Pollock et al., 1995; Wright & Fozard, 1988).

**Figure 3.3** Mechanisms of endothelin-1-mediated smooth muscle contraction and relaxation.

Endothelin-1 (Et-1) released from the endothelium can activate ET<sub>A</sub> and ET<sub>B</sub> receptors on smooth muscle and induce contraction. Et-1 can also act on ET<sub>B</sub> receptors on the endothelium to induce release of endogenous relaxing agents. Abbreviations: cAMP; cyclic adenosine monophosphate, cGMP; cyclic guanosine monophosphate, ET<sub>A/B</sub>; endothelin receptor A/B, MLCK; myosin light chain kinase, NO; nitric oxide, PGI<sub>2</sub>; prostacyclin.

Et-1 has been implicated in a range of diseases affecting both the airways and pulmonary circulation, including asthma and PAH (Fagan et al., 2001). Importantly, antagonism of Et-1 pulmonary arterial contraction with dual ET receptor antagonist bosentan is a current treatment used in PAH patients (Clozel et al., 1994; Rubin et al., 2002). Although the majority of airway reactivity studies investigate MCh only, Et-1 may be an important mediator to characterise, as it elicits rapid bronchoconstriction in asthmatics in vivo.
(Chalmers et al., 1997), and levels are markedly increased in steroid-refractory asthma (Pegorier et al., 2007).

### 3.1.6.3 Rosiglitazone

Given the limitations of current treatments to oppose airway and vascular contraction in asthma and PAH, alternative therapies need to be identified and characterised. The expression of the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR)-γ is up-regulated in asthmatic epithelium, and has been correlated with asthmatic inflammatory and remodelling processes (Benayoun et al., 2001). Rosiglitazone (RGZ) is a PPAR-γ agonist that can directly dilate mouse intrapulmonary airways in lung slices, where is it a superior dilator to β-adrenoceptor agonists (Bourke et al., 2012; Donovan et al., 2014). PPARγ is also expressed in VSM and vascular endothelial cells, and RGZ is able to inhibit hypoxia-induced PA remodelling as well as dilate isolated human pulmonary arteries (Crossno et al., 2007; Kozlowska et al., 2013). Further studies are required to assess the potential of RGZ in the context of lung diseases.

### 3.1.7 Applications of the lung slice technique in mouse models of lung disease

The lung slice technique provides opportunities to investigate specific changes in the distal lung in experimental models of acute and chronic disease (Sanderson, 2011), and to assess changes in contraction and sensitivity to therapy. Although these important advantages are recognised, studies to date using lung slices from models of disease have been limited in their assessment of airway responsiveness and notably lacking in terms of examining artery reactivity.

Altered in vitro airway reactivity in mouse lung slices has been investigated following in vivo exposure to ovalbumin (OVA) allergen challenge (Chew et al., 2008; Donovan et al., 2013) or cigarette smoke exposure (Donovan et al., 2016; Donovan et al., 2015b). These two stimuli are known to promote
airways hyperresponsiveness (AHR) when reactivity is assessed in vivo, and have now been shown to alter airway contractile responses in the small airways in vitro. Specifically, OVA can induce AHR to MCh in vivo, which is maintained in isolated tracheal segments in vitro, however sensitivity to MCh is reduced in lung slices in vitro, which may reflect differential interactions of small airways with the surrounding parenchyma following allergen challenge (Donovan et al., 2013). In mice, short-term cigarette smoke exposure in vivo alters airway contraction to serotonin in lung slices and reduces airway responsiveness to β-adrenoceptor agonists (Donovan et al., 2016; Donovan et al., 2015b).

Additionally, a number of novel therapeutic agents, such as RGZ, have been screened in lung slices, eliciting small airway relaxation in mouse, rat and human lung slices (Bourke et al., 2013; Donovan et al., 2014; Sturton et al., 2008). Novel vasodilators levosimendan (Ca²⁺ sensitizer) and milrinone (phosphodiesterase III inhibitor) have also been described in PA and veins in guinea pig and human lung slices (Rieg et al., 2013; Rieg et al., 2014). Furthermore, some physiological responses can be maintained in vitro, such as hypoxic pulmonary vasoconstriction, which has been observed in small pulmonary arteries in lung slices (Paddenberg et al., 2006).

### 3.1.7.1 In vitro assessment of airway reactivity following acute respiratory infection

The role of respiratory infections in exacerbations of asthma and progression of disease has been discussed extensively in 1.4.1. Small airway reactivity in vitro has only recently been evaluated in a single model of acute respiratory viral infection. In this study, mice in the resolution phase following infection with influenza A virus displayed impaired β-adrenoceptor relaxation responses in vitro (Donovan et al., 2016). Whilst there are presently no studies describing small airway reactivity specifically following acute bacterial infection, it has been shown that bacterial endotoxin lipopolysaccharide (LPS) treatment does not alter intrapulmonary airway
responses to various constrictors or dilators following LPS infection in vivo or in vitro (Donovan et al., 2015a).

3.1.7.2 *Streptococcus pneumoniae and influenza A virus*

*Streptococcus pneumoniae* (SP) is the major cause of bacterial pneumonia, characterized by shortness of breath, however its contribution of peripheral airway dysfunction is poorly characterized due to technical constraints in measuring small airway function during acute infection. Virus-induced bronchiolitis is also a major cause of persistent wheeze in children and also known to be an emerging and important risk factor for developing asthma (Holt & Sly, 2002). In order to test the applicability of the lung slice technique in models of respiratory infection, two major respiratory microbes were used: SP and influenza A virus (IAV).

3.1.7.3 *In vitro assessment of airway reactivity in genetically modified animals*

Genetically modified murine models have been extensively used to characterize mechanisms of disease progression and severity; however the assessment of lung slices from genetically modified animals is limited. Differential airway responsiveness to MCh in different inbred mouse strains has previously been reported (Duguet et al., 2000). Insight has been gained into cholinergic mechanisms of small airway contraction utilizing lung slices from muscarinic receptor or acetylcholine receptor knockout mice, confirming the role of the M2 and M3 receptors (Kummer et al., 2006; Struckmann et al., 2003). The assessment of small airway dilator responses in lung slices from genetically modified mice may permit modelling of disease-like changes in small airway responsiveness that cannot be readily achieved using isolated tissue preparations.

Extracellular superoxide dismutase (EC-SOD) is a biological enzyme that is abundant in the lung lining and bound to the matrix where it enzymatically converts superoxide into water and hydrogen peroxide. EC-SOD is essential
in the conversion of free radicals to non-toxic metabolites that would otherwise be damaging to the lung. Disruption of this process by EC-SOD polymorphism has been linked to reduced lung function (Dahl et al., 2008), and its expression is reduced around peripheral airways in chronic airway disease (Regan et al., 2011). A loss in EC-SOD activity occurs rapidly during antigen-induced asthmatic response, which in turn contributes to airway inflammation, as there is increased formation of nitrogen species (Comhair et al., 2000). The loss of SOD activity observed in these studies has subsequently been linked to excessive oxidative and nitrative stress and downstream events typifying asthma, importantly AHR (Comhair et al., 2005). EC-SOD is also known to regulate the degree of lung damage during acute respiratory infection, as over-expression conferred protection in an IAV mouse model (Suliman et al., 2001). Experimentally, EC-SOD knockout (SOD3 KO) mice fail to efficiently clear SP as compared to wild type mice (unpublished data). Given this, genetic knockout of this enzyme may worsen infection-induced changes in airway reactivity and therefore lead to AHR as is observed clinically. The contribution of the small airways to altered reactivity and AHR in SOD3 KO mice is not known.

3.1.8 Aims

In this chapter, the aims were methodological in nature;

• To compare airway reactivity with different experimental temperatures and buffer conditions in mouse lung slices

• To apply the lung slice technique to develop methodology for the preparation of rat lung slices with viable airways and arteries, and to characterize their reactivity

• To apply the lung slice technique to characterize contractile responses in airways from mouse lung slices following acute respiratory infection in vivo and in a genetically modified mouse strain
3.2 Methods

3.2.1 Animals and ethics

All experimental procedures in rats (approval #1212630) and mice (approval #1212485, #1111986) were approved by the Animal Ethics Committees of the University of Melbourne. Rats (Sprague-Dawley) and mice (BALB/C and C57BL/6 wildtype) were obtained from Animal Resources Centre, Western Australia. EC-SOD (SOD3) KO mice were bred in the Animal Facility, School of Biomedical Sciences, University of Melbourne.

3.2.2 Euthanasia

Male rats (350 – 400 g) were anaesthetised in an enclosed box containing a constant flow of isoflurane gas (5% in O2) for 5 minutes, and tested for absence of pedal reflex prior to decapitation. All mice (male and female, 6 – 8 weeks, BALB/C, C57BL/6 wildtype and SOD3 KO) were euthanized by overdose of sodium pentobarbitone (0.20 ml of 30 mg/ml i.p; Cenvet, Australia).

3.2.3 Buffer solutions and stock drug solutions

1 X HBSS/HEPES buffer was used in all rat and most mouse lung slice experiments. Hank’s Buffered Salt Solution (HBSS) (10 X stock, GIBCO/Invitrogen, AU) was diluted ten-fold with H2O and supplemented with 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid solution (HEPES) (GIBCO/Invitrogen, AU) and adjusted to pH 7.4. Krebs buffer was made in the laboratory and was used in selected mouse lung slice experiments (59 mM NaCl, 2.3 mM KCl, 0.69 mM MgSO4.7H2O, 2.5 mM CaCl2.6H2O, 0.6 mM KH2PO4, 10 mM EDTA, 25 mM NaHCO3, 6 mM D-glucose, pH 7.4) (Sigma-Aldrich, AU).

Stock solutions of RGZ (10⁻¹ M), BQ123 (10⁻³ M), BQ788 (10⁻³ M), iloprost (10⁻² M) were prepared in dimethyl sulphoxide (DMSO; Ajax Finechem) and
Et-1 (Genscript only, $10^{-4}$ M) was prepared in N-N-dimethylformamide (DMF; Sigma Aldrich). All other stock solutions were prepared in water.

### 3.2.4 Preparation of mouse and rat lung slices

All rats and mice were culled on experimental day 0. Mouse lung slices were prepared as detailed in 2.4.1.

Rat lung slices were prepared as per mouse lung slices (2.4.1), with minor modification. Following euthanasia, the trachea was pinned out and secured using silk thread to allow an IV catheter containing two ports (20 G Intima, Becton Dickinson) to be inserted, and the chest cavity opened. Heparinized buffer (~35 ml, 500 IU in 1 X HBSS/HEPES at 44°C; Pfizer, Australia) was injected via a winged cannula (25 G, Terumo, Australia) into the right ventricle to clear the lungs of blood. Warm agarose (~16 ml, 2% in 1 X HBSS/HEPES at 44°C) was then injected via the tracheal cannula to inflate lungs, followed by a bolus of air (~1.5 ml), to push agarose into the alveolar space. The agarose was solidified initially by washing tissue with ice cold 1X HBSS/HEPES, then tissue was kept at 4°C for 30 min.

Heart and lungs were then removed en bloc (Figure 3.4), and tissue cores (~8 mm$^2$) were prepared from left or right lung lobes and adhered with superglue to the mounting piece of a vibratome. The tissues were bathed in 1 X HBSS/HEPES at 4°C as sequential slices (150 μm) were cut starting from the lung periphery. Lung slices were transferred into a 24-well plate with each well containing 1 ml DMEM, supplemented with 1% penicillin-streptomycin solution, 1 slice per well, and incubated at 37°C and 5% CO$_2$ for 2 h before media was changed, then overnight, prior to experiments on day 1 or 2.
Figure 3.4 Isolated rat lungs following heparinised buffer perfusion and inflation with agarose.

### 3.2.5 Lung slice perfusion experimental protocols

See 2.4.2 - 2.4.3 for details of imaging and analysis of lung slice experiments. All drugs were prepared in 1 X HBSS/HEPES and perfused over slices at 5 min intervals on Day 1-2 unless otherwise stated.

#### 3.2.5.1 Characterisation of airway contraction to MCh and 5-HT in mouse lung slices

MCh (3 – 1000 nM; Sigma-Aldrich, Australia), 5-HT (1 – 1000 nM, Sigma-Aldrich, Australia) and Et-1 (0.3 – 300 nM; Auspep/Genscript, Australia, 10 min perfusion intervals) concentration-response curves were performed in mouse lung slices with 5 min perfusion intervals.

#### 3.2.5.2 Influence of temperature and buffer selection on airway contraction and relaxation in mouse lung slices

Airway contractile responses to MCh (3 – 1000 nM) were investigated in mouse lung slices, and following MCh pre-contraction (300 nM) dilator responses to ISO (3 – 3000 nM; Sigma-Aldrich). Experiments were performed at ambient room temperature (~21 °C) and at 37 °C, in 1 X HBSS/HEPES buffer and in KREBS buffer (+/- carbogen gas; composition as described in 3.2.3). For experiments performed at 37 °C, an in-line temperature heater (Warner Instruments, SDR Scientific, AU) was attached to the single outflow
needle, and a heating plate (Tokai-Hit, Japan) inserted into the microscope stage in order to heat the perfusion chamber, where temperature at input and outflow could be monitored in real time.

### 3.2.5.3 MTT assay for rat lung slice viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay was performed on rat lung slices on Day 1, or Day 2 +/- media change on Day 1. 100 µl (10 % of total well volume) of MTT reagent (2 mg/ml MTT in sterile phosphate buffered saline; Sigma-Aldrich) was added to a well containing one rat lung slice and returned to incubator (37 °C with 5% CO₂) for 2.5 h. During this incubation time, the yellow coloured MTT reagent is reduced by metabolically active cells within the lung slice, and turns the cells a deep purple colour, which can be solubilized and quantified by a spectrophotometer. After incubation, all media and MTT reagent was decanted out of each well, and DMSO (200 µl) added and the plate incubated at RT for 5 min. 180 µl of this solution was transferred to a well in a 96-well plate and the absorbance read at 595 nm on a plate reader (Multiskan Ascent, MTX Lab Systems, USA).

### 3.2.5.4 Characterisation of airway and artery contractile responses to 5-HT and Et-1 in rat lung slices

Contractile responses in rat airways to 5-HT (3 – 3000 nM) in lung slices were compared on Day 1 and Day 2, and then in rat lung slices containing both airways and arteries. Et-1-mediated contractile responses (0.3 – 100 nM) were compared in rat airways and arteries in lung slices. The potency of Et-1 (0.3 – 100 nM) sourced from two different suppliers (Auspep and Genscript, Australia) was compared in rat airways. The effects of the solvent DMF (used to prepare Genscript Et-1) was assessed in airways in rat lung slices; airways were contracted with 5-HT 100 nM before and after perfusion with DMF (0.0001 – 0.01 %, time-matched to Et-1 concentration-response experiment).
3.2.5.5 Characterisation of ET-receptors mediating Et-1 contraction in rat airways and arteries

Rat lung slices were incubated for 10 min with dual ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist bosentan (10 µM; a kind gift from A/Prof Christine Wright) prior to Et-1 concentration-response curves (3 – 300 nM). In other experiments, rat lung slices were incubated for 10 min with BQ123 (3 µM; Sigma-Aldrich, Australia), BQ788 (3 µM; Sigma-Aldrich) before perfusion with a single, sub-maximal concentration of Et-1 (10 nM). Airway and arterial contractile response to ET<sub>B</sub>-selective agonist sarafotoxin S6c (0.1 – 100 nM; Auspep, Australia) were also explored.

3.2.5.6 Reversibility of Et-1-mediated airway and artery contraction in rat lung slices

To investigate the reversibility of Et-1, airways and arteries in rat lung slices were contracted with a sub-maximal concentration of Et-1 (10 nM) for 15 min, followed by washout for 60 min.

3.2.5.7 Dilator responses in rat lung slices – pilot experiments

In a pilot experiment, an airway and artery in a rat lung slice was pre-contracted with Et-1 (10 nM) followed by increasing concentrations of dilator SAL (0.3 – 10 µM; Sigma-Aldrich, Australia). Following 5-HT concentration-response, the airway and artery in a rat lung slice was exposed to a single concentration of RGZ (100 µM, Cayman Chemical, USA).

3.2.6 Model of acute respiratory infection in mice

3.2.6.1 Preparation of infections

*S. pneumonia* EF3030 (serotype 19F, a clinical isolate; SP) and influenza A virus HKx31 (H3N2; IAV) was used in an acute respiratory infection pilot study, see 2.2 for bacterial and viral strain culture conditions.
3.2.6.2 Mouse treatment protocol

Female C57BL/6 mice of wild type (WT) or SOD3 knockout (SOD3 KO) were used in the pilot study of acute respiratory infection (Figure 3.5; University of Melbourne ethics approval #1111986). SP (final dose \( \sim 10^5 \) CFU) and IAV (final dose \( \sim 10^4 \) PFU) treatments were diluted in sterile D-phosphate buffered saline (PBS; GIBCO/Invitrogen, Australia) to delivery volume 35 \( \mu l \). Mice were placed individually into an airtight plastic induction chamber with 1.0 – 2.0 L/min flow rate of \( O_2 \) containing 2 – 3 % isoflurane (Henry Schein Isothesia, Provet AU) to induce light anaesthesia, as tested using the righting reflex. Anaesthetized mice were restrained by hand whilst the treatment was applied slowly (using a pipette) onto the top of the nostril to allow the mice to inhale the suspension. Mice were monitored until anaesthesia resolved (~1 min), and weighed daily during the study. There were three treatment groups in total, as detailed below. Mice infected with SP were culled 2 days post infection, at peak pneumococcal inflammatory response, and those infected with IAV were culled 6 days post infection, at which time there is significant lung inflammation as assessed by previous histological data from the laboratory (Gualano et al., 2008). Lung slices were prepared from these mice as detailed in 2.4.1.

3.2.6.3 Airway reactivity following acute respiratory infection

Airway contractile responses to MCh (3 – 3000 nM) and Et-1 (0.3 – 100 nM) were investigated in mouse lung slices following acute SP respiratory infection \textit{in vivo}. In dilator studies, airways were pre-contracted with MCh (300 nM) before perfusion with increasing concentrations of dilators ISO (10 – 1000 nM) or SAL (0.1 – 10 \( \mu M \)). MCh-mediated airway contraction was assessed in SOD3 KO mice.
Figure 3.5 Schematic of adult mouse infection protocols.

This model produced 3 treatment groups in total. Saline (35 µl administered intranasally on Day 0), *Streptococcus pneumoniae* Day 0 (in 35 µl delivery volume administered intranasally on Day 0) were culled on day 2, whilst saline + influenza A virus (saline 35 µl administered on Day 0 and influenza A virus administered on Day 1) were culled on day 7. Model applied in female C57BL/6 mice with WT or SOD3 KO background and lung slices were prepared from these mice.

3.2.7 Statistical analysis

Statistical analyses were carried out as detailed in 2.9. Specifically, grouped data of intrapulmonary airway and artery responses to constrictors and dilators were averaged over the last min of the perfusion period, whilst representative traces show data for each individual experimental frame, recorded at 2 s intervals. Where possible, data were fitted using non-linear regression (three parameter) to derive EC$_{50}$ values and maxima, and comparisons made using unpaired Student's t-test and one-way ANOVA with Bonferroni post hoc where appropriate. All data are expressed as mean ± SEM, and $n$ represents one artery or airway per animal from an independent experiment. P < 0.05 was considered statistically significant. Analyses were carried out using Graph Pad Prism™ (version 5.0).
CHAPTER 3: OPTIMISATION OF LUNG SLICE PROTOCOL

3.3 Results

3.3.1 Optimisation of lung slice experimental protocols

Using the standard protocol in our laboratory, lung slices were prepared from 6–8 week old male BALB/C mice, incubated in DMEM for 24 hours prior to experimentation on Day 1, as outlined in 2.4. Experiments were conducted at room temperature, and drug solutions made with 1XHBSS/HEPES buffer. Contractile responses in small airways were initially characterized using the cholinergic agonist MCh. Alternative contractile agonists, including 5-HT and Et-1 were also used, as these have additional disease relevance in terms of PAH models, and these agents are able to contract both airway and vascular smooth muscle.

Specialised computer software (VideoSavant, IO Industries, Canada) captured images at 2 sec intervals. Each concentration of drug (e.g. MCh dissolved in 1XHBSS/HEPES buffer) was perfused for 5-10 min intervals across the surface of the lung slice mounted in a custom-built perfusion chamber (Figure 3.6). Following greyscale analysis of the changes in airway lumen area, a time course trace of the experiment was generated. This data was normalized and expressed in terms of initial airway lumen area, rather than raw pixel values (Figure 3.6). The data from the last minute of each perfusion period was then averaged to generate grouped concentration response curves, and permits calculation of pEC$_{50}$ value for the agonist (Figure 3.6).

Under these conditions, the potency of MCh and 5-HT were comparable (pEC$_{50} = 7.1 \pm 0.1$ and $7.3 \pm 0.1$ respectively), but MCh appeared slightly more efficacious, eliciting ~50% contraction of small airways compared with only ~25% with 5-HT. Et-1 was the most potent (pEC$_{50} = 8.5 \pm 0.1$, $p<0.05$ compared to 5-HT and to MCh, one-way ANOVA with Bonferroni’s post hoc of fitted EC$_{50}$ values), but similarly efficacious to MCh (Figure 3.6, ).
Figure 3.6 Airway contractile responses under standard conditions in mouse lung slices.

Lung slices were generated from 6 – 8 week old male BALB/C mice. Representative images (A) show methacholine (MCh)-induced contraction in a small airway in a mouse lung slice. Images are collected every 2 s and greyscale analysis of airway lumen area is normalized to % initial lumen area to derive representative time course trace showing small airway contraction (B). Grouped data compares contraction to MCh to alternative constrictors serotonin (5-HT) and endothelin-1 (Et-1) (n = 11, 4, 5 respectively).

Having established contractile responses to several agonists, we then assessed the influence of experimental temperature and buffers on airway contraction and relaxation. Mouse lung slices were prepared (as 2.4.1), and concentration-response curves to MCh (Figure 3.7) and the β-adrenoceptor
agonist isoprenaline (ISO; Figure 3.8) were performed at room temperature (~21 °C), or with a heating element applied to the perfusion system to raise experimental temperature to 37 °C. In these experiments, drugs were dissolved in either 1 X HBSS/HEPES or in KREBS buffer (composition as 3.2.3). Given that KREBS buffer is routinely used in myograph studies where the baths are heated to 37 °C and continuously bubbled with carbogen gas (95% O₂, 5% CO₂), we also investigated the influence of bubbling carbogen gas through this buffer during lung slice experimentation.

Figure 3.7 Influence of temperature and buffer on airway contraction to methacholine in mouse lung slices.

Representative traces (A) show small airway contraction to increasing concentrations of MCh perfused at room temperature (21 °C) and 37 °C in 1 X HBSS/HEPES. Data are expressed as % initial airway lumen area. Grouped data shows contraction in small airways in lung slices with MCh drug solutions perfused in 1 X HBSS/HEPES buffer (B, n = 4) and KREBS buffer with addition of carbogen gas bubbling (C, n = 4).
Table 3.2 Comparative potency and efficacy of methacholine in varying mouse lung slice experimental conditions.

\( \text{pEC}_{50} \) and maxima were derived from non-linear curve fitting. Maxima data are expressed as maximal % reduction in lumen area (\( n = 4 \) per group).

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<th>HBSS/HEPES</th>
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<th>KREBS</th>
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<tr>
<td></td>
<td>( \text{pEC}_{50} )</td>
<td>MAX reduction in lumen area (%)</td>
<td>( \text{pEC}_{50} )</td>
<td>MAX reduction in lumen area (%)</td>
</tr>
<tr>
<td>MCh</td>
<td></td>
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<tr>
<td>21 °C</td>
<td>7.4 ± 0.4</td>
<td>78.9 ± 9.5</td>
<td>7.9 ± 0.2</td>
<td>70.6 ± 9.3</td>
</tr>
<tr>
<td>37 °C</td>
<td>7.4 ± 0.1</td>
<td>75.7 ± 6.4</td>
<td>7.4 ± 0.1</td>
<td>81.8 ± 6.7</td>
</tr>
<tr>
<td>37 °C + gas</td>
<td></td>
<td></td>
<td>7.5 ± 0.1</td>
<td>69.7 ± 9.0</td>
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MCh induced a concentration-dependent contraction in mouse airways in both buffers at 21 °C and at 37 °C, with airway contraction appearing transient at the higher temperature (Figure 3.7A). Whilst the efficacy of MCh was also similar at both 21 and 37 °C, the reversal of the contraction to MCh during washout appeared more rapid at the higher temperature. The potency of MCh was similar irrespective of temperature, choice of buffer, or the presence of carbogen gas (Figure 3.7, Table 3.2, \( p = 0.5 \), one-way ANOVA of fitted \( \text{pEC}_{50} \) values). The influence of temperature and buffer on dilator responses in small airways was also assessed. In these experiments, airways were perfused with increasing concentrations of ISO (Figure 3.8A) after pre-contraction with a sub-maximal concentration of MCh (300 nM). The rate of contraction to a single sub-maximal concentration of MCh (300 nM) was assessed in 1 X HBSS/HEPES buffer at room temperature and at 37 °C (Figure 3.8B). The contractile response to MCh reached a plateau in less than 2 minutes of perfusion at 37 °C, less than half the time taken at room temperature (\( p<0.05 \)). The onset of relaxation responses to ISO appeared slightly faster at 37 °C (Figure 3.8A).

Representative traces and grouped data show that ISO only caused partial relaxation in mouse airways with similar potency and maximum relaxation at either temperature in either buffer (Figure 3.8A, D, F). However, relaxation was generally poor in all settings, only reversing contraction by 20 – 30%, and this level of relaxation was not always well maintained (Figure 3.8, Table 3.3).
Figure 3.8 Influence of temperature and buffer on airway relaxation to dilator agent isoprenaline in mouse lung slices.

Representative traces (A) show small airway responses to methacholine (MCh) pre-contraction, followed by increasing concentrations of isoprenaline (ISO) perfused at room temperature (21 °C) and 37 °C in 1 X HBSS/HEPES. Data are expressed as % initial airway lumen area. Grouped data (B) shows time (min) to plateau contractile response following initiation of perfusion of MCh (300 nM) at 21 °C and 37 °C (n = 5). Grouped data shows MCh pre-contraction expressed as % reduction in airway lumen area in 1 X HBSS/HEPES (C, n = 4 – 5) and KREBS (E, n = 3 – 5), and subsequent small airway relaxation responses (D, F) expressed as % relaxation of relevant pre-contraction.*p<0.05, unpaired Student’s t test.
Table 3.3 Comparative potency and efficacy of isoprenaline in varying mouse lung slice experimental conditions.

*pEC*<sub>50</sub> and maxima were derived from non-linear curve fitting. Maxima data are expressed as maximal % relaxation of relevant methacholine pre-contraction (n = 4 – 5 per group).

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<tr>
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<th>HBSS/HEPES</th>
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<tr>
<td>ISO</td>
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<tr>
<td>21 °C</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>7.4 ± 0.2</td>
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<tr>
<td></td>
<td>max relax (%)</td>
<td>19.1 ± 3.8</td>
</tr>
<tr>
<td>37 °C</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>7.8 ± 0.4</td>
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<tr>
<td></td>
<td>max relax (%)</td>
<td>30.1 ± 11.9</td>
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<tr>
<td>37 °C + gas</td>
<td>8.1 ± 0.2</td>
<td>24.7 ± 5.4</td>
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As no significant differences in contraction or relaxation were observed in mouse lung slices when using different buffers or temperatures, all the following experiments were performed at room temperature, in 1 X HBSS/HEPES, as is the standard, published protocol in our laboratory.

### 3.3.2 Applications of the lung slice technique: Preparation of rat lung slices with airways and arteries

The preparation of rat lung slices is a newly established technique in the laboratory that requiring significant development. A number of steps in the procedure outlined in 3.2.4 were systematically altered, including the volumes of heparinized buffer, agarose and air bolus used, the duration of tissue washing with cold 1 X HBSS/HEPES prior to cutting, the selection of lung lobe used for slicing, the thickness of slices, the number and timing of media changes on day 0, the number of slices incubated per well, and assessment of viability and reactivity on experimental day 1 and 2.

Pilot experiments involved preparation of rat lung slices with airways only, using agarose to inflate lungs. Rat lung slices were assessed for viability by visualisation of beating cilia in airways, as well as MTT viability assay for mitochondrial activity. Rat lung slices were viable over days 1 – 2 when
maintained in culture, and airways were similarly reactive to 5-HT (Figure 3.9, NS).

**Figure 3.9 Rat lung slices are viable and reactive for 2 days in culture.**

Rat lung slices cultured for 24 hr (Day 1) or 48 hr (Day 2, with or without culture media change on Day 1) were assessed for viability via MTT assay (A, n = 5 – 9). Grouped data shows small airway contractile responses to serotonin (5-HT) on Day 1 and Day 2 rat lung slices (B, n = 3 – 10).

Following validation of rat lung slice viability, slices were prepared using the modified protocol with airways and blood vessels for experimentation. The relatively low success rate (~20%) of inflated arteries in rat lung slices was a limiting factor in experimentation. The following results were obtained from rat lung slices with well-inflated airways and arteries of ~350 µm and ~100 µm diameter, respectively (right panel, Figure 3.10).

5-HT-induced smooth muscle contraction was investigated in rat intrapulmonary airways and arteries in lung slices (Figure 3.11). 5-HT elicited both bronchoconstriction and vasoconstriction in rat lung slices, and caused transient contractions in airways. The threshold contraction was observed at 10 – 30 nM in airways and 100 nM in arteries, and 5-HT was approximately 30-fold more potent in airways (Figure 3.11C, Table 3.4, p<0.0001). Although a maximal response was not reached for arteries, estimated maximum from fitted curves suggested that similar reductions in area would be achieved in both airways and arteries. Contractile responses to
5-HT at the highest concentration tested appeared to be readily reversible in airways and arteries.

**Figure 3.10 Phase-contrast images of rat lung slices.**

Representative phase-contrast images depict poorly inflated (left) and well inflated (right) of intrapulmonary arteries in rat lung slices. Scale bar 200 μm.

**Figure 3.11 Serotonin elicits broncho- and vasoconstriction in rat lung slices.**

Representative images (A) and representative traces (B) show airway and artery contraction in response to increasing concentrations of serotonin (5-HT) in the same rat lung slice. Grouped data (C) shows contractile responses to 5-HT in airways and arteries expressed as % initial lumen area (n = 9 and n = 3, respectively).
Contractile responses to Et-1 in airways and arteries in rat lung slices were compared (Figure 3.12, Table 3.4). Et-1 elicited potent broncho- and vasoconstriction with comparable potency and efficacy. Maximal contractile responses to each Et-1 concentration were reached within 10 min of perfusion, with threshold response in both airways and arteries observed at 1 nM and maximal contraction at 30 – 100 nM Et-1.

Figure 3.12 Endothelin-1 potently contracts intrapulmonary arteries and airways in rat lung slices.

Representative images (A) and representative traces (B) show airway and artery contraction in response to increasing concentrations of endothelin-1 (Et-1) in the same rat lung slice. Grouped data (C) shows contractile responses to Et-1 in airways and arteries expressed as % initial lumen area (n = 5 and n = 6, respectively).
Table 3.4 Comparative potency and efficacy of serotonin and endothelin-1 in rat lung slices.

$pEC_{50}$ and maxima were derived from non-linear curve fitting of lung slice experiment data. Maxima data are expressed as maximal % reduction in lumen area. ***$p<0.001$ compared with airway $5-HT$ $pEC_{50}$, $^\gamma p<0.01$ cf artery Et-1 $pEC_{50}$, unpaired Student’s $t$ test.

<table>
<thead>
<tr>
<th></th>
<th>airway</th>
<th>5-HT artery</th>
<th>airway</th>
<th>Et-1 artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>7</td>
<td>3</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>$pEC_{50}$</td>
<td>7.4 ± 0.1</td>
<td>5.9 ± 0.2$^\gamma$</td>
<td>8.1 ± 0.2</td>
<td>8.6 ± 0.1</td>
</tr>
<tr>
<td>Maximum reduction in lumen area (%)</td>
<td>83 ± 3</td>
<td>78 ± 13</td>
<td>80 ± 6</td>
<td>66 ± 8</td>
</tr>
</tbody>
</table>

Additional validation experiments involved investigation of comparative potency of Et-1 from different suppliers (Auspep, Genscript). This also involved assessment of dimethylformamide (DMF) solvent, used in preparation of Genscript Et-1 stock solution, in rat intrapulmonary airways (Figure 3.13). Et-1 potency was comparable between Et-1 manufacturers. In time-matched control experiments, DMF solvent did not have any effect on basal airway tone, nor did DMF exposure alter airway contractions to sub-maximal concentration of $5-HT$ (Figure 3.13B, C).

Contraction to Et-1 in rat lung slices was characterized in the presence of the non-selective ET-receptor antagonist bosentan. The $pA_2$ of bosentan in rat tissues has been reported as 6.0 (Clozel et al., 1994), and so lung slices were incubated for 10 min with 10 μM bosentan prior to perfusion with increasing concentrations of Et-1.

Bosentan (10 μM) inhibited Et-1-induced smooth muscle contraction in both intrapulmonary airways and arteries (Figure 3.14, Table 3.5). Bosentan caused a rightward-shift in the Et-1 concentration-response curve in rat intrapulmonary airways, reducing Et-1 potency 10-fold ($p < 0.01$, Figure
3.14A, Table 3.5). Bosentan also appeared to antagonise Et-1 responses in intrapulmonary arteries; however this did not reach significance (Figure 3.14B). Et-1 was still able to elicit comparable maximum contraction in the presence of bosentan compared to control experiments in both airways and arteries. Interestingly, the onset of Et-1 contraction was notably transient in airways in 3 of 5 experiments (see representative experimental trace, Figure 3.14A).

![Figure 3.13 Validation of endothelin-1-induced airway contraction from different suppliers and effect of dimethylformamide solvent in rat lung slices.](image)

Comparison of rat airway responses to Auspep and Genscript endothelin-1 (Et-1) (A; n = 8 and n = 4, respectively), and effect of 60 min dimethylformamide (DMF) solvent perfusion on basal airway tone (B) and on 5-HT sub-maximal airway contraction (C, n = 3). Data are expressed as % initial airway lumen area.
**Figure 3.14** Bosentan antagonises endothelin-1-induced intrapulmonary airway and artery contraction in rat lung slices.

Representative traces and grouped data show endothelin-1 (Et-1) concentration-response experiments in intrapulmonary airways (A) and arteries (B) in the absence (black) and presence (blue) of bosentan (10 μM) in rat lung slices. Bosentan was perfused for 10 min to allow equilibration prior to initiation of Et-1 (Genscript) perfusions (n = 3 – 5).

From these experiments, an approximate pA₂ value for bosentan in intrapulmonary airways and arteries in rat lung slices could be calculated, assuming that the interaction between Et-1 and bosentan was competitive and ideal (Table 3.5) (Arunklakshana & Schild, 1959). Bosentan was almost 4-fold more potent in arteries than airways, however in order to gain a better estimate of pA₂, multiple concentrations of bosentan must be assessed in this system.

To determine the specific ET receptor responsible for contraction, selective ET receptor antagonists were used. Lung slices were incubated for 10 min with ETₐ-receptor antagonist BQ123 or ET₈-receptor antagonist BQ788, before application of a single, sub-maximal concentration of Et-1 (10 nM, as defined in Figure 3.12) (Figure 3.15). Reported pA₂ values for BQ123 and
BQ788 are 6.9 – 7.4 and 7.4, respectively (Davenport & Battistini, 2002), and so 3 µM of antagonist was used in these experiments.

**Table 3.5 Comparative potency of endothelin-1 in the absence and presence bosentan in airways and arteries in rat lung slices**

pEC\(_{50}\) and maxima were derived from non-linear curve fitting from lung slice experiment data. Maxima data are expressed as maximal % reduction in lumen area. pA\(_2\) values were derived from the x axis intercept on Schild plot, assuming interactions were competitive and ideal (Arunklakshana & Schild, 1959). **p<0.01 compared with control airway pEC\(_{50}\), unpaired Student’s t test.

<table>
<thead>
<tr>
<th>Et-1</th>
<th>airway control</th>
<th>airway bosentan</th>
<th>artery control</th>
<th>artery bosentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>pEC(_{50})</td>
<td>8.4 ± 0.2</td>
<td>7.2 ± 0.1**</td>
<td>8.6 ± 0.2</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>Maximum reduction in lumen area (%)</td>
<td>52 ± 11</td>
<td>63 ± 11</td>
<td>68 ± 13</td>
<td>78 ± 12</td>
</tr>
<tr>
<td>pA(_2)</td>
<td>~6.2</td>
<td>~5.6</td>
<td>~5.6</td>
<td>~5.6</td>
</tr>
</tbody>
</table>

Airways appeared to be transiently contracting during pre-incubation with BQ788 (as shown in representative trace, Figure 3.15A). However, neither BQ7883 nor BQ123 significantly inhibited airway contraction to a single, sub-maximal concentration of Et-1 (Figure 3.15A). Et-1-induced vasoconstriction appeared strongly ET\(_B\)-mediated, as pre-treatment with BQ788 completely inhibited arterial contraction to Et-1 in 3 of 4 experiments performed in different mice, while BQ123 was not inhibitory (Figure 3.15B).
Figure 3.15 Effect of selective ET<sub>A</sub> and ET<sub>B</sub> antagonists on endothelin-1-induced intrapulmonary airway and artery contraction in rat lung slices.

Representative traces (upper) and grouped data (lower, n = 4 – 5) showing intrapulmonary airway (A) and artery (B) contraction to a single concentration of endothelin-1 (Et-1; 10nM) following incubation with dimethylsuphoxide (DMSO) vehicle (0.03 %; control), ET<sub>A</sub> selective antagonist BQ123 (3 µM) or ET<sub>B</sub> selective antagonist BQ788 (3 µM). **p<0.01 compared with artery control, one-way ANOVA with Dunnett’s Multiple Comparison test (n = 4 – 5).

Sarafotoxin S6c is a highly selective ET<sub>B</sub>-receptor agonist (Williams <i>et al.</i>, 1991). In a single pilot experiment, sarafotoxin contracted both intrapulmonary airway and artery in a rat lung slice (Figure 3.16). Sarafotoxin appeared to be more potent in the rat artery, causing complete closure of the artery with 1 nM, which was consistent with the ET<sub>B</sub>-dependent Et-1-induced arterial contraction observed with BQ788 studies.
Figure 3.16 Sarafotoxin contracts an intrapulmonary airway and artery in a rat lung slice.

A single experiment yielded lumen area values that could be obtained from manual analysis of images from a single experiment, and shows lumen area (expressed as %initial) at end of perfusion period for each concentration of sarafotoxin (0.1 – 100 nM).

Before characterizing dilator responses in rat lung slices following Et-1 pre-contraction, the potential reversibility of Et-1 in this setting was assessed. Et-1 induced pseudo-irreversible contraction in both intrapulmonary airways and arteries, with contraction unable to be reversed following 55 min washout (n = 4 total, Figure 3.17). Consequently, any airway or artery relaxation following Et-1 pre-contraction could be attributed to the dilator agent rather than waning of Et-1 contraction.

Figure 3.17 Endothelin-1 induces pseudo-irreversible arterial and airway contraction.

Representative time-course traces show sub-maximal endothelin-1 (Et-1, 10 nM) contraction in arteries and airways is sustained following 55 min washout (representative of n = 3 replicates).
Pilot experiments involved screening a number of dilators for efficacy in rat intrapulmonary airways and arteries. Following pre-contraction with Et-1, the $\beta_2$-adrenoceptor agonist, salbutamol (SAL), partially relaxed a rat intrapulmonary airway, but as expected, did not elicit arterial relaxation (Figure 3.18). Other screening experiments suggested that prostacyclin analogue iloprost, a vasodilator used in treatment of PAH, had no dilatory effect in rat airways nor arteries in lung slices, nor did prostaglandin-$E_2$ (PGE$_2$), which has been previously shown to elicit mouse airway relaxation in lung slices (FitzPatrick et al., 2014) (data not shown).

**Figure 3.18 Salbutamol relaxes an airway, but not artery in a rat lung slice.**

Time-course trace shows airway and artery sub-maximal contraction to endothelin-1 (Et-1, 10 nM) followed by perfusion with increasing concentrations of salbutamol (SAL). Data expressed as % initial lumen area.

In addition to these dilators, the effect of PPAR$\gamma$ agonist rosiglitazone (RGZ) was investigated. A pilot experiment revealed that RGZ was able to relax a maximal 5-HT contraction in both an airway and artery from a single rat lung slice, eliciting $\sim 70\%$ relaxation in the airway and $\sim 25\%$ relaxation in the artery (Figure 3.19). RGZ was the only agent in this series of experiments that was able to elicit arterial relaxation in rat lung slices.
Representative phase contrast images shows intrapulmonary airway and artery in a rat lung slice during perfusion with 1X HBSS/HEPES, then when maximally contracted with serotonin (5-HT, 1000 nM) and with addition of rosiglitazone (RGZ, 100 μM) Images taken at end of perfusion period for each agent.

3.3.3 Applications of the lung slice technique: airway reactivity following acute respiratory infection in mice

The effect of acute bacterial or viral infection in vivo on airway reactivity in lung slices in vitro was explored. Lung slices were made from control and bacteria or virus-infected mice at peak lung inflammation (day 2, day 7 for bacteria and virus respectively). All data in this section are from female C57BL/6 mice, following acute respiratory infection in vivo (3.2.6).

Initially, constrictor and dilator agents were assessed in lung slices prepared following acute bacterial infection in WT mice (Figure 3.20). Contractile agonists MCh and Et-1 elicited ~65% and ~50% reduction in initial airway lumen area respectively, in both untreated and infected mice. Fitted $EC_{50}$ values were comparable between treatment groups for MCh (~200 nM) and Et-1 (~1 nM) (Figure 3.20B, C).

Following matched sub-maximal MCh pre-contraction, relaxation responses to ISO were quite variable, but were also unchanged following in vivo respiratory infection, reaching approximately 50% in airways from both saline and SP-treated mice (Figure 3.20D). Overall, the potency and efficacy of all agents tested, both constrictor and dilator, were not significantly affected following acute SP infection.
Figure 3.20 Small airway reactivity is unaltered following acute bacterial infection in vivo.

Female C57BL/6 mice were infected with saline or *Streptococcus pneumoniae* (SP) on day 0 and lung slices were made on day 2. Representative trace (A) and grouped data (B) show airway contractile responses to MCh, and to Et-1 (C). Dilator responses to ISO were investigated following sub-maximal pre-contraction with MCh (300 nM, D) with grouped data expressed as % relaxation of relevant pre-contraction (E) (n = 3 – 4 per group).

The potential for infection-induced airway hyper-responsiveness to be exacerbated in SOD3 KO mice was explored. Full concentration-response curves to MCh were obtained in lung slices generated from WT and SOD3 KO
mice treated with saline, SP or IAV infection (Figure 3.21). Structurally, airways in lung slices from SOD3 KO mice looked identical to those from WT mice, and exposure to SP or IAV infection also did not alter the gross appearance of airways (Figure 3.21A). In representative traces from n = 2 experiments in each group, the potency of MCh appeared to be reduced ~3-fold (0.5 log unit) in SOD3 KO mice relative to WT mice (Figure 3.21B, C, D). This was not altered by infection with either SP or IAV, but the WT mice treated with IAV showed a similar loss of MCh potency (Figure 3.21D). The reduction in airway lumen area in response to the highest concentration of MCh tested was ~70% in WT and SOD3 KO treated with saline or SP, however the efficacy of MCh appeared reduced to only ~30% in SOD3 KO (Figure 3.21D). The present data is preliminary, and so in order to determine whether infection-induced AHR is evident in WT or SOD3 KO mice, further experiments are required.
Figure 3.21 Comparative structure and reactivity to methacholine in lung slices from wildtype and SOD3 knockout mice exposed to acute infection

Female C57BL/6 mice were infected with saline, *Streptococcus pneumoniae* (SP) or influenza A virus (IAV) on day 0 and lung slices generated from these mice on day 2 (saline/SP) or day 7 (IAV). Representative images (A) show morphology of intrapulmonary airways in wildtype (WT) and superoxide dismutase 3 knockout (SOD3 KO) mice. Representative time-course traces show small airway contraction to increasing concentrations of MCh in WT and SOD3 KO mice treated with saline (B), SP (C) and IAV (D) (lower, representative of n = 2/group) showing contraction to MCh in WT compared to SOD3 KO mice following saline treatment, acute SP infection or IAV infection.
3.4 Discussion

In this methodological chapter, it was demonstrated that the experimental temperatures tested did not significantly affect small airway reactivity in mouse lung slices. The introduction of a new technique to the laboratory enabled investigation of comparative airway and arterial pharmacology in rat lung slices. Whilst contraction could be characterized, it was difficult to elicit airway or arterial relaxation in this species. Lastly, mouse lung slices were made following respiratory infection in vivo, and from genetic knockout mice, however this pilot study did not provide strong evidence of airway hyper-responsiveness in vitro in this setting.

The present data suggest that MCh-mediated small airway contraction was not significantly affected by the temperatures or choice of buffer tested. This is in accordance with a single previous study of this nature. In that study, the potency and efficacy of MCh and 5-HT did not differ between 22 °C and 37 °C in HBSS/HEPES buffer in airways in mouse and rat lung slices (Bai & Sanderson, 2008). However, the authors reported that MCh-induced Ca²⁺ oscillations increased substantially at the higher temperature, but small changes observed in airway reactivity were associated with high inherent Ca²⁺ sensitivity in rat, and so changes were deemed temperature independent (Bai & Sanderson, 2008).

There is often minimal justification as to the choice of experimental temperature in lung slice publications, where experiments are conducted at ambient temperature or involving heating apparatus. There is also inconsistency in experimental temperatures within species, for example in human lung slices, where earlier studies were conducted at 37 °C (Ressmeyer et al., 2006; Sturton et al., 2008) and later, by the same group, at room temperature (Ressmeyer et al., 2010). The earliest lung slice publications involved heating apparatus (Martin et al., 1996), yet this has not been consistently replicated by other groups. Experiments conducted at 37
0°C may better maintain physiological enzymatic or metabolic activity contributing to smooth muscle contraction and relaxation; however, significant differences in reactivity remain to be demonstrated. Typically, lung slice experimental protocols do not exceed 90 min duration. Myograph experiments may be > 4 h, and so temperature and carbogen gas supply may influence tissue viability more significantly in such lengthy protocols as compared to lung slice preparations. Nevertheless, a lack of protocol standardisation results in difficulty in comparing published findings. Additionally, it is not yet known whether temperature or buffer selection affects arterial reactivity in lung slices.

As we did not observe changes in reactivity with increased experimental temperature, or with different experimental buffers, remaining experiments were conducted at room temperature, using 1 X HBSS/HEPES buffers as previously published in our laboratory (Donovan et al., 2014; FitzPatrick et al., 2014). This is also consistent with previous studies reporting comparative pharmacology of intrapulmonary airways and arteries in lung slices were conducted at room temperature (Perez & Sanderson, 2005a; Perez-Zoghbi & Sanderson, 2007).

A protocol for the preparation of rat lung slices with viable and reactive airways and arteries required extensive development from previously published methods, where multiple steps were systematically altered. Initial studies applied the method published by Perez & Sanderson in mice, where warmed porcine gelatin (0.3 ml of 6% in 1 X HBSS/HEPES, 37 °C) was injected into the right ventricle to inflate pulmonary arteries, following standard agarose inflation (Perez-Zoghbi & Sanderson, 2007). The volume of gelatin was increased to account for increased size of rats compared to mice (varying volumes tested up to 3 ml). According to their protocol, the gelatin within mouse PAs in lung slices dissolved during overnight incubation, leaving PA unobstructed and viable. However, in our laboratory, this
previously established method did not successfully inflate PA in rat lung slices.

An alternative method was assessed, changing the order to have the agarose inflation after gelatin injection. However, this did not result in patent PAs. An additional step, where the lungs were firstly inflated with air (various volumes 1.5 – 3 ml) via tracheal cannula to facilitate the perfusion of lung vasculature, did not result in improved PA inflation. To determine if the gelatin was dissolving out of PA too quickly following initial preparation, cold 1 X HBSS/HEPES tissue-washing times (prior to slice cutting) were extended, again to no avail. The use of heparinized buffer was investigated, as this had successfully been applied in the preparation of mouse and guinea pig lung slices with PA (Martin et al., 1996; Wright & Churg, 2008). Ultimately, this method resulted in the highest success rate of all methodologies assessed, however the successful preparation of reactive intrapulmonary arteries was still relatively low compared to airway preparation, which was more reliable.

Other groups routinely perform a dual-inflation (airways and arteries) technique in mouse (Paddenberg et al., 2013) or guinea pig (Ressmeyer et al., 2006). The rationale for using rat in this study was to support the investigation of altered intrapulmonary airway and artery reactivity in rat models of PAH, which is preferentially modelled in this species as more pronounced vascular remodelling is evident than in mouse (Stenmark et al., 2009). The challenges experienced in reliably obtaining reactive rat lung slices with patent arteries was consistent with anecdotal evidence in a review of the technique by Sanderson, where the author suggests that whilst airways remain open following lung slice cutting procedures, vessels often collapse or tear away from the parenchyma, regardless of whether they are filled with gelatin (Sanderson et al., 2010). Publications incorporating arterial reactivity in lung slices, and in particular rat lung slices, are extremely limited. There is only one published report of comparative airway and artery reactivity in rat lung slices (Moreno et al., 2006). In this study, the authors
demonstrated endothelium-dependent dilator responses in arteries in lung slices by incubating slices with single concentrations of endogenous agents. Compared to the present findings, the authors used a different strain of rat (Wistar), which were bled via vena cava prior to instillation of agarose, and experiments involved static application of pharmacological agents, with responses measured at timed intervals. As such, the present findings are the first to compare concentration-response curves in airway and arteries in rat lung slices to multiple agents in real time. In these experiments, we characterised contractile responses in rat lung slices to various agonists, confirming comparable airway contraction to Et-1 and 5-HT. Additionally, we have demonstrated that Et-1 induces more potent arterial contraction than 5-HT.

The efficacy and potency of Et-1 was comparable in rat airways and arteries. Et-1 EC$_{50}$ has previously been reported as 22 nM in intrapulmonary airways of equivalent size in rat lung slices (Martin et al., 2000b), similar to the present result. 5-HT elicited more potent contraction in rat intrapulmonary airways than arteries. Rat pulmonary artery contraction is mediated by both 5-HT$_{2A}$ and 5-HT$_1$ receptors (Morecroft et al., 2005) while the 5-HT$_{2A}$ receptor subtype mediates contractile responses in rat airways (Cazzola & Matera, 2000; MacLean et al., 1996b). Thus, actions through 5HT$_1$ receptors, or through 5HT$_{2A}$ receptors, that may be expressed at relatively higher levels in intrapulmonary airways than arteries in the distal lung, may explain the difference in sensitivity to this contractile agonist.

Whilst Et-1 and 5-HT both bind G-protein coupled receptors, there was a vast difference in potency of the two agonists in rat intrapulmonary arteries. This could be attributed to different receptor densities and/or G-protein coupling, as ET receptors typically bind G$_o$ and 5-HT receptors bind G$_i$/G$_o$ (Figure 3.1). Additionally, the arterial contraction induced by these two agonists may rely on differing contributions of Ca$^{2+}$ oscillations and Ca$^{2+}$ sensitivity. 5-HT induced contraction in airways and in arteries in mouse lung slices relies on
CHAPTER 3: OPTIMISATION OF LUNG SLICE PROTOCOL

the initiation of Ca\(^{2+}\) oscillations (Perez & Sanderson, 2005a; Perez & Sanderson, 2005b), whereas Et-1 induced contraction involves both increased Ca\(^{2+}\) oscillations and increased Ca\(^{2+}\) sensitivity (Perez-Zoghbi & Sanderson, 2007). Perhaps Ca\(^{2+}\) sensitivity apparatus plays a larger role in mediating arterial relaxation in the rat lung, and as such 5-HT is not able to induce as potent a contraction in rat arteries as is observed for Et-1.

The efficacy of the non-selective ET\(_A/ET_B\) antagonist bosentan in opposing Et-1-mediated contraction in small airways and arteries was confirmed in rat lung slices. Bosentan significantly antagonised Et-1-induced airway contraction at a concentration of 10 µM, with a similar trend observed in arterial response. A potent vasodilator, bosentan used to improve haemodynamics in patients with PAH (Channick et al., 2001; Clozel et al., 1994), however the effects of bosentan on the small airways is less well characterised, and is of importance given the close structural relationship of intrapulmonary airways and arteries. A previous study has demonstrated the efficacy of bosentan in inhibiting Et-1-induced bronchoconstriction in rat lung slices, where 100 µM bosentan caused ~1 log unit shift in the Et-1 concentration response curve, and 10 µM bosentan did not have any effect on the development of Et-1 contraction (Martin et al., 2000a). The authors did not report bosentan efficacy in intrapulmonary arteries in lung slices. The slightly reduced sensitivity of intrapulmonary rat airways to the antagonistic effects of bosentan compared to the present findings could be due to thicker lung slices (220 µm) or strain of rat used (Wistar). Ultimately, we have demonstrated that bosentan is able to inhibit the potent, pseudo-irreversible Et-1-induced contraction in both rat airways and arteries.

Given the efficacy of dual ET\(_A/ET_B\) antagonist bosentan in both rat intrapulmonary airways and arteries, we sought to elucidate the relative contributions of each receptor subtype in Et-1-induced contractile responses. From the use of specific ET-receptor antagonists (BQ123 and BQ788), it appeared that airway contractile responses were reliant on both receptor
subtypes, as neither antagonist inhibited a sub-maximal Et-1 contraction at the concentration tested. In contrast, it appeared that Et-1 contraction in arteries in rat lung slices was primarily $\text{ET}_B$-mediated where a specific $\text{ET}_B$ receptor antagonist (BQ788) ablated Et-1 induced arterial contraction. This was supported by observation of potent arterial contraction in response to $\text{ET}_B$-selective agonist sarafotoxin in a pilot experiment. The ratio of $\text{ET}_A$: $\text{ET}_B$ receptor density in alveolar wall of the peripheral rat lung has been reported at 52:43 (Goldie et al., 1996), whilst $\text{ET}_B$ receptors mediate contraction in rat pulmonary resistance arteries (McCulloch et al., 1998), which is consistent with the present findings. Whilst we have developed a setting in which Et-1 contractile mechanism could be explored further, the receptor distribution within human airways differs from rat, as it contains a higher population of $\text{ET}_B$ receptors, and comparable distribution of $\text{ET}_A$/$\text{ET}_B$ receptors in distal pulmonary arteries (Davie et al., 2002; Knott et al., 1995). This important species difference must be considered when translating findings from rat lung slices to clinical observations.

Contraction to Et-1 in airways and arteries was pseudo-reversible, whilst 5-HT-induced contractions could be reversed in minutes with washing. This finding had implications for the selection of contractile agonist for assessment of dilator reactivity. It proved difficult to elicit relaxation against Et-1 in intrapulmonary arteries in rat lung slices. This may have been due not only to the difficulty in reversing Et-1 induced contractile responses, but also to the dependence of some dilators on endothelial-derived factors to cause arterial relaxation, as these factors are likely to be absent in this system due to constant perfusion of the slices. In a previous study of rat lung slices, arterial relaxation was observed in response to acetylcholine (ACh). This required pre-contraction of the artery with thromboxane mimetic U46619, and static incubation of lung slices for 5 min with ACh present, which the authors state was sufficient for accumulation of epithelial-derived hyperpolarising factor and nitric oxide, which mediated the observed relaxation (Moreno et al., 2006). Conversely, in the perfused experimental
set-up used in the current study, accumulation of epithelial-derived factors is not achieved, which may be required to observe relaxation responses in this setting. Prostacyclin is a potent endogenous vasodilator and acts on the prostacyclin (IP) receptor, and its analogs, including iloprost, are used clinically to overcome vasoconstriction in PAH (Hoeper et al., 2000). Despite its clinical efficacy, iloprost has previously been shown to have poor affinity for the IP receptor, and displays high affinity for EP₃ receptor, which mediates arterial contraction, and this contrary binding affinity may interfere with any dilator responsiveness in rat intrapulmonary airways in the current study (Kuwano et al., 2008). Surprisingly, PGE₂ was not able to elicit airway nor arterial relaxation, despite its previously displayed efficacy in intrapulmonary airways in mouse lung slices (FitzPatrick et al., 2014).

A number of novel agents have previously been screened in lung slices, and have successful elicited small airway relaxation in mouse, rat and human lung slices (Bourke et al., 2013; Donovan et al., 2014; Sturton et al., 2008). Novel vasodilators including levisomendan and milrinone have also been described in PA and veins in guinea pig and human lung slices (Rieg et al., 2013; Rieg et al., 2014). Airway relaxation in rat lung slices to SAL was achieved, and was comparable to that previously observed in mouse lung slices under similar pre-contraction with MCh (FitzPatrick et al., 2014). However, the dilator agents (iloprost, PGE₂) screened in rat lung slices in this chapter produced little airway or arterial relaxation. The only effective agent tested was RGZ, a dilator that had previously been shown to elicit superior bronchodilation to β-adrenoceptor agonists in mouse airways in lung slices (Donovan et al., 2014). In the present study a single experiment suggested that a high concentration of RGZ elicited both airway and arterial relaxation, following maximal pre-contraction with 5-HT. RGZ was not tested against Et-1 due to its pseudo-irreversibility, but it may be more clinically relevant to PAH to test whether RGZ can inhibit the development of Et-1-mediated contraction, rather than reverse an established pre-contraction. Ultimately, more extensive characterisation of dilator agents in rat lung slices is required.
to demonstrate potential to oppose airway contraction in asthma and vascular contraction in PAH.

Whilst the implications of respiratory bacterial and viral infections on asthma causation and exacerbation are emerging (1.4.1), little is known about the effects of acute infection on small airway function specifically. In a proof of principle study, a mouse model involving respiratory infection in vivo was used to investigate potential changes in airway reactivity in vitro, as assessed by the lung slice technique. Models of acute respiratory bacterial or viral infection were used, which have previously been validated in the laboratory, and are characterised by elevated lung inflammation, which is further increased in SOD3 KO mice (Satzke et al., 2014).

This pilot study showed no altered airway reactivity in vitro to constrictor or dilator agents following SP infection in vivo. Whilst there are presently no studies reporting small airway reactivity following pneumococcal infection in vivo, a previous study exploring effect of lipopolysaccharide (LPS; a bacterial endotoxin) infection similarly showed no changes in small airway responsiveness in mouse lung slices, despite evidence for pronounced lung inflammation (Donovan et al., 2015a). No changes were observed when mice received LPS treatment in vivo, when lung slices were treated with LPS in vitro, or both.

Additional pilot experiments presented here suggest that in vivo IAV (Mem71 strain) infection in WT C57BL/6 mice may reduce MCh potency in the small airways. In contrast, a separate study undertaken concurrently with the work in this thesis showed that the intrapulmonary airways of BALB/C mice infected in vivo with IAV were hyperresponsive to MCh, whilst the potency of MCh was unchanged (Donovan et al., 2016). Although the reduced MCh potency remains to be confirmed in the current relatively underpowered study, these contrasting results may reflect differences in strains of mice and their sensitivity to the effects of viral infection. Of note, Donovan et al showed
that airways in lung slices from IAV-infected mice did not display impaired bronchodilator responsiveness, but when infection was combined with short-term cigarette smoke exposure, bronchodilator responsiveness was completely ablated (Donovan et al., 2016). It appears that despite these acute respiratory infections causing lung inflammation, this does not always translate to hyperresponsiveness or impaired bronchodilator responses in isolated lung slices in vitro. For this reason, insight into infection-driven changes in airway reactivity may be better addressed by utilising alternative in vivo techniques, such as the Flexivent. Moreover, the effect of respiratory co-infection has not been assessed in vitro in the lung slice or in vivo, as exposure to two pathogens may initiate a greater inflammatory response in the lung culminating in altered airway reactivity across multiple generations of airways.

EC-SOD is abundant in the lung lining where it enzymatically converts damaging free radicals to non-toxic metabolites. Clinically, SOD polymorphism has been associated with reduced forced vital capacity lung function parameter in COPD patients, and SOD inactivation linked to AHR in asthmatics (Comhair et al., 2000; Dahl et al., 2008). SOD3 KO mice are more sensitive to lung injury as they are unable to mount a proper protective responses (Carlsson et al., 1995). Conversely, overexpression of SOD in a mouse model is protective against IAV-induced lung pathology and inflammation (Suliman et al., 2001). In the current study, acute IAV infection in combination with SOD3 KO did not result in AHR in vitro, with small airways appearing hyporesponsive to MCh in initial observations. This trend that was observed in both saline vehicle-treated and SP-infected SOD3 KO mice, where MCh potency appeared reduced. Additional experiments are required to extend these pilot observations in order to elucidate altered reactivity in the small airways in this model, and the implications of reduced protection from IAV-induced lung damage with SOD3 deletion.
3.4.1 Conclusions from Chapter 3

Results from this chapter were used to define the experimental conditions applied for the rest of this thesis. Findings indicated that temperature and buffer selection did not significantly alter airway reactivity in mouse lung slices, and so experiments from this chapter onwards were conducted at room temperature and using 1 X HBSS/HEPES buffers as is standard, published laboratory protocol. Whilst the preparation of rat lung slices with airways and arteries was established as a new technique in the laboratory, results indicated that this approach requires continued development in order to improve reliability. Given this, focus was shifted away from proposed studies in rat models of PAH towards preparing lung slices from mouse models of respiratory infections relevant to acute and chronic diseases. Since this was successfully applied here in a pilot study to mice that had been acutely infected with bacterial and viral respiratory pathogens *in vivo*, this approach was then utilised in Chapter 4.

Furthermore, as bacterial and viral infections mount a complex immunological response that involves migration and recruitment of circulating immune cells directly impacting deleterious airway remodelling, *in vivo* assessment of airway function was subsequently employed in this thesis. In the following chapters, complex *in vivo* models of neonatal respiratory infection are developed to investigate the early life origins of asthma. In addition, a multidisciplinary approach is used to elucidate disease mechanisms involving *in vitro* and *in vivo* techniques to reveal new insight into molecular asthma phenotypes with novel therapeutic implications.
CHAPTER 4

Influenza A infection facilitates neonatal pneumococcal colonisation and alters lung function in adulthood
CHAPTER 4: VIRAL-INDUCED PNEUMOCOCCAL COLONISATION

4.1 Introduction

4.1.1 Viral infection in children

Following the elderly, the second highest rates of influenza-associated hospitalization occur in children younger than 5 years old. It is estimated that 20 million cases of influenza infections occur yearly worldwide in this age group (Thompson et al., 2004). This accounts for 13% of paediatric cases of acute lower respiratory tract infections (Nair et al., 2011). In Australia, it has been estimated that around 1,500 children are admitted to hospital for laboratory confirmed influenza each year (Newall & Scuffham, 2008). Within this group, infants <1 year old are particularly vulnerable, with more frequent influenza-associated hospitalisations as compared with other childhood age groups (Zhou et al., 2012). This is consistent with the very low level vaccination rates in infants, due to modest efficacy and limited availability of vaccines during influenza season, and children under the age of 6 months being too young for vaccination (Belshe et al., 2007). Immunological profiles of influenza strains have been extensively characterised and clinically, with the response to different strains ranging from mild disease to severe pneumonia when pandemic strains can initiate an exaggerated inflammatory response resulting in severe lung injury (Tumpey et al., 2005).

Viral-induced lung injury primarily occurs at the respiratory epithelium, where the virus invades epithelial cells and replicates rapidly, initiating a pro-inflammatory cascade. The kinetics of this response have been investigated in murine models, where an influx of neutrophils peaks in the first few days following infection, followed by monocyte, eosinophil and lymphocyte infiltration 7 – 10 days post infection, at which time epithelial cell regeneration commences (Buchweitz et al., 2007). Recently, the potent regenerative capacity of a rare lung stem cell population has been identified (Vaughan et al., 2015; Zuo et al., 2015). This population, which characteristically express transformation related protein 63 (Trp63) and
keratin 5, can undergo proliferative expansion and assemble at sites of injury to initiate repair processes. Selective ablation of these stem cells prevents lung regeneration, resulting in pre-fibrotic lesions and compromised oxygen exchange (Zuo et al., 2015). Mature epithelial cells play a very limited role in lung repair following viral injury (Vaughan et al., 2015), and instead regeneration requires a discrete “back-up” population of progenitor cells to recover epithelial integrity (Rawlins, 2015). Additionally, mucus cell metaplasia and enhanced mucus production can persist weeks following infection, after the inflammatory response has dissipated (Buchweitz et al., 2007).

**4.1.2 *Streptococcus pneumoniae* colonisation in early life**

In addition to viral infections, young children represent a major reservoir for *Streptococcus pneumoniae* (SP) carriage (Adegbola et al., 2001). SP is an extracellular bacterium and is the predominant microbe responsible for bacterial pneumonia, sepsis and meningitis. SP infection accounts for 1 million deaths in < 5 year-olds worldwide per year (O’Brien et al., 2009). Pneumococcal carriage rates are highest in situations of crowding, such as day care centres (O’Brien et al., 2009). As children represent a major reservoir for SP carriage, they are thought to contribute significantly to the dissemination of pneumococcal strains within the community (Leiberman et al., 1999).

Neonatal upper airway colonisation is also associated with increased risk of bronchiolitis in early life, which is independent of concurrent asthma (Vising et al., 2013). There are also emerging epidemiological data to suggest that asymptomatic upper airway colonisation is associated with persistent wheeze in children. The prevalence of asthma at 5 years of age was significantly increased in the children colonised as neonates with microbes including SP in the hypopharyngeal region (Bisgaard et al., 2007). Similarly, a recent study of the infant nasopharyngeal microbiome in the first year of life identified SP colonisation as a strong predictor of asthma (Teo et al., 2015).
The detection of colonising bacteria occurs frequently during significant bronchiolitis episodes; and so the common terminology of ‘viral wheeze’ may not accurately reflect the aetiological nature of many acute events in children under the age of 5 years (Carlsson et al., 2015; Thorburn et al., 2006). Additionally, colonising bacteria that disseminate into the lower respiratory tract are associated with reduced lung function and asthma into adulthood (Chan et al., 2015). Whilst mild infection may help to build immunity, severe early life infection with SP may result in deleterious effects on lung structure and function that are associated with the development of chronic respiratory illnesses in later life (Starkey et al., 2013b).

4.1.2.1 Pneumococcal vaccines

Vaccine strategies have been developed to prevent community-acquired pneumonia. The pneumococcus is a complex pathogen, with over 90 serotypes identified. There are currently 2 types of pneumococcal vaccines available; the polysaccharide vaccine targets 23 of the most common serotypes associated with invasive pneumococcal disease, and protects against 85-90% of strains present in the community, however is not suitable for infants or the elderly (Bogaert et al., 2004). A conjugate vaccine has since been developed that specifically targets 7 serotypes that have been identified as responsible for >80% of infections in children. This vaccine is suitable for infants (< 2 years of age), and can be administered up to 5 years of age if there is significant risk of infection (Bogaert et al., 2004). The conjugate vaccine has reduced the incidence of invasive pneumococcal disease in infants by over 80%, and one factor thought to contribute to this is the ‘herd immunity’ generated by the vaccine, protecting non-vaccinated siblings and adults (Miller et al., 2011). As young children are a reservoir of pneumococcus, elimination of this carrier status reduces the risk of transmission and spread of virulent strains within the community. However, there has been a documented increase in prevalence of invasive disease to
non-vaccine serotypes that escape immune defences and appear to resist antimicrobial agents (Kyaw et al., 2006).

4.1.3 Influenza infection drives bacterial colonisation

There are several environmental, host and microbiological factors that increase an individual’s susceptibility to pneumococcal disease. Notably, infection with influenza A virus (IAV) has been associated with increased pneumococcal burden (O’Brien et al., 2000), increasing susceptibility to pneumococcal pneumonia 100-fold (Shrestha et al., 2013). Clinically, up to 40% of infants hospitalised with severe viral bronchiolitis were co-infected with bacteria in the lower airways, and as such were at high risk for bacterial pneumonia (Thorburn et al., 2006). In a separate study, children with severe bacterial pneumonia had experienced IAV illness in the preceding month, suggesting that prevention of IAV infections may also prevent pneumococcal disease (O’Brien et al., 2000). Importantly, concurrent IAV infection is essential for the transmission of SP from colonised mice to their naive co-housed littermates, as inhibiting the viral replication prevented transmission (Diavatopoulos et al., 2010).

It is well established that IAV infection can reduce immunity to bacterial pathogens, and secondary bacterial infections are a leading cause of influenza-associated illness and mortality. Antibiotic use has little effect in influenza-associated bacterial pneumonia (McCullers & English, 2008). Mechanistically, this “lethal synergism” may be occurring in two ways, as virus impairs pneumococcal clearance and the pneumococcus enhances viral release from infected cells (Smith et al., 2013). Impaired pneumococcal clearance may be driven specifically by the interferon (IFN)-\(\gamma\) produced during viral lung inflammation, and as such IFN-\(\gamma\) suppresses innate protection against bacterial pathogens in the lung and increases susceptibility to more invasive infections (Nakamura et al., 2011; Sun & Metzger, 2008). The extent of this interaction and subsequent severity of bacterial and viral synergy has
been correlated with pneumonia and inflammation, but not bacteraemia (Smith et al., 2007).

4.1.4 Altered airway reactivity following neonatal respiratory infection

It has been established that influenza and other viruses can acutely alter lung function and lead to airway hyperresponsiveness (AHR) and induce greater non-specific AHR in asthmatic patients compared to healthy controls. Viral-induced AHR is driven by a multitude of factors, which extend through to the small airways where damage may exert serious functional consequences (Folkerts et al., 1998).

Viral pathogens cause epithelial cell necrosis, which may result in increased exposure of sensory nerve endings present in the epithelium to inhaled irritants. Upon activation, these nerve endings release bronchoconstricting substances (substance P, neurokinin A) that activate cholinergic nerve fibres to release acetylcholine (ACh). ACh binds to a high-affinity muscarinic M₃ receptor on the airway smooth muscle (ASM) to mediate contraction; this action is normally terminated by negative feedback actions of pre-junctional receptors. Viral infection, specifically respiratory syncytial virus (RSV), impairs this negative feedback loop thereby increasing cholinergic contraction of ASM (Dakhama et al., 2005). In addition to directly altering ASM contraction, epithelial necrosis also physically adds cellular debris in the lumen. Alongside impaired mucociliary clearance and decreased production of epithelial-derived bronchodilator substances (prostaglandin-E₂, nitric oxide), this collectively contributes to virus-induced AHR (Hegele et al., 1995). Viral infections also increase vascular permeability leading to submucosal oedema that intrinsically narrows airways, and whilst this has little effect on baseline lung function, any subsequent ASM contraction is markedly enhanced (Hegele et al., 1995).

Clinically, the resolution of viral-associated AHR varies considerably between adults and children. Experimentally, heightened responsiveness to both
inhaled and intravenous constrictor agonist methacholine (MCh) has been observed in adult mice during the acute phase of IAV infection, assessed using a low-frequency forced oscillation technique (FOT). This IAV-associated AHR was not related to an intrinsic smooth muscle defect, but rather an increase in permeability of the alveolar-capillary barrier, which was facilitating greater MCh access to ASM (Bozanich et al., 2008). This AHR was transient in nature, as lung function normalised with resolution of inflammation and tissue injury in adult mice (Bozanich et al., 2008). In contrast, physiological and inflammatory responses to IAV infection were assessed in infant mice, where residual AHR persisted for 21 days, when inflammation and alveolar–capillary permeability had fully resolved (Larcombe et al., 2011). This AHR can persist beyond this time point and into advanced adulthood (You et al., 2008). This closely replicates the virus-associated AHR observed in both atopic and non-atopic children, which can persist for beyond 5 weeks following clearance of a single upper respiratory viral infection (Xepapadaki et al., 2005).

4.1.5 Aim

Whilst the majority of childhood cohort studies have investigated asthma risk in terms of early life viral infection, there is increasing evidence that bacterial pathogens may play a role in the development of asthma. Pneumococcal infection is preceded by asymptomatic, often transient, colonization of the upper airways, which has been associated with persistent wheeze and increased asthma risk. Co-infection with a respiratory virus can exacerbate bacterial burden in young children and susceptible adults, and the effect of this on subsequent adult lung health is unknown. Using a mouse model of neonatal respiratory co-infection, the aim of this study was to assess the effect of early life co-infection with SP and IAV on mouse lung health, immunity and structure in adulthood.
4.2 Methods

4.2.1 Animal ethics and monitoring

All experimental procedures performed using mice were approved by the Animal Ethics Committee of the University of Melbourne (approval #1111986 & #1413288). Advanced pregnant C57BL/6 dams were obtained from Animal Resources Centre, Western Australia. Dams were housed separately and monitored for births, with minimal disruption. Infant mice were weaned and at 4 weeks of age, separated by sex and were housed 1 – 4 per cage. Mice were monitored and weighed a minimum of twice weekly. All pups within a litter were allocated to the same treatment group.

4.2.1.1 Preparation of infections

SP (EF3030, serotype 19F, a clinical isolate) and IAV (HKx31, H3N2) were used in infant co-infection studies as described in 2.2.

4.2.2 Model of infant co-infection

Male and female C57BL/6 wildtype mice were used in the study of infant respiratory co-infection used in this chapter (Figure 4.1). SP (2000 CFU/ml) and IAV (7000 PFU/ml) treatments were prepared in sterile PBS with a delivery volume of 3 µl. At 5 days of age, infant mice received intranasal administration of SP (or saline vehicle), followed by IAV (or saline vehicle) at 12 days of age. Infant mice were restrained by hand, without anaesthesia, whilst the infection was applied slowly to the nostril to allow mice to inhale the suspension. There were 4 treatment groups in total; vehicle, SP, IAV and SP/IAV. Outcomes were measured at 6 – 8 weeks of age, with exception of measurement of bacterial burden and viral load, which was measured at 20, 30 or 40 days of age.
CHAPTER 4: VIRAL-INDUCED PNEUMOCOCCAL COLONISATION

Figure 4.1 Model of infant co-infection.

This model was performed using C57Bl/6 mice, male and female, and yielded 4 treatment groups; saline, Streptococcus pneumoniae (SP) alone, influenza A virus (IAV) alone, and co-infection (SP + IAV). Infant mice were monitored on a daily basis for signs of illness or distress as detailed in General Methods 2.1.1. Weaned mice (3 – 4 weeks of age) were weighed twice weekly.

4.2.3 Collection and measurement of specimens

BAL and lung tissue was collected as detailed in 2.5. Following neonatal challenge with SP and/or IAV, mice were allowed to recover and assessed starting at 6 – 8 weeks of age in early adulthood. At endpoints (6 – 8 weeks of age), mice were euthanized by overdose of sodium pentobarbitone (0.20 ml of 60 mg/ml i.p.). BAL was performed by tracheotomy and total and differential BAL cell counts determined as per 2.5.1. At time points of Day 20, 30, 40 of life, nasopharyngeal and lung tissues were collected, homogenised and serial dilutions of tissue homogenates were prepared in RPMI media (Sigma Aldrich) and cultured on horse blood agar plates (Medical Preparation Unit, Department of Microbiology & Immunology, University of Melbourne, VIC, AU) to determine bacterial load as per 2.6.

4.2.3.1 Determining viral titre in homogenised mouse tissues

Viral titre of infectious virus in mouse lung and nasopharyngeal tissue homogenates was measured by standard plaque assay in Madin-Darby canine kidney (MDCK) cells (ATCC, In Vitro Technologies, Australia). This involved the use of two media solutions. Complete media consisted of DMEM
supplemented with 10% fetal calf serum (FCS, heat-inactivated), 1% non-essential amino acids, 20 mM HEPES and 30 µg/ml gentamycin (Life Technologies, Thermo Fisher Scientific, Australia). Maintenance media was identical to complete media, but did not contain FCS.

The infectivity of mouse homogenized tissue samples were determined via titration in MDCK cells as the number of PFU per ml. 6-well plates were prepared such that there was 1.8 – 2.0 X 10^6 cells per well, and incubated at 37 °C under 5 % CO₂ until they reached confluence. Confluent cells were incubated under the same conditions for 1 h with 200 µl of neat, 1:3, 1:30 or 1:300 dilution of homogenized tissue sample (diluted in maintenance media). After 1 h, each well of cells was overlaid with 3 ml of 0.9 % agarose (Sigma-Aldrich) in 1 X overlay medium (Leibovitz’s L-15 medium, GIBCO) containing trypsin (0.0025%; Sigma-Aldrich) which was warmed to 37 °C, and plates were incubated at 37 °C under 5 % CO₂ for 3 days.

Cells were fixed at room temperature with 2 ml 2% formaldehyde (Australian Biostain, Australia) in 0.9% saline overnight. Fixative was rinsed off with H₂O before the agarose plug was carefully flicked off. Cells were stained with ~ 1 ml 0.5 % crystal violet (Sigma-Aldrich) in 100% methanol and placed on a rocker for 1 h. Stain was washed off under running water and plates allowed to air dry before counting plaques using a light box.

4.2.3.2 Measurement of total protein in bronchoalveolar lavage fluid

Total BALF protein was determined using Pierce BCA Protein Assay Kit (Thermo Scientific). BALF samples were thawed on ice and assayed in duplicate as per 2.5.3.

4.2.4 Measurement of airway reactivity in vivo

Mice were anaesthetized with ketamine/xylazine as per 2.3. In vivo airway responsiveness in 6 – 8 week female mice was assessed at baseline and following MCh challenge (1, 3, 10, 30 mg/ml) as per 2.3.
4.2.5 Measurement of airway reactivity \textit{in vitro}

4.2.5.1 Preparation of mouse lung slices

Lung slices were prepared with viable intrapulmonary airways from 6 – 8 week old female mice as detailed in 2.4. Lung slices were used in experiments on Day 1 and 2.

4.2.5.2 Characterisation of contractile and dilator responses in mouse lung slices

MCh (3 – 3000 nM) or Et-1 (0.3 – 100 nM) concentration-response curves were performed in mouse lung slices prepared from adult mice exposed to infant infections. For dilator studies, small airways were sub-maximally precontracted with MCh (300 nM), before perfusion of isoprenaline (ISO; 3 – 1000 nM) or chloroquine (CQ; 1 – 100 µM). All experiments were conducted with 5 min perfusion intervals with the exception of concentrations of Et-1, which were 7 min, to permit plateau of each contractile response.

4.2.6 Immunohistochemistry

4.2.6.1 Immunohistochemical staining procedures

The left lobe of lung was removed immediately \textit{post mortem} and fixed in 10\% neutral-buffered formalin. Tissues were paraffin-embedded and cut at a thickness of 5 µm. Sections were stained with Haemoxylin & Eosin (H&E), Masson's trichrome (MT) for assessment of epithelial and sub-epithelial collagen content or with Alcian blue–periodic acid-Schiff (Ab-PAS) for assessment of goblet cell transdifferentiation. These staining procedures were carried out by Monash University Histology Department. \(\alpha\)SMA staining was performed as per 2.7.1.

4.2.6.2 Morphometric analysis

Images of stained slides were captured by Monash University Histology Department and analysed using Aperio ImageScope software (Leica
Biosystems, Australia). Morphometric evaluation of lung tissue sections was performed as described previously (Mookerjee et al., 2006). A minimum of five bronchi selected according to size (150 – 350 μm luminal diameter) were analysed per mouse. The thickness of the bronchial epithelial layer in MT-stained slides was measured by tracing around the basement membrane and the luminal surface of epithelial cells and calculating the area between these lines. Total smooth muscle thickness in α-SMA stained slides was similarly measured by tracing around the outer extent of the airway smooth muscle layer in the submucosal region and around the basement membrane and the area between these lines calculated. Total areas were calculated by subtracting the inner area from the outer area. These areas were expressed per length (μm) of basement membrane to account for variation in bronchial diameters.

4.2.7 Quantitative Real-time PCR for gene expression analysis of lung tissue

RNA and subsequently cDNA was purified from mouse lung tissue samples as per 2.8 using β-mercaptoethanol. All qRT-PCR experiments were conducted using the same preparation of cDNA (Table 4.1). Taqman ® PCR primers (Life Technologies) were used to perform Quantitative RT-PCR. Samples were assayed with reaction volume of 7.5 μl in a 384 well optical well plate, in duplicate. The reaction volume in each well contained 2.5 μl cDNA, 0.375 μl Taqman ® primer, 3.75 μl Taqman Fast Advanced Master Mix and 0.875 μl RNA/DNase free water. The plate was sealed and spun at 400 X G for 1 min. qRT-PCR was performed on Quantstudio 7.
Table 4.1 Specific primers used in qRT-PCR experiments in Chapter 4.

<table>
<thead>
<tr>
<th>Experiment 1: Epithelial integrity markers</th>
<th>Experiment 2: Th2 immunity markers</th>
<th>Experiment 3: Surfactant protein markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZO-1 intracellular tight junction protein</td>
<td>IL-5</td>
<td>Trp63 transformation related protein 63; epithelial integrity</td>
</tr>
<tr>
<td>Muc5ac mucin marker</td>
<td>IL-13</td>
<td>Stfpc pulmonary-associated surfactant protein C</td>
</tr>
<tr>
<td>Ocel-1 occludin-1; tight junction protein</td>
<td>IL-33</td>
<td>Stfpd pulmonary-associated surfactant protein D</td>
</tr>
<tr>
<td>CDH1 cadherin-1; transmembrane protein</td>
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</tbody>
</table>

4.2.8 Statistical analysis

Statistical analyses were carried out as detailed in 2.9. Data were pooled from five independent experimental cohorts of mice and all data are expressed as mean ± SEM, where n represents one mouse. Comparisons were made using unpaired Student’s t-test and one-way ANOVA with Bonferroni post hoc where appropriate. P < 0.05 was considered statistically significant. Analyses were carried out using Graph Pad Prism™ (version 5.0).
4.3 Results

4.3.1 Neonatal co-infection facilitates long-term nasopharyngeal *Streptococcus pneumoniae* colonisation

There is emerging epidemiological data to suggest that neonatal pneumococcal colonisation can predispose to asthma in later life, due to detrimental effects on the developing immune system. The mechanisms by which respiratory virus can promote long term pneumococcal carriage are yet to be fully elucidated. To address this, an established model of neonatal co-infection was used, where IAV is known to disrupt clearance of SP leading to a transient increase in upper and lower airway bacterial carriage (Diavatopoulos et al., 2010). Neonatal mice were inoculated intranasally with vehicle, SP, IAV or SP/IAV, and outcomes were assessed in early adulthood (6 – 8 weeks) as described in Figure 4.1. Mice from all treatment groups were weighed twice weekly from age of weaning until endpoint. Early life infection with SP and/or IAV did not have any long lasting effects somatic growth or adult body weight in male and female mice across all groups (Figure 4.2). Male mice were slightly larger than female mice, however infections did not elicit any deficits in behaviour, grooming, socialisation and were well tolerated with no litter rejection by the dam following administration of infections.
Figure 4.2 Infant co-infection does not alter somatic growth.

Male and female C57BL/6 wildtype mice were infected intranasally *Streptococcus pneumoniae* (SP; 2000 CFU/ml or saline vehicle) at day 5 of life, and co-infected with influenza A virus (IAV; 7000 PFU/ml or saline vehicle) at day 12; resulting in vehicle, SP, IAV and SP/IAV treatment groups. Mice were weighed twice weekly from age of weaning (day 23 of life) (n = 5 – 9/group over several cohorts).

To determine the kinetics of SP carriage in the upper and lower airways, bacterial load in nasopharyngeal and lung homogenates were assessed at time points of 20, 30 and 40 days of life (Figure 4.3). In infant mice infected with SP alone, nasopharyngeal carriage was transient, and steadily declined to negligible levels by day 40. In contrast, co-infected mice displayed higher levels of SP upper airway carriage that persisted to Day 40 (39128 ± 11785 CFU in nasopharynx), and with a significant 1-log increase in load relative to mice infected with SP alone (1545 ± 1489 CFU in nasopharynx) (Figure 4.3A). In the lung, SP levels were negligible at all time points in both treatment groups (Figure 4.3B).
Figure 4.3 Nasopharyngeal colonisation following infant co-infection persists into adulthood.

Nasopharyngeal (A) and whole lung (B) homogenates were assessed for colony-forming units (CFU) at Day 20, 30 and 40 of life. Homogenates were serially diluted and incubated on horse blood agar plates overnight at 37 °C and CFU recorded from highest-countable dilution. Saline (sal; PBS treated) control values were obtained from untreated mice at Day 40 of life. *p<0.05, unpaired Student’s t test (n = 6 – 9).

The viral load in upper and lower airways was also assessed following neonatal IAV or co- infection by counting PFU in MDCK cells infected with nasopharyngeal or lung homogenates (Figure 4.4). At Day 20 of life, IAV was effectively cleared in co-infected mice, whilst negligible levels remained in the upper and lower airways of mice infected with IAV alone (Figure 4.4A, B).

Figure 4.4 Virus is cleared by Day 20 following early life infection.

Viral titres were assessed in nasopharyngeal (A) and lung (B) homogenates at Day 20 of life (8 days post IAV infection) as assessed by plaque-forming units (PFU) assayed in Madin-Darby canine kidney (MDCK) cells (n = 5 – 6).
The number of inflammatory leukocytes in the bronchoalveolar lavage fluid (BALF) was assessed in adult mice following infant infection (Figure 4.5). Total cell counts were comparable across all groups (p=0.07, one-way ANOVA), and levels were indicative of the inflammatory process being fully resolved (Figure 4.5A). Cells were mostly macrophages (> 99%), with low-level neutrophils (0 – 2 %), and these cell numbers were not altered between groups (Figure 4.5B, C). There was a significant 2-fold increase in lymphocyte counts in co-infected mice compared to vehicle control mice (Figure 4.5D).

![Graph A: Total cells](image)
![Graph B: Macrophages](image)
![Graph C: Neutrophils](image)
![Graph D: Lymphocytes](image)

**Figure 4.5 Elevated lymphocytes in bronchoalveolar lavage fluid following early life co-infection.**

Bronchoalveolar lavage (BALF) was assessed for total (A) cell counts at Day 40 using ethidium bromide nuclear stain, and differential (B, C, D) cell counts performed with DiffQuik staining. *p<0.05 compared with vehicle, one-way ANOVA with Dunnett’s post hoc (n = 5 – 11).
To determine whether the increase in BALF lymphocytes was associated with classical induction of T\textsubscript{H}2 immune response, gene expression of specific T\textsubscript{H}2 cytokines interleukin (IL)-5, IL-13 and IL-33 were measured via Taqman qRT-PCR in lung tissue. There were no significant alterations in expression of these cytokines following early life co-infection (Figure 4.6).

![Graphs showing gene expression of IL-5, IL-13, and IL-33](image)

Figure 4.6 Infant co-infection does not induce classical T\textsubscript{H}2 cytokine immune profile.

Taqman qRT-PCR was performed on lung tissue samples for assessment of gene expression of interleukin (IL)-5 (A), IL-13 (B) and IL-33 (C) following neonatal infections normalized to GAPDH housekeeping gene and expressed as fold-increase from vehicle treated control (n = 6 per group).

Consistent with the BALF immune profile and resolution of inflammation in adult mice, H&E revealed no obvious inflammatory cell infiltration into peribronchial or perivascular regions of lung tissue (Figure 4.7).
Figure 4.7 Infant co-infection does not induce persistent airway inflammation.

Haemoxylin & Eosin (H&E) stain images were captured on Olympus BX 52 microscope at 10X zoom (scale bar 200 µm) and are representative of n = 5 – 11 per group. AW = airway, BV = blood vessel.

4.3.2 Co-infection increased central airways resistance and hysteresivity

To assess whether early life infection resulted in functional impairment, *in vivo* lung function testing with a nebulised bronchoconstrictor MCh challenge was performed in adult mice (~7 weeks of age) that had been infected as neonates (Figure 4.8 – Figure 4.11). Infant co-infection did not significantly alter baseline lung function, however early life IAV infection reduced tissue elastance (Figure 4.8C). Newtonian resistance (Rn), which is equivalent to central airways resistance, was increased at the higher doses of MCh, peaking at 30mg/mL (Figure 4.9A) in co-infected mice. This was not accompanied with any significant changes at baseline in additional measures of lung function tissue damping or tissue elastance (Figure 4.9B, C).
Figure 4.8 Infant co-infection does not alter baseline lung function.

Lung function testing was performed using SCIREQ® Flexivent to assess baseline central airway resistance (Rn) (A), tissue damping (G) (B) and tissue elastance (H) (C). *p<0.05 compared with vehicle, one-way ANOVA with Bonferroni’s Multiple Comparison test (n = 10 – 12).
Figure 4.9 *In vivo* airway hyperresponsiveness in adulthood following exposure to co-infection in infancy.

Lung function testing was performed using SCIREQ® Flexivent to assess central airway resistance (Rn) (A), tissue damping (G) (B) and tissue elastance (H) (C) at baseline, with nebulised saline and MCh (3, 10, 30 mg/ml). *p<0.05* compared with vehicle Rn at 30 mg/ml, one-way ANOVA with Bonferroni's Multiple Comparison test (n = 3 – 11).
Central airways resistance to maximal methacholine challenge is increased following neonatal co-infection.

Lung function testing was performed using SCIREQ® Flexivent to assess maximal MCh-induced airways resistance (Rn) (A), tissue damping (G) (B) and tissue elastance (H) (C) is expressed as % change from nebulised saline values; dotted line represents vehicle mouse response to nebulized saline. *p<0.05 compared with vehicle, one-way ANOVA with Bonferroni’s Multiple Comparison test (n = 5 – 7).

Maximal MCh responses (30 – 100 mg/mL) were compared across all groups and presented as the percentage change above nebulised saline response (Figure 4.10). Following early life co-infection, Rn increased 2-fold at maximal MCh as compared to vehicle control mice (419 ± 48 % compared to 211 ± 35 %), and there was a trend for increased G in co-infection group (Figure 4.10A, B). At maximal MCh response, H was not altered in co-infected
mice relative to vehicle, however was reduced in mice treated with SP or IAV alone (Figure 4.10C).

Airway hysteresivity ($\eta$) is a measure of ventilation heterogeneity and peripheral function, and is defined as the ratio of G/H, therefore is not directly related to central airways resistance (Fredberg & Stamenovic, 1989). Hysteresivity remained constant with MCh challenge in vehicle-treated mice (88 ± 9%), but was significantly elevated at high MCh following exposure to IAV (196 ± 39 %) or co-infection in early life (176 ± 30 %) (Figure 4.11A,B).

Figure 4.11 Airway hysteresivity is increased following early life influenza A virus infection.

Lung function testing was performed using SCIREQ® Flexivent to assess hysteresivity ($\eta$), calculated as the ratio tissue damping / tissue elastance, at baseline, with nebulised saline and methacholine (MCh; 3, 10, 30 mg/ml) (A). Data also expressed as % change from nebulised saline values; dotted line represents vehicle mouse response to nebulized saline (B). *p<0.05 compared with vehicle, unpaired Student’s t test (n = 4 – 5).

4.3.3 Neonatal co-infection does not induce airway remodelling in adult mice

To assess whether altered airway responsiveness observed in vivo was due to remodelling processes occurring in the lung, a comprehensive histological analysis of lung tissue performed. Mucus and smooth muscle were quantified histologically and complemented with gene expression analysis of lung
tissue. Ab-PAS staining revealed no observable increase in goblet cells across all groups (Figure 4.12A), and the lack of goblet cell expansion was consistent with mucin marker Muc5ac gene expression being unchanged following neonatal infections (Figure 4.12B).

Figure 4.12 Neonatal co-infection is not associated with mucus hypersecretion.

Alcian blue-Periodic Acid Schiff (Ab-PAS) staining was performed on 5μm-thick lung sections from adult mice co-infected in infancy to assess goblet cell metaplasia (A, image representative of n = 5 – 6). Taqman qRT-PCR was performed on lung tissue samples for assessment of gene expression of mucin marker Muc5ac, normalized to housekeeping gene GAPDH and expressed as fold-increase from vehicle treated control (B, n = 6).
Quantification of αSMA-positive stain area surrounding airways (expressed relative to basement membrane area) revealed no significant alterations to smooth muscle bulk across the treatment groups (Figure 4.13). The size of airways selected for quantification analyses (5 airways/lung section) were consistent across all treatment groups (diameter >150 μm, mean 191 μm).

Figure 4.13 Early life infection does not alter smooth muscle bulk.

α-smooth muscle actin (αSMA) staining was performed on 5 μm-thick lung sections from adult mice co-infection in infancy to assess smooth muscle bulk (A, images representative of n = 5 – 11). AW = airway, BV = blood vessel. Quantification of brown αSMA-positive stain in sections pooled across multiple cohorts of mice was carried out on Aperio ImageScope software, and is expressed as area of positive stain (μm²) relative to basement membrane length (μm) (B).
Airway reactivity *in vitro* was investigated in PCLS, where perfused drug solutions can access the airway smooth muscle layer directly, in a manner that is similar to aerosolised MCh delivery *in vivo*. Lung slices were made from 6 – 8 week old female mice from each treatment group and small airway responses to contractile and dilator agents were assessed (Figure 4.14, Figure 4.15). Consistent with the absence of increased α-SMA-positive stain area, intrapulmonary airway responses to MCh *in vitro* were unaltered between treatment groups. MCh (3000 nM) induced a ~60% reduction in airway lumen area in all airways tested (Figure 4.14C). Small airway responses to an alternative constrictor agonist, endothelin-1 were similar across all groups, where Et-1 induced a comparable maximal reduction in lumen area as MCh but with greater potency (Figure 4.15A). Dilator response to chloroquine (CQ), a bitter taste receptor agonist routinely used in the laboratory, following a sub-maximal pre-contraction with MCh were also assessed (Figure 4.15B, C). CQ dilated small airways in lung slices from all treatment groups, with a slightly variable level of relaxation at the highest concentrations tested (Figure 4.15C).
Figure 4.14 Neonatal co-infection does not alter small airway reactivity to methacholine in vitro.

Precision-cut lung slices (PCLS) were prepared from adult mice co-infected in infancy. Representative images (A), representative time-course trace (B) and grouped data (C) for methacholine (MCh) concentration-response experiments in small airways in PCLS show reduction in airway lumen area, expressed as % initial airway lumen area, to increasing concentrations of MCh (n = 3 – 5).
Figure 4.15 Early life infections do not alter small airway contractile or dilator responses in precision-cut lung slices.

Precision-cut lung slices (PCLS) were prepared from adult mice co-infected in infancy. Small airway contractile responses to endothelin-1 (Et-1; A, n = 4 – 5) are expressed as % initial airway lumen area. Dilator responses to chloroquine (CQ) (C, n = 4 – 5) are expressed as % relaxation of relevant sub-maximal methacholine (MCh; 300 nM) pre-contraction (B).

Since mucin or smooth muscle abnormalities were not detected via histological and/or gene expression analyses, the potential for epithelial disruption as a factor contributing to increased airway responsiveness in vivo was considered. Histologically, the epithelium appeared normal (Figure 4.7, Figure 4.16A) and when quantified, the epithelial thickness was comparable in all treatment groups (Figure 4.16B).
Figure 4.16 Infant co-infection does not alter epithelial thickness.

Masson's trichrome (MT) staining was performed on 5 µm-thick lung sections from adult mice co-infected in infancy to assess epithelial thickness (A, images representative of multiple cohorts). AW = airway, BV = blood vessel. Quantification of pink MT-positive stained epithelium (B) was carried out on Aperio ImageScope software and is expressed as area of positive stain (µm²) relative to basement membrane length (µm) (n = 5 – 11 per group).

Gene expression of epithelial integrity markers including ZO-1, Occludin-1 and E-cadherin was also investigated as a potential cause of AHR (Figure 4.17). There were no significant differences in expression of these markers across infant infection treatment groups relative to vehicle treated mice, although there was a small trend towards a reduction in ZO-1 and Occludin-1.

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in co-infected mice (Figure 4.17A, B). Total protein concentration in BALF was determined by standard protein assay and was found to be unchanged following infant co-infection (Figure 4.17D).

Finally, the potential for neonatal co-infection to alter the composition of the cellular makeup of the respiratory epithelium using markers of type II cells or the basal cell marker Trp63 was assessed. Gene expression of surfactant protein C (SP-C; Stfpc), D (SP-D; Stfpd) and Trp63 in lung tissue were unchanged across all treatment groups (Figure 4.18), suggesting that the cellular architecture of the lung is not permanently altered by early life co-infection.

Figure 4.17 Infant co-infection does alter markers of epithelial integrity.

Taqman qRT-PCR was performed on lung tissue samples for assessment of gene expression of epithelial integrity markers ZO-1 (A), occludin-1 (B) and E-Cadherin (C) was normalized to GAPDH housekeeping gene and expressed as fold-increase from vehicle treated control (n = 6). Protein concentration was assessed in bronchoalveolar lavage (BALF) via standard ELISA and is expressed as µg/ml (D, n = 6 - 7).
Figure 4.18 Infant co-infection does not alter surfactant protein markers.

Taqman qRT-PCR was performed on lung tissue samples for assessment of gene expression of surfactant protein markers pulmonary-associated surfactant protein C and D (Stfpc; A Stfpd; B) and transformation related protein 63 (Trp63; C) was normalized to GAPDH housekeeping gene and expressed as fold-increase from vehicle treated control (n = 6).
4.4 Discussion

In this study, early life co-infection resulted in persistent upper airway SP carriage into adulthood. This was accompanied by a small but significant increase in central airways resistance following aerosolised MCh challenge in adult co-infected mice, in contrast to mice infected with a single respiratory pathogen. An increase in airway hysteresivity was observed in co-infected mice, which appeared to be driven by early life IAV infection. A significant increase in BALF lymphocytes was not associated with altered a classical Th2 cytokine response. Airway hyperresponsiveness in vivo was not maintained in lung slices in vitro, nor was it associated with a thickened or leaky epithelium, goblet cell hyperplasia, altered epithelial marker expression or increased smooth muscle bulk. Critically, neonatal IAV facilitated long-term pneumococcal carriage resulting in airway dysfunction, and may represent a significant early life event contributing to the development of asthma. Importantly in this model, due to logistical reasons, all mice within a litter were allocated to the same treatment group, which may have introduced bias in interpretation of results, as the groups may not be truly independent. One method to circumvent this was to repeat this model multiple times, and as such all data presented in this chapter are pooled from five independent cohorts of treated mice.

Prospective childhood cohorts have now identified viral respiratory infections as a major risk factor for developing persistent wheeze and asthma in individuals with co-existing aeroallergen sensitization (Sly et al., 2010). Post natal lung growth represents an important susceptibility period where the combination of viral infections and aeroallergen sensitisation in children can lead to persistent pathological changes and respiratory dysfunction (Holt & Sly, 2012). The role of co-infection in this context is less clear; however there is emerging data to suggest that nasopharyngeal colonisation may increase the risk of developing asthma. In a recent study, the presence of bacterial pathogens including SP in the nasopharyngeal compartment during
infancy was identified as a significant risk determinant for febrile lower respiratory infections and later asthma development (Teo et al., 2015). The pneumococcal vaccine can reduce risk; however eliminating vaccine-targeted serotypes creates a niche where other serotypes and pathogenic bacteria can invade, and suggests a potential causal link between the use of antibiotics and development of asthma (Huffnagle, 2010; Teo et al., 2015).

There are over 90 serotypes of SP, with high genetic diversity. SP commonly colonises the upper airways, where it can lead to persistent carriage, particularly in infants where colonisation rates in excess of 60% have been reported (Bogaert et al., 2004). This colonisation, although common, is often transient and asymptomatic. There are multiple risk factors for impaired SP clearance, however co-existence of respiratory viruses markedly increase pneumococcal load during the acute phase of infection and also facilitates SP transmission (Diavatopoulos et al., 2010). Clinically, there is a strong but short-lived association between SP and viral infection, where influenza infection can increase one's susceptibility to secondary SP infection by 100-fold (Shrestha et al., 2013). This effect appears not to be virus-specific, as RSV infection can enhance pneumococcal virulence (Smith et al., 2014). Here, in an infant model of co-infection, IAV facilitates long-term nasopharyngeal colonisation that persists into adulthood, in contrast to mice infected with SP alone, where the pneumococcus was cleared before day 40.

Pneumococcal infection is associated with a robust acute inflammatory response, and clearance of SP is dependent on recruitment of leukocytes to the airways and engagement of humoral-mediated immunity (Wilson et al., 2015). It has been established that during co-infection with IAV and SP, interferon-dependent anti-viral responses can alter acute cell-mediated immunity to SP leading to a marked reduction in pneumococcal clearance (Sun & Metzger, 2008). Here, infant IAV infection has lasting consequences on upper airway pneumococcal colonisation into adulthood. There was no evidence for significant invasive dissemination of SP into the lower airways.
Additionally, IAV was effectively cleared before adulthood, as lung viral titres were negligible by day 20 of life, consistent with a similar previous study (Diavatopoulos et al., 2010).

Lung hysteresivity is a term used to reflect the mechanical coupling between energy dissipative forces and tissue-elastic properties in the parenchyma. Remodelling of the tissue increases hysteresivity in both emphysema and fibrosis in a way that correlates with the volume proportion of collagen, however the physical basis of this phenomenon is not well understood (Suki & Bates, 2011). Elevated hysteresis has been observed following acute IAV infection in adult mice (Larcombe et al., 2011). The present data suggests that there may be microstructural changes contributing to ventilation heterogeneity. However, there were no changes in baseline lung function, suggestive that impaired alveolar growth was not contributing to altered reactivity. Additionally, there was no evidence for altered size of small airways as quantified during histological analyses and lung slice experiments. Preliminary histological assessment indicated that alveolar size was not grossly altered; alveolar enlargement in adulthood can be driven by neonatal (24 h old pups) Chlamydophila pneumoniae respiratory bacterial infection (Horvat et al., 2010).

In order to provide mechanistic insight into in vivo AHR, we assessed a number of parameters that are known to contribute to altered airway responsiveness. Histologically, there was no evidence for remodelling of the airways, with no increase in goblet cells or Muc5ac gene expression, nor increase in ASM bulk. Functionally, ASM appeared to be normal as assessment of MCh constrictor responses in isolated airways in precision-cut lung slices were not altered following infant co-infection. Dilator responsiveness to CQ was assessed as an alternative bronchodilator to isoprenaline, which induces low levels of small airway relaxation (Chapter 3, Figure 3.8), however dilator responsiveness appeared unaltered following early life co-infection. IAV-induced disruption of the airway-epithelial barrier
has previously been reported to promote AHR due to increased MCh accessibility to smooth muscle (Bozanich et al., 2008). The potential for altered expression and/or distribution of MCh receptors was not assessed in the present study, but may provide further insight into altered airway responsiveness observed in vivo but not in vitro. Additionally, co-infection with virulent strains of IAV and SP has been shown to lead to greater loss of basal epithelial cells and poor re-establishment of the normal airway epithelium during reparative processes (Kash et al., 2011). There was no evidence for disruption of the epithelial barrier surrounding airways, and expression of the epithelial integrity markers (ZO-1, occludin-1 and E-cadherin) were not altered following neonatal co-infection. The strain of virus used in this study is considered to be moderately virulent, and so may not promote long term lung damage as is seen with more virulent pandemic strains.

Surfactant protein D (SP-D) is epithelial-derived immune modulator that is highly expressed in the lung. SP-D can alter macrophage activity to promote pathogen elimination and also regulate pro-inflammatory mechanisms. SP-D knockout mice characteristically display chronic lung inflammation and worsening emphysema with age (Forbes & Haczku, 2010). It has been suggested that reduced SP-D production results in heightened airway responsiveness in vivo (Atochina et al., 2003), however there were no significant changes in gene expression of SP-C or SP-D in the present study, although there was a trend for reduction of these markers following IAV infection.

Residual lung function defects in adulthood have previously been observed in BALB/C mice that were infected with IAV in early life, long after viral clearance (Larcombe et al., 2011). The dissimilar response to IAV alone in the present study may reflect mouse and viral strain differences, where BALB/C mice inoculated with Mem/71 were previously been used, in contrast to this study where C57BL/6 mice infected with HKx31 were assessed. BALB/C
mice are known to be more sensitive than C57BL/6 mice to developing AHR to MCh challenge in asthma models (Takeda et al., 2001). Conversely, mice infected with SP alone are protected against hallmark features of models of allergic airways disease, thereby reducing AHR (Preston et al., 2011).

Acute inflammatory responses to IAV are also known to transiently alter lung function. Interestingly, mice deficient in neutrophils display impaired lung function associated with an increase in airway resistance, tissue damping and tissue elastance as a consequence of impaired viral clearance and more severe histopathology (Tate et al., 2009). Hence, an appropriate acute inflammatory response can prevent significant structural changes underlying respiratory functional abnormalities. Infection of neutropenic mice was also characterized by an increase in lung oedema (Tate et al., 2009). Consistent with this study, an increase in permeability of the alveolar–capillary barrier was related to AHR and this response was shown to be transient in adult mice as lung function normalised with resolution of inflammation, oedema and tissue repair (Bozanich et al., 2008). In the present study, inflammation had resolved with the exception of a small but significant increase in BAL lymphocytes in co-infected mice. The increase in lymphocytes was not associated with an increase in IL-5 or IL-13 expression; hence co-infection did not induce a persistent classic Th2 response. One limitation of this study was the lack of comprehensive flow cytometric analysis of this increase in lymphocytes, which may have yielded insight into the changes occurring within the T cell compartment. SP is known to induce expansion of multiple T lymphocyte subsets including mucosal innate T cells that contribute to control of pneumococcus, and the current data suggest that co-infection with IAV promotes lymphocyte accumulation. However, the phenotype of lymphocyte subsets that may be contributing to pneumococcal carriage remains to be assessed. It has previously been shown that bacterial components of SP can initiate the accumulation of T regulatory cells, which suppress allergic airways disease in response to ovalbumin sensitisation and challenge (Thorburn et al., 2012). In the present model, the presence of SP
did not confer protection to AHR, possibly as an allergic adjuvant was not used to initiate lung function abnormalities.

### 4.4.1 Conclusions from Chapter 4

In summary, co-infection caused a small but significant increase in change in central airway resistance that was not associated with conventional airway remodelling such as mucus overproduction, smooth muscle thickening or epithelial leakage. An increase in hysteresivity was observed in both IAV and co-infected mice. Hence, it is possible that co-infection in infancy is causing distinct structural changes that contribute to ventilation heterogeneity, which persists into adulthood. The implications of the lasting effects of early life co-infection in combination with aeroallergen sensitisation will be comprehensively investigated in Chapter 5.
CHAPTER 5

Neonatal pneumococcal lung colonisation facilitated by acute viral infection induces severe asthmatic phenotype in adulthood


5.1 Introduction

5.1.1 Viral infection and allergic sensitisation in early life

It is now evident that the development of asthma in childhood is multifactorial, driven by genetic predisposition and multiple environmental exposures including allergic sensitisation and viral respiratory infections. Viral infections in early life have the capacity to cause significant lung injury and inflammation, and infants represent a particularly vulnerable group for virus-associated hospitalisations (Tumpey et al., 2005; Zhou et al., 2012). However, there is mounting evidence that virus-associated wheeze in early life alone is insufficient to initiate asthma, particularly as almost all children have experienced viral respiratory tract infection by 2 years of age (Blomqvist et al., 2002). Moreover, large cohort studies have revealed that early life sensitisation to cat or house dust mite (HDM) allergens is not sufficient to cause asthma, wheeze, or bronchial hyperresponsiveness (Lau et al., 2000), and there is no consistent evidence that allergen alone is an independent risk factor for asthma development on a population scale (Pearce et al., 2000).

Holt & Sly have proposed that early life exposure to both respiratory viral infection and aeroallergen synergistically disturbs lung development resulting in a uniquely potent ‘two-hit’ risk factor for asthma (Figure 5.1) [reviewed in (Holt & Sly, 2012)]. Severe, persistent asthma is 10– to 30-fold more likely to develop in children who suffer a significant viral lower respiratory tract infection (LRI) in the background of pre-existing aeroallergen sensitisation (Kusel et al., 2007; Kusel et al., 2012).
Importantly, this elevated risk is dependent upon the aeroallergen and infectious exposures occurring in the first few years of life. The Perth Childhood Asthma Study (CAS) cohort study showed early life viral infection was associated with an increased risk of asthma only in children who had aeroallergen sensitization before 2 years of age (Kusel et al., 2007). This finding was mirrored in the Childhood Origins of Asthma (COAST) study where the strongest asthma risk was identified in children who had viral wheeze and were also sensitised with aeroallergen during first 3 years of life (Jackson et al., 2008). Notably, elevated asthma risk was only observed where viral wheeze occurred on a pre-existing allergic sensitisation, suggesting a causal relationship where early sensitisation is a risk factor for developing viral wheeze and asthma thereafter (Jackson et al., 2012; Oddy et al., 2002). Furthermore, in children with confirmed asthma, exacerbation of disease requiring hospitalisation is most likely when viral infection and high allergen exposure occurs in unison (Murray et al., 2006).

As well as the evidence for a causal relationship between allergy and virus within the ‘two-hit’ hypothesis, asthma risk appears also to be affected by the
genetics and virulence of the wheeze-inducing virus. Historically, respiratory syncytial virus (RSV) has been considered the most important respiratory pathogen in asthma causation. RSV has only 2 serotypes, and so whilst a primary infection can invade the lower airways, an antibody response can be mounted and re-infections are less severe and more likely to be limited to the upper respiratory tract (Borchers et al., 2013; Henderson et al., 1979). Conversely, there are ~180 rhinovirus (RV) serotypes so primary RV infections can occur again and again, particularly with highly virulent strains, such as RV-C (human rhinovirus group C), which has been identified in the majority of children hospitalised with an acute episode of moderate-severe asthma (Bizzintino et al., 2011). RV has been increasingly recognised and repeatedly implicated as a highly significant pathogen in childhood wheezing that is an early, strong predictor of asthma (Jackson et al., 2008; Jackson et al., 2016; Lemanske et al., 2005).

5.1.2 Allergen and viral infection worsen type 2 inflammation

There are multiple mechanisms by which early life allergic sensitisation and viral infections synergistically worsen Th2 inflammation and predispose children to more severe asthma. Persistence of inflammation in response to a respiratory virus is thought to contribute to the accumulation of pathological remodelling in individuals with pre-existing aeroallergen sensitisation (Holt & Sly, 2012). Clinically, underlying allergic inflammation can directly enhance virus-associated airway hyperresponsiveness (AHR) (Gern et al., 1997). However, the interactions between anti-viral and atopic pathways are complex, and much of the current understanding of these interactions is derived from murine models of disease (Subrata et al., 2009). In experimental allergic airways models, rhinovirus infection was shown to exacerbate Th2 immunity associated with interleukin (IL)-4 and IL-13, increased mucus production and AHR in adult mice sensitised to ovalbumin (Bartlett et al., 2008). In an alternate model, influenza A virus (IAV) was shown to enhance eosinophilic inflammation via expansion of Th2 cells in
adult mice subsequently exposed to HDM (Al-Garawi et al., 2009). Enhanced Th2 immunity in response to IAV infection was associated with increased IL-5, but not IL-13 levels in this model. Neonatal IAV infection also exacerbated robust allergen-specific immunity and lung remodelling in mice exposed to repeated HDM challenge, which persisted into adulthood (Al-Garawi et al., 2011).

Th2 inflammation can in turn reduce tight junction interactions and compromise epithelial barrier function, increasing susceptibility to viral infection (Hammad & Lambrecht, 2015). Furthermore, primary epithelial cells from atopic children mount improper antiviral responses when exposed to RSV and produce low amounts of type I interferon (IFN), with higher viral shedding compared to healthy controls (Spann et al., 2014). Additionally, both viral infections and allergens can enhance production of IL-33 by epithelial cells, thereby promoting Th2 inflammation and remodelling processes. Importantly, the effects of IL-33 are steroid-resistant and associated with a more severe phenotype of asthma (Jackson et al., 2016).

Th17 cells are functionally distinct from Th2 cells, and have recently been associated with more severe, steroid-insensitive forms of asthma (Al-Ramli et al., 2009). Th17 responses following production of IL-17A from multiple cellular sources initiate recruitment of neutrophils to the airways, induce mucus cell metaplasia and have pleotropic effects on airway smooth muscle (ASM) culminating in airway narrowing (Newcomb & Peebles, 2013). Acute RSV infection in neonates has been associated with elevated Th17 cells (otherwise absent in healthy counterparts) in the airways and blood (Stoppelenburg et al., 2014). The role of Th17 responses in early life allergen sensitisation and viral infections that culminates in asthma risk remains to be fully elucidated.
5.1.3 Role of early life bacterial infections

In contrast to respiratory viruses, commensal bacteria were considered to play a protective role in development of asthma. This proposal was supported by the hygiene hypothesis, where exposure to a broad range of environmental microbes appeared to confer protection (Strachan, 1989). It is now emerging that the protective effects may be coordinated by the developing infant gastrointestinal microbiota which can educate the immature immune system (Holgate et al., 2015).

The role of bacterial respiratory pathogens in asthma risk is less clear, and appears to be dependent upon the type of pathogen and aeroallergen exposure (Stein et al., 2016), however there is emerging evidence that disruption to the airway microbiota may predispose individuals to airways disease in adulthood (Gollwitzer et al., 2014). Early life infection with Chlamydia muridarum has been shown to increase the severity of ovalbumin-induced AHR associated with an increase in mucus hypersecretion (Horvat et al., 2007). In contrast, Haemophilus influenzae infection suppressed ovalbumin-induced AHR through down-regulation of T\textsubscript{H}2 immunity, however increased neutrophilic inflammation via IL-17A dependent mechanisms (Essilfie et al., 2011). Streptococcus pneumoniae (SP) infection and components of SP including conjugate vaccine were also shown to suppress T\textsubscript{H}2 immunity and AHR to ovalbumin challenge by inducing T regulatory cells (Preston et al., 2011; Thorburn et al., 2010). However, the potential protective effects of bacterial components may be influenced by bacterial pathogenicity, asthmatic phenotype as well as type of aeroallergen sensitisation. A significantly higher prevalence of asthma has been reported in children colonised in infancy with SP, M. catarrhalis, H. influenzae or combination in the hypopharyngeal region (Bisgaard et al., 2007). SP carriage specifically has been detected in 50% of asthmatic children, independent of vaccination status (Esposito et al., 2016). Taken together, this data suggests that protection against colonisation by pneumococcal conjugate vaccine may
diminish over time, or that bacterial clearance is impaired in asthmatics. This observation may also reflect serotype replacement in response to vaccination.

5.1.4 Bacterial and viral co-infection may worsen outcomes

The presence of colonising bacteria in the respiratory tract can influence asthma risk, with mounting evidence for worsened outcomes with bacterial and viral co-infection, as discussed in 1.4.2 (Teo et al., 2015). Respiratory viruses are known to impair immunity to bacteria leading to bacterial outgrowth, changing the composition of the airway microbiome, hence it is likely that both the virus and secondary changes in airway bacteria contribute to respiratory symptoms and airway obstruction (Jackson et al., 2016). A recent prospective study screened for common airway pathogenic bacteria and viruses during acute wheezy episodes in the first 3 years of life and identified both viruses and bacteria in 55% of episodes, which exceeded the prevalence of episodes caused by virus or bacteria alone (Carlsson et al., 2015). Additionally, the CAS cohort has associated transient nasopharyngeal bacterial colonisation following acute viral infection with spread to the lower airways and severity of inflammatory symptoms, and importantly, has shown that early life asymptomatic SP colonisation was a strong predictor of asthma (Teo et al., 2015). In Chapter 4, neonatal co-infection resulted in persistent nasopharyngeal SP carriage associated with AHR into adulthood. The role of neonatal co-infection in the setting of allergic sensitisation is not yet defined and may provide novel insight into clinically relevant pathology.

5.1.5 Modelling allergic airways diseases experimentally with house dust mite

Laboratory animals do not naturally develop allergic asthma, and so airway dysfunction and inflammatory processes must be initiated via administration of allergen, infection or combination. Whilst many murine models utilise ovalbumin (OVA), a glycoprotein obtained from chicken eggs, this allergen is
not associated with human allergy, and requires intraperitoneal sensitisation with a chemical adjuvant prior to OVA challenge (Kumar et al., 2008). Mice can become tolerant to prolonged OVA exposures, where allergen-specific AHR reduces and inflammation resolves (Kumar et al., 2008). The relatively short life span of mice may reflect the more acute nature of these models, as most lack signs of chronic inflammation within the airway wall, remodelling in the epithelium, sub-epithelial fibrosis and long term AHR, with exception of those requiring months of allergen challenges. Whilst the OVA model has its limitations, it is a strong inducer of \( T_H2 \) immunity and remains a robust model to evaluate mediators that can counteract that actions of \( T_H2 \) mediated remodelling processes. However, \( \sim 50\% \) of asthmatics suffer from atopic asthma without evidence of \( T_H2 \) response (McGrath et al., 2012), and so mouse models with alternate inflammatory profiles are required to better understand asthma in this population of non-\( T_H2 \) patients.

More recently, there has been focus on the more clinically relevant allergen HDM, as up to 85% of young asthmatics are allergic to HDM and have elevated HDM-specific IgE (Gregory & Lloyd, 2011). Clinically, the allergenic potential of HDM lies within the mites themselves, as well as their faecal pellets, which both contain proteolytic enzymes such as cysteine proteases. Importantly, HDM is biochemically complex and has the potential to activate both the innate and adaptive immune system (Gregory & Lloyd, 2011). HDM allergen challenge is usually administered intranasally or intratracheally, for days, weeks or months, the latter resulting in severe eosinophilia, collagen deposition and AHR that was not reversed following HDM cessation (Johnson et al., 2004). Neonatal IAV infection primes the lung to develop a HDM-induced asthmatic phenotype consisting of pronounced inflammation, AHR, goblet cell hyperplasia and peri-bronchial collagen deposition that persists into adulthood (Al-Garawi et al., 2011). Additionally, these changes that mimic hallmark features of paediatric asthma develop in HDM-challenged mice in parallel, in the first few weeks of age (Saglani et al., 2009).
Whilst more clinically relevant, HDM mouse models of allergic airways disease are not without their shortcomings. There are clear differences in lung size, morphometry and posture between humans and mice, and branching patterns may affect the deposition of aerosol allergens in the lung leading to skewed analysis of airway dysfunction. Some have speculated whether the inflammation caused by intranasal HDM challenge is due to the allergen itself, or merely a consequence of repeated nasal insult with an inflammatory agent, and so validation with systemic administration of the allergen may be required (Birrell et al., 2010). Nonetheless, the use of HDM in mouse models has yielded important insights into human asthma, including how epithelial cells and dendritic cells communicate to drive allergic airway inflammation (Hammad et al., 2009).

5.1.6 Aim

The aim of this study was to establish the role of neonatal bacterial and viral co-infection on susceptibility to allergen-induced airway dysfunction. We sought to develop and characterise a novel experimental model of nasopharyngeal neonatal co-infection with SP and IAV, superimposed onto subsequent repeated exposure to the aeroallergen, HDM.
5.2 Methods

5.2.1 Animal ethics and monitoring

Male and female BALB/C and C57BL/6 mice were used in this study as approved by the Animal Ethics Committee of the University of Melbourne (#1413288). Advanced pregnant BALB/C or C57BL/6 dams were obtained from Animal Resources Centre, Western Australia. The housing and monitoring of dams and pups are detailed in 2.1. Infant mice were weaned and at 3 weeks of age, separated by sex and were housed 1 – 4 per cage. Mice were monitored and weighed a minimum of five times weekly.

5.2.2 Preparation of infections

SP (EF3030, serotype 19F, a clinical isolate) and IAV (HKx31, H3N2) were used in infant co-infection and aeroallergen sensitisation studies, see 2.2 for bacterial and viral strain culture conditions.

5.2.3 Preparation of house dust mite

HDM (*Dermatophagoides pteronyssinus*) was obtained from Greer Laboratories (Charlotte, NC, USA) as lyophilized powder, which was dissolved in sterile PBS to protein concentration 5.25 mg/ml and aliquots were stored at -80 °C. Allergen was diluted in sterile PBS to permit administration of 10 µg HDM (protein content) in a 10 µl delivery volume.

5.2.4 Model of infant co-infection and aeroallergen sensitization

To control for potential variability caused by gender and litter size, only female BALB/C mice born to litter sizes restricted to 5 – 7 pups were used. Male BALB/C and C57BL/6 male and female mice of any litter size were used in the initial pilot experiments only (5.2.4.1). SP (2000 CFU) and IAV (500 PFU) were prepared in sterile PBS to delivery volume 3 µl. At 8 days of age, all infant mice within the litter received intranasal administration of SP (or PBS vehicle), followed by IAV (or PBS vehicle) at 15 days of age. Infant mice
were restrained, without anaesthesia, whilst the infection was applied slowly to the nostril to allow mice to inhale the suspension. 5 – 6 days following IAV, at 20 – 21 days of age, female mice were weaned and male mice were culled. Weaned female mice were sensitized to 10 µg HDM aeroallergen 5 days per week for 3 weeks. Mice were placed into an airtight plastic induction chamber with 1.0 – 2.0 L/min flow rate of O$_2$ containing 2 – 3% isoflurane (Henry Schein Isothesia, Provet AU) to induce light anaesthesia, as tested using the righting reflex. Anaesthetized mice were restrained by hand whilst the HDM treatment (or sterile saline vehicle) was applied slowly (10µl volume, using a pipette) onto the top of the nostril to allow the mice to inhale the suspension. Mice were monitored until anaesthesia resolved (~1 min), and weighed daily during the study. Mice received HDM treatments for ≥ 3 consecutive days prior to end point, which was assessed 24 hours after final HDM administration (Figure 5.2).

**Figure 5.2 Model of infant co-infection and house dust mite allergen sensitization.**

This model yielded 8 treatment groups in total; saline, *Streptococcus pneumoniae* (SP) alone, influenza A virus (IAV) alone, and co-infection (SP + IAV) and subsequently sensitised with saline or house dust mite (HDM). Mice were monitored for signs of distress and weighed 5 times weekly throughout the study. This model was carried out in female BALB/C mice with all outcomes assessed 24 hours following final saline or HDM administration.
5.2.4.1 Development of model – pilot cohorts

Prior to establishing the above described model, C57BL/6 mice were used and the infant co-infection protocol applied as detailed in 4.2.2, where 5 day old C57BL/6 pups were infected with SP (~2000 CFU), co-infected with IAV (~7 PFU) and combined with 5 µg HDM treatments commencing at day 17–18 of age, 3 times weekly, for 4 weeks. As this treatment regimen failed to initiate a robust HDM-specific immune response, the model was altered so that SP treatment occurred at 8 days of age, IAV infection was elevated to 500 PFU administered at 15 days of age, following which 10 µg HDM treatments commenced at day 20–21 of age, 3 times weekly, for 4 weeks (Figure 5.3). This cohort failed to display a robust IAV-induced HDM exacerbation, which is in line with C57BL/6 strain reported as “less allergic”, and so an ethics amendment was made to permit this model to be completed in BALB/C mice, which readily develop IgE-specific allergic responses to HDM exposures (Shinagawa & Kojima, 2003).

5.2.5 Collection of specimens

All outcomes were assessed 24 hours following final HDM treatment (or saline vehicle, 14–16 treatments in total), when mice were approximately 6 weeks of age.

5.2.5.1 Measurement of airway reactivity in vivo

Mice were anaesthetized with ketamine/xylazine as per 2.3. In vivo airway responsiveness was assessed at baseline and in response to nebulized saline and a single, maximal methacholine challenge (MCh, 30 mg/ml, as defined in Chapter 4) as per 2.3.

5.2.5.2 Bronchoalveolar lavage

Immediately following lung function testing, mice were removed from the ventilator and euthanized via overdose of sodium pentobarbitone (0.20 ml of 60 mg/ml i.p.). Bronchoalveolar lavage (BAL) fluid was collected as detailed
in 2.5. Total and differential cell counts were performed and remaining BAL was separated into BALF and BAL cell pellet and stored at -80°C until use as per 2.5.2.

5.2.5.3 Lung tissue collection

Following BAL collection, the left lobe was isolated, placed into a cassette and stored in neutral buffered formalin (10%) overnight, before being transferred into 70% EtOH prior to embedding and sectioning. Remaining lung tissue was placed in an eppendorf tube, snap frozen in liquid nitrogen and stored at -80°C until use.

5.2.5.4 Collection of nasopharyngeal tissue for colony-forming unit assay

Nasopharyngeal tissue was homogenized and serial dilutions of homogenate cultured overnight on horse blood agar (HBA) plates to determine colony-forming units (CFU; bacterial load) as detailed in 2.6, using HBA plates containing 5 µg/ml gentamycin to inhibit growth of contaminant microbes (Medical Preparation Unit, University of Melbourne).

5.2.5.5 Assessment of tibia lengths

The right tibia bone was isolated and stored in sodium hydroxide (1 M) solution overnight at 37 °C to digest remaining tissue, then blotted dry and the length measured (blinded) using digital callipers (Traceable ISO 17025, Fisher Scientific, UK).

5.2.6 Lung tissue analyses

5.2.6.1 Quantitative reverse transcription polymerase chain reaction for gene expression analysis of lung tissue

RNA and subsequently cDNA was purified from mouse lung tissue samples using Trizol RNA extraction procedure as per 2.8. All qRT-PCR experiments were conducted using the same preparation of cDNA (Table 5.1). Taqman ®
PCR primers (Life Technologies) were used to perform Quantitative RT-PCR. Samples were assayed with reaction volume of 7.5 µl in a 384 well optical well plate, in duplicate. The reaction volume in each well contained 1.5 µl cDNA, 0.375 µl Taqman ® primer, 3.75 µl Taqman Fast Advanced Master Mix and 1.875 µl RNA/DNase free water. The plate was sealed and spun at 400 X G for 1 min. qRT-PCR was performed on Quantstudio 7. GAPDH was used as housekeeping gene.

Table 5.1 Specific primers used in qRT-PCR experiments in Chapter 5.

<table>
<thead>
<tr>
<th>Q-PCR genes</th>
<th>Description</th>
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</thead>
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<tr>
<td>Muc5ac</td>
<td>mucin markers</td>
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<tr>
<td>Muc5b</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>Key interleukins (IL) that drive classical Th2 immune response</td>
</tr>
<tr>
<td>IL-13</td>
<td></td>
</tr>
<tr>
<td>IL-33</td>
<td>granulocyte-macrophage colony stimulating factor (GM-CSF)</td>
</tr>
<tr>
<td>CSF2 (GM-CSF)</td>
<td></td>
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<td>interleukin 17-α</td>
</tr>
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</tr>
<tr>
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<td>granulocyte colony stimulating factor (G-CSF)</td>
</tr>
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<td>chemokine (C-X-C motif) ligand 1 and 2</td>
</tr>
<tr>
<td>CXCL2</td>
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5.2.6.2 Quantitative real-time PCR for Streptococcus pneumoniae in lung tissue

After the aqueous phase lysate was removed from each tube following Trizol extraction protocol as detailed in 2.8.1, the remaining phase was used for DNA extraction. 0.3 ml 100 % ethanol was added to each tube and mixed well by vortexing and left at RT for 2 – 3 min. Samples were centrifuged for 10 min at full speed at 4°C, and the supernatant discarded. The remaining pellet was washed with 1 ml 0.1M sodium citrate (Merck) in 10 % ethanol for 30 min at RT, mixing well with vortex several times over the 30 min. Samples were centrifuged for 10 min at full speed at 4°C and supernatant discarded. This wash and centrifuge step was repeated, following which the pellet was resuspended in 1.5 ml 70% ethanol and left at RT for 20 min. Samples were
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centrifuged for 10 min at full speed at 4°C and supernatant discarded and remaining pellet air dried until no ethanol was visible (5 – 10 min). The pellet was resuspended in 100 µl diethylpyrocarbonate-treated water (Thermo Fisher) and incubated for 30 min at 65 °C using a dry block heater, gently mixing several times over the 30 min. Samples were centrifuged for 10 min at full speed at 4°C and the DNA-containing supernatant transferred to a new set of microcentrifuge tubes.

Microbial DNA qRT-PCR was performed on these samples using Qiagen kit for SP. A serial dilution of standards was prepared using the kit SP DNA standard (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, equivalent to 47000CFU, 4700CFU, 470CFU, 47CFU, 4.7CFU respectively). Samples were assayed in duplicate in 384 well plate with total reaction volume 8.33 µl. The reaction volume for samples to be assessed contained 4.17 µl microbial qRT-PCR mastermix, 0.33 µl microbial DNA qRT-PCR assay and 3.83 µl genomic DNA sample. The plate was sealed and spun at 400 X G for 1 min. QuantStudio™ Real-Time PCR software was set up to run the following PCR experiment: activation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 2 min. The CFU value (S) given from the software was used to calculate total CFU for each sample. Total CFU = S/3.83×100. This value was normalized to tissue weight and the total CFU by bacterial load expressed as CFU/100mg.

5.2.6.3 Myeloperoxidase assay

10 mg ground frozen lung tissue was resuspended in myeloperoxidase (MPO) extraction buffer MEB (50mM Potassium Phosphate (pH 6), 0.5% HTA-B [hexa-decyl-trimethyl ammonium bromide, Sigma], and 10mM EDTA) to a final concentration of 50mg/mL. The tissue was homogenised by drawing up with 1 ml pipette tip 10 times, followed by two freeze/thaw cycles by snap freezing in liquid nitrogen and thawing in 37 °C water bath. Thawed samples were immediately transferred to centrifuge and spin at
16,000 XG at 4 °C for 20 min. 10 µl of the supernatant from each sample was plated into each well of a 96 well plate in duplicate. ECL western reagent (Pierce, Thermo Scientific AU) was prepared by mixing equal volumes of A and B reagent and was protected from light. Immediately prior to initiating assay plate read, 50 µl ECL western reagent was added to each well using a multipipette for rapid addition. The plate was read using Flexstation III (Molecular Devices, CA, USA) set to measure chemiluminescence every 60 sec over a 15 min period. The change in relative fluorescence units (RFU) was calculated by subtracting values obtained at $t = 3 \text{ min} - t = 2 \text{ min}$ and data expressed as RFU/mg of tissue ($1 \times 10^3$).

5.2.7 Bronchoalveolar lavage analyses

5.2.7.1 Total bronchoalveolar lavage fluid protein assay

Total BALF protein was determined using Pierce BCA Protein Assay Kit (Thermo Scientific, AU). BALF samples were thawed on ice and diluted 1:2 in sterile PBS, and assayed in duplicate as per 2.5.3.

5.2.8 Immunohistochemistry

5.2.8.1 Immunohistochemical staining procedures

H&E, Ab-PAS and MT stains on these sections were performed by School of Biomedical Sciences Histology Facility, University of Melbourne. αSMA staining for smooth muscle was carried using Dako EnVision ® + Dual Lunk System-HRP (DAB+) kit as per 2.7.1. Myeloperoxidase (MPO) staining was carried out in the same protocol with minor modification. Polyclonal rabbit anti-human MPO antibody (Dako) was diluted 1:500 in sterile PBS and primary antibody incubation time reduced to 30 min. Secondary antibody incubation (labelled polymer-HRP) time was also reduced to 30 min. DAB stain was added for 3 min prior to rehydration and cover-slipping.
5.2.8.2 Morphometric analysis

Whole lung sections were scanned (200X zoom) using slide scanner (Olympus VS-120) and morphometric analysis was performed using CellSens Dimensions software (Olympus). Morphometric evaluation of lung tissue sections was performed on a minimum of four bronchi per mouse section selected according to size (100 to 350 µm luminal diameter) as previously described (Al-Garawi et al., 2009). A 30 µm band was selected around the subepithelial layer of randomly selected bronchioles per section and the percentage positive stain area analysed from α-SMA, AB-PAS and MT stained slides using standardised threshold values. Airway inflammation in H&E sections were graded, blinded, as an average of the entire lung section score with the average scores of five individual airways as previously described (Coomes et al., 2016). For entire lung section; presence of perivascular inflammation = 1, inflammation around 3 or more bronchioles = 2, dense inflammatory foci 3 cells deep= 3 and loss of lung architecture= 4.

5.2.9 Statistical analysis

Statistical analyses were carried out as detailed in 2.9. All data are expressed as mean ± SEM, and n represents one mouse, with data pooled from 5 independent experiments unless otherwise stated. Comparisons were made using unpaired Student’s t-test and one-way ANOVA with Bonferroni’s Multiple Comparison or Dunnett’s post hoc where appropriate. P < 0.05 was considered statistically significant. Analyses were carried out using Graph Pad Prism™ (version 5.0).
5.3 Results

5.3.1 Optimisation of a novel model of neonatal co-infection and aeroallergen exposure

5.3.1.1 Strain of mice: C57BL/6 are not sensitive to repeated house dust mite challenge

A novel model was developed to assess the implications of neonatal bacterial and viral co-infection on HDM-induced airway dysfunction and inflammation. A pilot model was assessed in C57BL/6 mice based on early life co-infection treatment protocol described in Chapter 4, overlaid with a HDM allergen challenge protocol. The administration of bacterial and viral infections was delayed by 3 days (to Day 8 and Day 15) so that daily HDM treatment was initiated after age of weaning (Day 20 – 21), to reduce the potential for maternal rejection of pups due to handling. HDM sensitization was initiated within 6 days of early life IAV infection to assess the potential for IAV-induced exacerbation of HDM-induced allergic airways disease (Al-Garawi et al., 2011). A mild HDM regimen was chosen to permit stratified HDM-induced inflammation following early life exposure to IAV compared to co-infection. This regimen involved 10 µg HDM treatments, administered 3 times per week, for 4 weeks (12 total) (Figure 5.3). Outcomes were assessed 24 hours after the final HDM administration in order to capture an acute inflammatory response. In this pilot model, male mice were slightly larger than female mice, however exposure to infections and allergic sensitisation was well tolerated and did not adversely affect any aspect of behaviour, grooming, or socialisation (Figure 5.4).
Figure 5.3 Pilot model of infant co-infection and house dust mite allergen sensitization in C57BL/6 mice.

Infant male and female C57BL/6 mice were infected with SP at day 8 of life (2000 CFU in 3 µl sterile PBS, or saline vehicle) and co-infected with influenza A virus (500 PFU in 3 µl sterile PBS). Mice were weaned at 20–21 days, at which time HDM (10 µg in 10 µl sterile PBS, or saline vehicle) treatments commenced 3 days per week for 4 weeks, resulting in 12 total treatments. Outcomes were measured 24 hours following the final HDM treatment.

Figure 5.4 Infant co-infection and house dust mite allergen sensitisation does not alter somatic growth in male or female C57BL/6 mice.

Body weight was recorded 3 times weekly in male and female C57BL/6 mice during saline vehicle (VEH) or house dust mite (HDM) challenge protocols as per model described in Figure 5.3. Male (A) and female (B) mice were weighed immediately after challenge three times weekly from age of weaning (day 20–21 of life) (n = 2–7 from one experiment).
The number of inflammatory leukocytes in the BALF was assessed in adult male C57BL/6 mice exposed to IAV or co-infection in early life, followed by sensitisation to HDM allergen or saline vehicle (Figure 5.5). BALF was collected 24 hours following final HDM treatment, at which time total cell counts were comparable across the four treatment groups, indicative of very low level inflammation (Figure 5.5). Given the lack of significant lung inflammation in the pilot model of neonatal co-infection and HDM challenge in C57BL/6 mice, ethics was obtained to assess similar model in BALB/C mice.

Figure 5.5 Neonatal co-infection combined with house dust mite sensitisation did not induce bronchoalveolar inflammation in male C57BL/6 mice.

Bronchoalveolar lavage (BAL) was performed on adult male mice as per model described in Figure 5.3. BAL fluid (BALF) was collected 24 h after final vehicle (VEH) or house dust mite (HDM) treatment, and assessed for total cell counts using ethidium bromide nuclear stain (n = 3 – 5 from one experiment).

5.3.1.2 Sex of mice: male mice are less susceptible to house dust mite sensitisation

A model of early life co-infection combined with HDM allergen challenge was developed in BALB/C male and female mice as described in Figure 5.2. Initially, responses to HDM allergen alone were compared in male and female mice, in the absence of early life infection (SAL-VEH and SAL-HDM treatment
groups only). HDM sensitisation protocols did not affect the somatic growth of male or female BALB/C mice after weaning, and was again well tolerated (Figure 5.6A, C). Additionally, final body weight was comparable following HDM sensitisation (Figure 5.6B, D).

![Figure 5.6](image)

**Figure 5.6 House dust mite sensitisation does not alter adult body weight in male and female BALB/C mice.**

Male and female BALB/C mice from saline vehicle (SAL-VEH) and house dust mite (SAL-HDM) groups were weighed daily as per model defined in Figure 5.2. Daily weights are shown as % change in body weight at age of weaning (Day 20 – 21) (A, C) and absolute body weight at end point (B, D) (n = 9 – 15 pooled from 4 independent experiments).

BALF was collected from male and female BALB/C mice 24 hours following final HDM (or saline vehicle) administration and assessed for inflammatory leukocyte infiltration (Figure 5.7). HDM sensitisation caused a significant increase in total cell counts in both male and female mice, with a ~5-fold
increase observed in female mice (Figure 5.7A). HDM-associated eosinophils and lymphocytes were significantly elevated in both male and female mice, confirming HDM-mediated induction of T\(_{H2}\) inflammatory response in these mice (Figure 5.7B, C).

Given the evidence for residual lung inflammation following HDM sensitisation in this strain of mice, the potential for HDM-mediated functional impairment was assessed. In vivo lung function testing was performed in both male and female BALB/C mice (Figure 5.8). Central airways resistance (Rn) to MCh challenge appeared slightly increased in male and female mice following HDM sensitisation (Figure 5.8A, B). Additional parameters of airway mechanics were unchanged following HDM sensitisation in male mice, with no clear HDM-driven differences in tissue damping, tissue elastance or hysteresivity observed (Figure 5.8C, E, G). In female mice, HDM sensitisation caused a significant increase only in tissue elastance (Figure 5.8F). To reduce the potential for variability, this model was restricted to female mice, as they appeared to be slightly more susceptible to HDM-induced inflammation and airway reactivity, as is consistent with our reference model of neonatal infection and HDM challenge (Al-Garawi et al., 2011).
Figure 5.7 House dust mite sensitisation induced Th2 bronchoalveolar inflammation in adult male and female BALB/C mice.

Bronchoalveolar lavage (BAL) was performed on adult male and female mice repeatedly challenged with saline vehicle (VEH) or house dust mite (HDM) as per model described in Figure 5.2. BAL fluid (BALF) was collected 24 h after final HDM treatment, and assessed for total (A) cell counts using ethidium bromide nuclear stain, and differential (B, C) cell counts performed on cytospin slides prepared with DiffQuik staining. *p<0.05, **p<0.01, ***p<0.001 unpaired Student’s t test (n = 7 – 15 pooled from 4 independent experiments).
Figure 5.8 Repeated house dust mite allergen exposure does not alter lung mechanics in male BALB/C mice.

Lung mechanics were assessed on adult male (left panels) and female (right panels) mice repeatedly challenged with saline vehicle (VEH) or house dust mite (HDM) as per model described in Figure 5.2. Lung function testing was performed 24 h after final HDM treatment, via SCIReq® Flexivent to assess central airway resistance (Rn) (A & B), tissue damping (G) (C & D), tissue elastance (H) (E & F) and hysteresivity (η) (G & H) at baseline, with nebulised saline and MCh (30 mg/ml). *p<0.05, unpaired Student’s t test compared to VEH at 30 mg/ml MCh (n = 7 – 9 pooled from 4 independent experiments).
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5.3.1.3 Litter size affects somatic growth in control mice

Given the large variability in the number of pups born to each BALB/C dam (1 – 12), we investigated the potential for litter size to affect body weight at age of outcome. Female BALB/C mice that received saline vehicle at day 8 and day 15, followed by daily saline vehicle challenge (SAL-VEH group) were weighed from age of weaning to age of outcome. Mice within this group were then stratified into small litter size mice (coming from a litter with 1 – 5 pups) and larger litter size mice (6 or more pups in litter). Mice born into a large litter size (6+ pups) displayed accelerated growth after weaning (Figure 5.9A). However, mice born into a large litter were still significantly smaller at age of outcome compared to their small litter counterparts (Figure 5.9B). Given that litter size significantly affected growth and subsequently adult body weight in saline control groups, litter size was controlled (5 – 7 pups only) to reduce variability this may have caused in other parameters assessed in a novel model of neonatal co-infection combined with HDM sensitisation.
5.3.2 House dust mite sensitisation causes growth impairment and bacterial lung infection persisting into adulthood following neonatal co-infection

To control for variability related to litter size and gender, the following data were collected only from female BALB/C mice, born within litters of 5 – 7 pups. This model was adapted from the experimental model of neonatal co-infection with SP and IAV described in Chapter 4, superimposed onto subsequent repeated HDM allergen exposure as detailed in Figure 5.2. At 8 days of age, neonatal mice were infected intranasally with SP and 7 days later, at 15 days of age mice were co-infected with HKx31 influenza virus. A mild to moderate HDM treatment regime (10 µg in 10 µl or 10 µl saline vehicle) was chosen, with a total of 15 treatments over 3 weeks. Whilst somatic growth did not appear to be affected throughout the study (Figure 5.10A, B), neonatal co-infection significantly reduced final adult body weight.
by approximately 10% relative to saline control treatment group (Figure 5.10C). HDM challenge did not further exacerbate this body weight effect; however the combination did result in significantly smaller tibia length (4% reduction) relative to saline treated mice (Figure 5.10D). Tibia length has been shown to retain isometric relationship to body length and is widely considered as representative of long bone development (Lupu et al., 2001). Two-way ANOVA analysis revealed no significant difference in body weight or loss in tibia length in co-infected mice (+/- HDM), to indicate that co-infection is a variable predominantly responsible for the small reduction in somatic growth in this model.

Figure 5.10 A novel model of neonatal co-infection and house dust mite aeroallergen challenge results in growth impairment in adulthood.

Female BALB/C wildtype mice born into litters between 5 and 7 pups only were used in this study. Body weight (g) was monitored daily throughout the study in both vehicle-treated (VEH; A) and house dust mite (HDM)-treated (B) mice and compared at endpoint (C, Day 40 – 42 of age) (n = 5 – 15). Left tibia bone length was compared (D). *p<0.05, two-way ANOVA compared to SAL-VEH (n = 5 – 15).
To determine the chronicity of SP carriage in this model, bacterial load in the upper and lower airways was assessed in the nasopharynx and the lung, respectively (Figure 5.11). SP carriage was not present in mice not exposed to SP infection (data not shown). Upper airway carriage was similar across all SP-treated groups, with nasopharyngeal colonisation persisting into adulthood (Figure 5.11A). Neonatal mouse SP infections were prepared in a volume of 3 µl, and as such detection of SP in lower airways would likely represent dissemination of SP from upper to lower airways. SP was detected in lung tissue (448 ± 195 CFU/100 mg tissue), and this value was not significantly different to co-infected mice (Figure 5.11B; 749 ± 238 CFU/100 mg tissue). When neonatal SP infection was combined with HDM sensitisation, there was an increase in mean pneumococcal load to 2936 ±992 CFU/100 mg tissue, however this not reach statistical significance. In neonatal co-infected mice challenged with HDM, the dissemination of SP into the lung was more substantial, with a 1-log increase in bacterial lung load (Figure 5.11B; 6528 ±2368 CFU/100 mg lung tissue, p <0.05).

Figure 5.11 House dust mite sensitisation facilitates chronic lower airway Streptococcus pneumoniae colonisation into adulthood following neonatal co-infection.

SP carriage was assessed at endpoint in homogenised nasopharyngeal samples and in lung tissue. Freshly collected nasopharyngeal homogenates were serially diluted and incubated on horse blood agar plates overnight at 37 °C and colony forming units (CFU) recorded from highest-countable dilution (A) (n = 6 – 14). A sample of lung tissue that had been snap frozen at endpoint was homogenised and microbial DNA qRT-PCR carried out to
measure SPN CFU per 100 mg lung tissue based on manufacturer’s SP DNA standards (B). *p<0.05, one way ANOVA with Bonferroni’s Multiple Comparison post hoc compared to SP-VEH (n = 4 – 7).

5.3.3 Neonatal co-infection combined with house dust mite challenge increases central airway resistance associated with mucus hypersecretion

In female BALB/C mice co-infected with SP and IAV as neonates, adult baseline lung function was investigated in vivo with SCIREQ® Flexivent (Figure 5.12). Parameters of lung function including Newtonian resistance (Rn, equivalent to central airway resistance), tissue damping (G), tissue elastance (H) and hysteresivity (the ratio of G/H; η) were all unchanged at baseline in this model (Figure 5.12A-D).

Figure 5.12 Early life infection and house dust mite sensitisation does not alter baseline lung function.

Lung function testing was performed using SCIREQ® Flexivent to assess baseline central airway resistance (Rn) (A), tissue damping (G) (B) and tissue elastance (H) (C) and hysteresivity (η) (D), calculated as the ratio G/H (n = 5 – 15).
Assessment of *in vivo* responses to maximal MCh challenge (30 mg/ml, as defined in 4.3.2) showed that central airways resistance to MCh was not altered across treatment groups in the absence of HDM challenge (Figure 5.13A). Additionally, MCh-induced G, H and η were not altered in VEH-challenged mice (Figure 5.13D, E, F). However, Rn in response to maximal MCh challenge increased approximately 2-fold in the co-infection plus HDM group relative to VEH counterpart (Figure 5.13C). In addition, analysis by two-way ANOVA also confirmed that HDM alone did not increase Rn to MCh relative to VEH-challenged mice. Two-way ANOVA did show a significant increase in co-infected mice challenged with HDM compared to co-infected mice not challenged with HDM. This MCh-induced hyper-responsiveness was not observed in HDM-sensitised mice following neonatal infection with a single pathogen, nor was it associated with alterations in other lung function parameters measured (Figure 5.13C-E). HDM sensitisation alone caused a small but significant increase in tissue elastance that was a trend also observed in other HDM treatment groups (Figure 5.13E).
Figure 5.13 House dust mite sensitisation only drives airway hyperresponsiveness in mice co-infected in early life.

Lung function testing was performed using SCIREQ® Flexivent to assess airways resistance (Rn) at baseline, in response to nebulised saline and to maximal methacholine (MCh; 30 mg/ml) challenge in vehicle-treated (A) and HDM-treated (B) mice. Maximal MCh-induced Rn (C), tissue damping; G (D), tissue elastance; H (E) and hysteresivity; η (calculated as the ratio G/H) (F), are expressed as % change from nebulised saline values. Dotted line represents vehicle mouse response to nebulized saline. *p<0.05, one-way ANOVA compared to SAL-VEH (n = 5 – 15).
To investigate whether AHR observed in vivo in the co-infection plus HDM group was due to remodelling processes occurring in the airways, smooth muscle mass (Figure 5.14) and collagen deposition (Figure 5.15) were assessed histologically. Quantification of α-smooth muscle actin positive stain area surrounding the bronchioles (100 – 350 µm diameter) revealed no significant differences across treatment groups with infection or HDM (Figure 5.14B). AHR was not associated with increased collagen deposition, as there were no changes in % positive collagen stained area surrounding airways in Masson’s trichrome (MT) stained lung sections (Figure 5.15B). Bronchoalevolar lavage (BAL) fluid protein concentration was measured as a surrogate for epithelial-vascular integrity and there was no increase in protein concentration with either HDM sensitisation or neonatal infections (Figure 5.16).
Figure 5.14 Neonatal infection and house dust mite sensitisation does not alter smooth muscle area.

Representative images (A, 100X optical zoom) show area of positive α-smooth muscle actin staining (αSMA, brown) surrounding bronchioles. At least four airways were selected per mouse whole lung section within diameter range of 100 – 350 µm and the % positive stain area for αSMA within this region was then quantified (B) (n = 4 – 7).
Figure 5.15 Neonatal infection and house dust mite sensitisation does not increase collagen deposition.

Representative images (A, 100X optical zoom) show area of positive Masson's trichrome (MT; blue) stain surrounding bronchioles. At least four airways were selected per mouse whole lung section within diameter range of 100 – 350 µm and the % positive stain area for MT within this region was then quantified (B) (n = 4 – 7).
BALF samples were assayed for total protein concentration using standard BCA protein assay and is expressed in mg protein/ml BAL fluid (n = 4 – 7).

Given that there was no evidence for increased smooth muscle area, collagen deposition or epithelial leakage in this model, we assessed the potential for mucous hyper-secretion contributing to AHR observed in vivo. Analysis of AB-PAS sections revealed an expansion of mucus producing goblet cells following HDM challenge, with evidence for complete mucus plugging in the bronchioles of co-infected mice (Figure 5.17A). When quantified, the AB-PAS-positive stain area in lung sections was significantly increased in the co-infection plus HDM group, whilst inoculation with single respiratory pathogen did not alter HDM-mediated expansion of goblet cells (Figure 5.17B). This finding was in accordance with increases in gene expression of Muc5B and Muc5AC mucin genes, which were elevated in HDM-challenged mice, and this was only increased by neonatal co-infection (Figure 5.17C, D).

Peri-bronchiolar airway inflammation was assessed and scored blinded in lung sections stained with H&E (Figure 5.18). Whilst there was evidence of unresolved lung inflammation in all mice sensitised with HDM, there was a significant increase in airway inflammation scoring when HDM was combined with neonatal co-infection (Figure 5.18B).
Figure 5.17 House dust mite sensitisation drives airway mucus hyper-secretion and increased mucin gene expression following neonatal co-infection.

*Figure legend overleaf.*
Figure 5.17 House dust mite sensitisation drives airway mucus hypersecretion and increased mucin gene expression following neonatal co-infection.

Representative images (A) (100X optical zoom) show positive AB-PAS mucus staining (purple) surrounding bronchioles. At least 4 airways were selected with diameter 100 – 350 µm per mouse and % positive PAS stain area within individual airways was then quantified using Olympus CellSens software (n = 4 – 7). Taqman qRT-PCR was performed on lung tissue for assessment of Muc5B (C) and Muc5AC (D) mucin gene expression that was normalized to housekeeping gene GAPDH and expressed as a fold-increase (RQ) relative to SAL-VEH group. *p<0.05, one way ANOVA compared to SAL-HDM (n = 4 – 7).
Figure 5.18 House dust mite driven peri-bronchiolar inflammation is exacerbated by neonatal co-infection.

Representative images (A) (10X optical zoom) show H&E stain in lung sections. Intensity of peri-bronchiolar inflammation was scored blinded, for the entire lung section (B): presence of perivascular inflammation = 1, inflammation around 3 or more bronchioles = 2, dense inflammatory foci 3 cells deep = 3 and loss of lung architecture = 4. *p<0.05, one way ANOVA compared to SAL-HDM (n = 4 – 9).
5.3.4 Neonatal co-infection and house dust mite sensitisation promotes a neutrophilic/T\(_{H17}\) inflammatory profile in the lung

Since there was histological and molecular data to support unresolved airway inflammation and mucus hyper-secretion in this model, further characterisation of the inflammatory infiltrate was carried out (Figure 5.19). Total cell counts were elevated with HDM sensitisation, but were not affected by neonatal infections, and in the absence of HDM sensitisation cell counts were low (Figure 5.19A). Differential cell counting revealed that macrophage numbers were increased by HDM and this was not further increased by neonatal infection (Figure 5.19B). Whilst neutrophil counts were also elevated by HDM sensitisation, only neonatal co-infection prior to HDM exposure significantly increased BAL neutrophil numbers above HDM alone (Figure 5.19C). This was consistent with the increase in myeloperoxidase (MPO) activity in the lung, used as a measure of tissue neutrophil levels, where significantly increased MPO activity was also seen in co-infection plus HDM mice relative to HDM alone (Figure 5.19D).

The contribution of T\(_{H2}\)-associated inflammatory processes was also assessed (Figure 5.20). HDM sensitisation also increased eosinophil cell counts in the BAL, however this was unchanged by the different neonatal infections (Figure 5.20A). The gene expression of specific T\(_{H2}\) cytokines was also quantified and appeared relatively T\(_{H2}\)-neutral; HDM sensitisation alone increased expression levels of IL-33 and GM-CSF, but this was not evident with either single infection or co-infection (Figure 5.20B, C). In contrast, IL-13 and IL-5 level were highest in mice sensitised to HDM following neonatal viral infection only, with a significant increase in IL-5, and co-infection did not alter this increased IL-5 expression in response to IAV (Figure 5.20D, E).
Figure 5.19 Neonatal co-infection combined with house dust mite sensitisation drives a neutrophilic inflammatory phenotype.

Bronchoalveolar lavage was assessed for total (A) cells and macrophages (B) and neutrophils (C) using DiffQuik differential staining where number of leukocytes per mL of BAL is presented (n = 4 – 13). Myeloperoxidase (MPO) activity in lung tissue (MPO units/mg tissue) was quantified as a measure of neutrophil tissue content (n = 4 – 7). *p<0.05, one way ANOVA compared to SAL-HDM (C) or SAL-VEH (D).
Figure 5.20 House dust mite sensitization drives $T_{h2}$ type immune response that is altered by neonatal infection.

Bronchoalveolar lavage was assessed for eosinophil inflammation (A) using DiffQuik differential staining where number of eosinophils per mL BAL is presented ($n = 4 – 10$). Taqman qRT-PCR was performed on lung tissue for assessment of gene expression of $T_{h2}$ immunity markers interleukin (IL)-33 (B), granulocyte-macrophage colony stimulating factor (GM-CSF) (C), IL-13, (D) and IL-35 (E) and data normalized to housekeeping gene GAPDH was expressed as fold change relative to SAL-VEH group ($n = 4 – 7$). *p<0.05, one way ANOVA compared to SAL-VEH.
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Figure 5.21 Neonatal co-infection combined with house dust mite sensitisation drives a serum amyloid A and Th17-type immune phenotype.

Taqman qRT-PCR was performed on lung tissue for assessment of gene expression of neutrophil markers interleukin (IL)-17A (A), serum amyloid A (SAA) (B) and granulocyte colony stimulating factor (G-CSF) (C) and data normalized to housekeeping gene GAPDH was expressed as fold change relative to SAL-VEH group. *p<0.05, one way ANOVA compared to SAL-VEH (n = 4 – 7).

Given the consistently greater increases in both neutrophil BAL cell counts and MPO activity with co-infection, we sought to assess molecular drivers of this neutrophilic inflammation. A panel of neutrophil regulators was assessed via qRT-PCR on lung tissue (Figure 5.21). HDM alone increased expression of IL-17A, SAA and G-CSF and this was markedly increased only in mice that were co-infected in early life (Figure 5.21A-C). Additionally, the expression of the neutrophil chemokines CXCL1 and CXCL2 were determined. Neonatal infection did not exacerbate the HDM-induced expression of CXCL1 (Figure
Expression of CXCL2 was increased in IAV-infected mice exposed to HDM, and co-infection did not further alter response to IAV (Figure 5.22B).

Figure 5.22 House dust mite combined with neonatal co-infection increases expression of neutrophil chemokines.

Taqman qRT-PCR was performed on lung tissue for assessment of gene expression of neutrophil chemokines C-X-C motif chemokine ligand 1 and 2 (CXCL1, CXCL2). *p<0.05, one way ANOVA compared to SAL-VEH (n = 4 – 7).
5.4 Discussion

It is thought that asthma (including severe asthma) originates in early life, and there has been increasing interest in the role of viral infection during this developmental window, with little focus on the interaction between viruses and bacteria. In the present study, we developed a novel model of neonatal respiratory co-infection combined with repeated allergen exposure with a clinically relevant allergen HDM. Neonatal co-infection combined with HDM sensitisation lead to dissemination of SP into the lower airways and AHR to MCh challenge in vivo, which was mediated by mucin hyper-section rather than increased ASM bulk or other remodelling processes. Additionally, there was evidence for unresolved lung tissue inflammation with a strong neutrophilic inflammatory signal, and increases in IL-17A and SAA, suggestive of a more severe asthmatic phenotype being modelled.

Initially, a mild HDM treatment regimen was applied to the previously characterised model of neonatal SP and IAV co-infection in C57BL/6 mice (Chapter 4). Neonatal infection followed by repeated HDM challenge failed to induce a robust Th2 immune response, consistent with this strain of mice being less susceptible to allergen-induced inflammation (Sahu et al., 2010), however more recently it has been demonstrated that this strain of mice can develop AHR in adult HDM models utilising a more moderate dosing regimen (Byrne et al., 2017), this effect was not seen in pilot experiments. Using BALB/C mice instead, mild-moderate HDM sensitisation alone did not affect growth or adult body weight, and there was evidence for more pronounced HDM-induced lung inflammatory infiltrates in female mice. Previously, female mice have been shown to also have a greater propensity to develop AHR in response to lung inflammation (Larcombe et al., 2011), and so female mice were chosen for further study. Litter size significantly affected adult body weight of female mice in the absence of allergen or infection. This may have been an artefact of neonatal overfeeding in litters with fewer pups, which has been previously linked to elevated lung inflammation in the
absence of allergen challenge (Ye et al., 2012), and so to control for variability, litter size was also restricted in this model.

The present findings demonstrate that neonatal SP infection leads to persistent nasopharyngeal SP carriage into adulthood, and that IAV or repeated HDM challenge did not alter upper airway carriage rate. However, dissemination of bacteria into the lower airways was significantly increased in co-infected mice exposed to repeated HDM challenge. Suppression of mucociliary and immune function by respiratory viruses are known to reduce pneumococcal clearance. In secondary bacterial infection models, the transient depletion of alveolar macrophages represents a window of opportunity for respiratory pathogens such as SP to colonize the airways (Ghoneim et al., 2013). The IFN-γ produced during the recovery phase of a viral infection can inhibit lung anti-bacterial defenses (Sun & Metzger, 2008). Furthermore, HDM exposure has been shown to suppress anti-bacterial defenses in adult mice leading to more persistent SP lung colonization (Habibzay et al., 2012). In the present study, only the combination of co-infection plus HDM exposure led to a significant increase in invasive bacterial dissemination into the lung, suggesting that IAV and HDM co-operate to establish long-term lung colonization. Importantly, SP colonization was not associated with a reduction in leukocyte cell recruitment but rather an increase, suggesting that the recruited immune cell populations are phenotypically insufficient to clear the pathogen.

Increased bacterial burden in the lungs of co-infection plus HDM mice was associated with AHR to MCh challenge in vivo, whereas single pathogen challenge did not cause AHR. HDM challenge alone also did not increase Rn to MCh, which is reflective of the mild-moderate dosing regimen (Al-Garawi et al., 2009). Small airway reactivity via the lung slice technique was not investigated in this model due to limited material, however it is unlikely that altered reactivity would be evident given there was no evidence of increased smooth muscle staining. Elevated airway reactivity was not mediated by
hyper-proliferation of airway smooth muscle nor collagen deposition, but was associated with a marked increase in goblet cells producing mucus, accompanied by an increase in Muc5B and Muc5ac gene expression. Mucin over-production and hyper-secretion are prominent in fatal asthma where patients die from severe airflow obstruction caused by bronchoconstriction of airways occluded by mucous plugging (Aikawa et al., 1992). Whilst airway goblet cell hyperplasia is present in mild asthma, a greater increase in stored and secreted mucin occurs in moderate disease (Ordoñez et al., 2001). Furthermore, secretion of mucus from goblet cells in allergic airways models directly contributes to AHR in concert with airway smooth muscle contraction (Singer et al., 2004). In a more recent study, mucin was identified as a central effector of AHR following allergic stimuli, as confirmed by genetic knockout of Muc5ac. Importantly, allergen-induced inflammatory effects on airway smooth muscle alone were insufficient to cause AHR, implicating mucus occlusion as an essential mechanism (Evans et al., 2015). SP is also known to increase expression of Muc5ac (Lim et al., 2009), and our findings demonstrate that the combination of co-infection and HDM augments Muc5ac expression, which is likely mediated by persistent lung colonisation.

The hygiene hypothesis is supported by findings where pneumococcus and its components are capable of reducing TH2 immunity and AHR following ovalbumin allergen challenge (Preston et al., 2011; Thorburn et al., 2010). Phosphorylcholine epitopes found on the surface of the SP pathogen are also found in HDM, and anti-phosphorylcholine antibodies decrease pulmonary dendritic cell activation and TH2 immunity to HDM (Patel & Kearney, 2015). Additionally, GM-CSF production following HDM administration can drive TH2 immunity and eosinophilic inflammation (Cates et al., 2004). Our findings are consistent with these studies as nasopharyngeal colonisation reduced components of TH2 immunity, including HDM-induced expression of IL-33 and GM-CSF. However, it appears that AHR was driven by mucin overproduction independent of TH2 immunity in our model, as the reduction
in IL-13 and GM-CSF in co-infection plus HDM treated mice failed to reduce AHR.

Co-infection plus HDM challenge significantly increased neutrophilic inflammation. Neutrophil recruitment can promote goblet cell degranulation after antigen challenge in sensitized guinea pigs, and inhibition of adhesion molecules on neutrophils reduced goblet cell degranulation (Agusti et al., 1998; Takeyama et al., 1998). It is now recognised that asthma is a heterogeneous disease consisting of multiple phenotypes based on clinical, inflammatory and molecular profiles. Non-$T_h$2 asthma is likely to represent a large proportion of all asthma, and this patient population is more likely to be poorly controlled by corticosteroid therapies. Neutrophilic asthma has been proposed as a biological phenotype, as sputum neutrophilia has been associated with more severe asthma and high health care usage, although there is some controversy around whether this represents a true phenotype (Wenzel, 2012). What is evident is that the co-existence of eosinophils and neutrophils is prominent in more severe asthma (Hastie et al., 2010), and our model displays both eosinophilic and neutrophilic inflammation where neutrophilia was exacerbated by co-infection in the background of HDM challenge.

The use of asthma endotypes, where patients are stratified on the basis of dominant molecular pathways, may offer opportunities for more tailored treatments to overcome heterogeneity in responsiveness to current therapy (Anderson, 2008). Expression of IL-17A has been reported to be high in severe asthmatics with poor lung function, however the potential benefit of targeting IL-17 in neutrophil associated asthma remains unresolved in the clinical setting (Chakir et al., 2003). Experimentally, IL-17A produced by $T_h$17 cells enhances ASM contractile force and directly mediates AHR following allergen sensitisation (Kudo et al., 2012). In our study, co-infection in combination with HDM markedly increased expression of IL-17A and serum amyloid A (SAA). It has been shown previously that SAA can initiate
neutrophilic inflammation in the airways via IL-17A-dependent mechanisms by polarising macrophages towards a phenotype that produces more TH17-skewing cytokines (Anthony et al., 2014; Anthony et al., 2013; Bozinovski et al., 2012). Sputum levels of SAA were found to be increased in asthmatics and remained elevated after prolonged treatment with anti-inflammatory therapies (Ozseker et al., 2006). Furthermore, SAA can function as an adjuvant to sensitisise mice to allergen and subsequently enhance TH17 immunity (Ather et al., 2011). Taken together, the neutrophilic/TH17/SAA signal, which was accompanied by AHR and mucus hyper-secretion in the present study in early life co-infection plus HDM group suggests this model may be capturing key features of severe, steroid insensitive asthma.

5.4.1 Conclusions from Chapter 5

In summary, neonatal co-infection in the background of HDM sensitisation resulted in chronic lung colonisation, exacerbated neutrophilic inflammation, increased mucin production and AHR. Hence, pneumococcal colonisation does not protect against allergic airways disease which is predominately driven by mechanisms that are independent of TH2 immunity.
CHAPTER 6

General Discussion
Asthma is a remarkably heterogeneous disease where the airways are chronically inflamed and undergo significant structural changes collectively resulting in significantly impaired lung function. The underlying cause of asthma is yet to be determined, and current therapies are only able to control symptoms and combat inflammation, without cure or reversal of disease. Importantly, small airways dysfunction is present in the majority of asthmatics. This remains relatively undertreated by existing therapy and contributes considerably to the severity of disease. Asthma is presently the most common chronic disease affecting children, and it becoming clear that disease originates in early life as a result of complex synergistic interactions of various environmental exposures. There has been increasing interest in the role of neonatal infections; with evidence emerging that asymptomatic pneumococcal colonisation is a strong predictor of future asthma, which can be exacerbated by viral infection. It is therefore paramount that appropriate models of disease are developed in order to elucidate these complex immunological interactions that have profound pathogenic potential during this window of lung development.

The aims of this project centred on developing appropriate experimental methods to assess the understudied contribution of the small airways in severe asthma. The initial section of the project required optimisation of lung slice technique methodology in order to directly assess altered reactivity in animal models of acute infection. Critically, early life exposure respiratory infections and aeroallergens are major cause of small airway pathology, however the immunological and cellular mechanisms that drive this pathology are yet to be determined. With a focus on viral-induced pneumococcal colonisation, the long-term implications of neonatal co-infection were assessed, and a novel model involving aeroallergen sensitisation on a background of chronic colonisation was characterised.
6.1 Key findings and their significance

6.1.1 Validation and optimisation of lung slice technique protocols

The lung slice technique is a powerful *in vitro* tool that permits direct assessment of reactivity of intrapulmonary airways and arteries *in situ*, with their parenchymal attachments maintained. Despite its physiological relevance, its application in models of lung disease has been relatively limited, with only minimal justification for the choice of experimental conditions used when measuring contraction and relaxation. This project initially optimised conditions for measuring mouse airway reactivity by systematically comparing the effects of different temperature and buffer selections on responsiveness. These studies used methacholine as a clinically relevant constrictor agent and isoprenaline as the dilator agent due to its known efficacy in rodent airways. While it was shown that temperature and buffer selection did not significantly affect reactivity, these experiments provided validated conditions for further experiments.

This study then extended the use of this technique into rat lung slices, where intrapulmonary airway and artery contraction was comprehensively characterised. Continued difficulties in reliable preparation of rat lung slices with viable arteries were encountered despite altering various factors involved in methodology; limited publications in this area may suggest this problem is not exclusive to our laboratory. Nevertheless, comparisons of contractile responses revealed that endothelin-1 was similarly effective in rat airways and arteries despite mediating its effects via different endothelin receptor subtypes, while serotonin was markedly more potent in airways. While rat airways relaxed as expected to salbutamol, it proved difficult to elicit arterial relaxation despite testing a range of vasodilators. Due to the significant limitations in the successful preparation of rat lung slices, subsequent investigation of pulmonary vascular reactivity was not pursued.
Until recently, the application of the lung slice technique to models of lung disease, and particularly to those involving infection, has been limited. Assessment of the effects of acute bacterial infection \textit{in vivo} showed that intrapulmonary airway reactivity was largely unaffected, even in combination with deletion of the superoxide dismutase 3 gene and the loss of its protective antioxidant effects. Nevertheless, these pilot studies confirmed the potential for investigating changes in small airway reactivity in models of infection using validated experimental conditions; these comprised the remaining sections of the thesis.

### 6.1.2 Neonatal viral-induced pneumococcal colonisation impaired adult lung function

There is increasing epidemiological evidence to suggest that neonatal bacterial colonisation may increase risk of asthma and respiratory dysfunction in later life (Bisgaard \textit{et al.}, 2007), and viral infections are known to precipitate persistent colonisation. The mechanisms by which asymptomatic neonatal pneumococcal colonisation can impact adult lung health have not been. Using a previously established model of early life viral-induced pneumococcal colonisation, this study confirmed residual airway dysfunction despite resolution of inflammation. Only respiratory co-infection resulted in persistent airway hyperresponsiveness (AHR) in absence of any remodelling processes or mucosal epithelial abnormality. Importantly, this model utilised C57BL/6 mice which are relatively resistant to aeroallergen sensitisation (Sahu \textit{et al.}, 2010). Taken together, this data suggests that neonatal pneumococcal colonisation has long-standing deleterious effects on the development of normal lung function by affected ventilation heterogeneity, possibly accompanied by microstructural changes to the lung, and future studies should consider the impact of co-infection on extracellular matrix components that contribute to the parenchymal architecture. A limitation of this study was the lack of flow-cytometric analyses of the T cell compartment, given the small but significant increase in this cell type in the
BAL fluid of co-infected mice, and this may yield important information regarding which lymphocyte population/s may be expanded.

The broader implication of this study is that prevention of bacterial colonisation in infancy could contribute to the prevention of lower respiratory tract infections and persistent wheeze. However, this remains a challenging proposition clinically, as disruption of nasopharyngeal microbiome using current vaccine strategies can result in serotype replacement and colonisation with resistant strains that are potentially more pathogenic. Consequently, pneumococcal carriage rates remain notably high amongst the paediatric population, and further investigation of effective preventative approaches is paramount given its critical impact during lung development. Future studies will need to address whether chronic neonatal nasopharyngeal colonization with non-vaccine serotypes have a similarly deleterious effect on adult lung health.

6.1.3 Neonatal co-infection worsens outcomes following aeroallergen sensitisation

The final study presented in this thesis answered an unresolved question in asthma around how pneumococcal lung infection can worsen allergic airways disease. There are recent epidemiological studies which associate nasopharyngeal bacterial colonisation and acute viral infection with invasive spread of the bacterium into the lower airways, leading to more severe inflammatory symptoms and increased risk of asthma (Teo et al., 2015). By developing a novel experimental model, it was clearly demonstrated that neonatal bacterial and viral co-infection predisposed to persistent lung bacterial infection, and increased airway reactivity following aeroallergen sensitisation (Figure 6.1). The lung slice technique was not utilised in this model, as lung tissue was allocated to histological and molecular analyses, complemented by in vivo airway reactivity investigations. It was established that mucus hyper-secretion was the key driver of AHR, and importantly, neonatal co-infection represents a clinically relevant cause of mucus
overproduction in allergic airways disease. In the absence of co-infection, house dust mite (HDM) orchestrated a sub-clinical T\textsubscript{H}2 response. Critically, viral-induced neonatal pneumococcal colonisation facilitated more damaging inflammation in response to aeroallergen challenge, with a mixed eosinophilic and neutrophilic inflammatory profile, where neutrophils were exacerbated by co-infection and HDM. This study identified serum amyloid A, interleukin (IL)-17A and granulocyte-colony stimulating factor (G-CSF) as molecular markers for this phenotype, which parallels features of more severe asthma. This pathogenic synergism was observed within a model utilising only one strain of bacteria and virus, and requires validation with other common pathogens to clarify whether these effects are serotype-dependent. Additionally, this study utilised BALB/C strain of mice, which are more susceptible to allergic inflammation (Sahu \textit{et al.}, 2010), and also demonstrated the importance of litter size in these types of mouse models. Future studies should investigate antibody responses in this model, and given there was evidence for significant neutrophilia, the potential for steroid-resistance should be investigated. Further, it remains to be determined whether neutrophils are driving the mucus hyper-secretion observed in this model. This could be investigated by inhibiting neutrophilic inflammation using blocking antibodies to IL-17A or G-CSF. Alternatively, pro-resolving formyl peptide receptor 2 (FPR2) agonists such as lipoxin A\textsubscript{4}, which counteract the effects of SAA should be assessed for efficacy (Bozinovski \textit{et al.}, 2012). Collectively, this model and associated studies present novel opportunity to develop treatment strategies to circumvent a highly relevant cause of childhood asthma.
This thesis presents findings that suggest aeroallergen sensitisation alone initiates a sub-clinical $T_h2$ response accompanied by normal – mild changes in airway reactivity, with low level mucus and eosinophil counts in the airways. Conversely, when bacterial and viral co-infection is combined with aeroallergen sensitization, the pneumococcus invades the lower airways resulting in pneumococcal lung infection, which is accompanied by increased airway reactivity, mucus hyper-secretion, and a mixed neutrophilic/eosinophilic airway inflammation.
6.2 Concluding remarks

The potential of the lung slice technique is vast and remains under-utilised in animal models of disease, particularly where small airway dysfunction is marked. Neonatal viral-induced pneumococcal colonisation lead to AHR in adulthood, consistent with large cohort studies implicating colonisation as a risk factor for development of asthma. Furthermore, this was the first study to elucidate the pathogenic synergism of early life bacterial and viral co-infection in combination with aeroallergen exposure and confirmed the hypothesis of this thesis. Early life pneumococcal colonisation of the lung was significantly augmented by allergic sensitisation and viral infection and led to development of a more severe asthmatic phenotype associated with mucus hyper-secretion, AHR and TH17/SAA/neutrophilic inflammation. Critically, this model provides a unique opportunity to assess the efficacy of novel therapeutics that could target the pathogenic inflammatory mechanisms and restore lung function, and ultimately provide new treatment strategies for critically unwell and undertreated asthmatics.
References


REFERENCES


REFERENCES


Perez JF, Sanderson MJ (2005a). The contraction of smooth muscle cells of intrapulmonary arterioles is determined by the frequency of Ca2+ oscillations induced by 5-HT and KCl. J Gen Physiol 125(6): 555-567.


REFERENCES


Appendix
Neonatal pneumococcal colonisation caused by Influenza A infection alters lung function in adult mice

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There is emerging epidemiological data to suggest that upper respiratory tract bacterial colonisation in infancy may increase the risk of developing respiratory dysfunction later in life, and respiratory viruses are known to precipitate persistent colonisation. This study utilized a neonatal mouse model of Streptococcus pneumonia (SP) and influenza A virus (IAV) co-infection, where bronchoalveolar leucocyte infiltration had resolved by adulthood. Only co-infection resulted in persistent nasopharyngeal colonisation over 40 days and a significant increase in airway resistance in response to in vivo methacholine challenge. A significant increase in hysteresivity was also observed in IAV and co-infected mice, consistent with ventilatory heterogeneity and structural changes in the adult lung. Airway hyper-responsiveness was not associated with a detectable increase in goblet cell transdifferentiation, peribronchial smooth muscle bulk or collagen deposition in regions surrounding the airways. Increased reactivity was not observed in precision cut lung slices challenged with methacholine in vitro. Histologically, the airway epithelium appeared normal and expression of epithelial integrity markers (ZO-1, occludin-1 and E-cadherin) were not altered. In summary, neonatal co-infection led to persistent nasopharyngeal colonisation and increased airway responsiveness that was not associated with detectable smooth muscle or mucosal epithelial abnormalities, however increased hysteresivity may reflect ventilation heterogeneity.

Influenza-associated hospitalization rates in children younger than 5 years old are exceeded only by the elderly1. Globally, it has been estimated that around 20 million cases of influenza infections occur worldwide in children younger than 5 years, which accounts for 13% of paediatric cases of acute lower respiratory tract infections2. Infants aged less than one year have been reported to be even more susceptible to influenza-associated hospitalizations3, which is consistent with children under the age of 6 months being too young for vaccination. The clinical response to different viral strains can vary from mild disease to severe pneumonia, and immunological and inflammatory profiles have been extensively characterised, where pandemic strains are more likely to initiate an exaggerated inflammatory response that culminates in severe lung injury4. Young children also represent a major reservoir for Streptococcus pneumoniae (SP) carriage5, which is the predominant microbe responsible for bacterial pneumonia. Although there are multiple environmental, host and microbiological factors that increase an individual's susceptibility to pneumococcal disease, infection with IAV in particular, has been associated with an increase in pneumococcal burden6. Notably, concurrent IAV infection is essential for the transmission of SP from colonised mice to their naive co-housed littermates, as transmission...
could be prevented by inhibiting viral replication\(^1\). The detection of bacteria that can colonise the airways occurs frequently during significant bronchiolitis episodes, hence the common terminology of viral wheeze may not accurately reflect the aetiological nature of many acute events in children under the age of 5 years\(^2\). Neonatal upper airway colonisation is also associated with increased risk of bronchiolitis in early life independent of concurrent asthma\(^3\). There are also emerging epidemiological data to suggest that asymptomatic nasopharyngeal bacterial colonisation is associated with persistent wheeze in children, where the prevalence of asthma at 5 years of age was significantly increased in the children colonised as neonates in the hypopharyngeal region with microbes including SP\(^4\).

It has also been established that influenza and other viruses can acutely alter lung function and lead to airway hyper-responsiveness (AHR) in response to increasing concentrations of constrictor agonists, such as methacholine (MCH)\(^5\). Using a low-frequency, forced oscillation technique, heightened responsiveness to both inhaled and intravenous MCH has been observed during the acute phase of influenza A virus (IAV) infection in mice. In this study, no intrinsic smooth muscle defect was observed, but rather an increase in the permeability of the alveolar–capillary barrier was facilitating greater MCH access to airway smooth muscle\(^6\). Furthermore, increased AHR was shown to be transient in nature as lung function normalised with resolution of inflammation and tissue injury in adult mice\(^7\). In contrast to adult mice, physiological and inflammatory responses to IAV infection were assessed in infant mice, where residual AHR persisted for 21 days when inflammation and alveolar–capillary permeability had fully resolved\(^8\). In the current study, the effect of infant co-infection with SP and IAV on adult mouse lung function was assessed, revealing persistence of MCH-induced hyper-responsiveness that was independent of mucosal epithelial abnormalities.

**Methods**

**Animals and Model of Neonatal Co-infection.** Advanced pregnant C57BL/6 dams were obtained from Animal Resources Centre, Western Australia. Dams were housed separately and monitored for births, with minimal disruption. Upon birth, dams were housed with their litters in light-protected cages until weaning at 3–4 weeks of age. Mice were housed at 22°C under normal light:dark cycle, and given free access to a normal diet and water, and monitored for signs of illness or distress. All experimental procedures performed in mice were approved by the Animal Ethics Committee of the University of Melbourne (approval #1111986 & #1413288), compliant with the National Health and Medical Research Council (NHMRC) Australian Code of Practice and were carried out in accordance with the approved guidelines. Neonatal mice were infected with SP and/or IAV as previously described\(^9\) with the following minor modifications. At 5 days of age, C57BL/6 mice were infected intranasally without anaesthesia with vehicle (sterile PBS) or SP (serotype EF3000, 2 × 10\(^6\) CFU/mL) in a volume of 3 μl sterile PBS. At 12 days of age, mice were infected intranasally with vehicle (sterile PBS) or IAV (strain HK × 31, H3N2, 7 × 10\(^7\)PFU/mL) in a volume of 3 μl sterile PBS.

**Collection and Measurement of Specimens.** Following neonatal challenge with SP and/or IAV, mice were allowed to recover and assessed starting at 6–8 weeks of age in early adulthood. At time of analysis, mice were euthanized by overdose of sodium pentobarbitone and bronchoalveolar lavage (BAL) was performed by tracheotomy and total and differential BAL cell counts determined as previously described\(^10\). Briefly, at time points prior to 6–8 weeks, nasopharyngeal and lung tissues were collected, homogenised and serial dilutions of tissue homogenates were cultured on horse blood agar plates to determine bacterial load as previously described\(^11\).

**Measurement of Airway Hyperresponsiveness in vivo and in vitro.** Lung function was measured using a modification of the low-frequency forced oscillation technique and a small-animal ventilator (flexVent; Scireq, Montreal, QC, Canada) as described\(^12\). Respiratory impedance (Z\(_{aw}\)) was measured and partitioned into airway and parenchymal components to allow calculation of Newtonian resistance (Rn, equivalent to airway resistance (Raw) because of the high compliance of the chest wall), tissue damping (G) and elastance (H\(_t\)), as previously described\(^13\). Airway inertia (I\(_{aw}\)) was negligible after correcting for the tracheal cannula and is not reported. Tissue hysteresivity (\(\gamma\)) was calculated as the ratio G/H\(_t\). Baseline respiratory mechanics was recorded, followed by saline aerosol and increasing doses of MCh delivered by an ultrasonic nebuliser (MCh; 1, 3, 10, 30, 120 μg/mL). Baseline respiratory mechanics was recorded, followed by saline aerosol and increasing doses of MCh delivered by an ultrasonic nebuliser (MCh; 1, 3, 10, 30, 120 μg/mL). Differences in responsiveness were assessed at the maximum response to 30 μg/mL MCh challenge. In vitro small airway reactivity in response to MCh was assessed via the precision cut lung slice technique as previously described\(^14\). Briefly, low-melting point agarose (GIBCO/Invitrogen, Australia) was used to inflate lungs, sequential slices (150 μm) using a vibratome (VT 1000 C, Leicamicrosystems) were cut and lung slices containing intrapulmonary airways ranging from 150–300 μm in diameter were incubated at 37°C and 5% CO\(_2\) for 24 h prior to use in experiments.

**Histology and Immunohistochemistry.** The left lobe of lung was removed post mortem and immediately fixed in 10% neutral-buffered formalin. Tissues were paraffin-embedded and cut at a thickness of 5 μm. Sections were stained with H&E, Masson trichrome for assessment of epithelial and sub-epithelial collagen content or with Alcian blue–periodic acid–Schiff for assessment of goblet cell transdifferentiation. Morphometric evaluation of lung tissue sections was performed as described previously\(^15\). A minimum of five bronchi selected according to size (150 to 350 μm luminal diameter) were analysed per mouse. To assess the presence of airway smooth muscle bulk, primary antibody to anti-α smooth muscle actin (α-SMA, Dako, Glostrup, Denmark) was used and the thickness of labelled α-SMA per length of basement membrane measured in a minimum of 5 bronchi, as previously described\(^16\). Images of stained slides were captured using Aperio ImageScope software.

**Quantitative Real-time PCR for gene expression analysis of lung tissue.** RNA was purified from whole lung tissue using RNAeasy kit as per manufacturer’s instructions and cDNA was prepared as previously described\(^17\). Taqman PCR primers were used to perform Q-PCR, where all threshold cycle values (Ct) were...
normalized to control (glyceraldehyde phosphate dehydrogenase) and the relative fold change determined by the \( \Delta\DeltaCT \) value as previously described\(^2\).

Data analysis. Data and presented as the mean ± standard error of mean (SEM) for \( n \) mice. All data were statistically analysed using GraphPad Prism 5.0 (Graphpad, San Diego, CA). Where detailed and appropriate, one-way analyses of variance (ANOVA) with Bonferroni or Dunnett’s post-hoc tests were used. In other cases, unpaired Student’s t-tests were used to analyse data. \( p < 0.05 \) was considered to be statistically significant.

Results

Neonatal co-infection facilitates long term nasopharyngeal SP colonisation. An established co-infection model was used, where IAV is known to disrupt clearance of SP leading to a transient increase in nasopharyngeal and lower airway bacterial carriage in adult mice\(^7\). In our study, neonatal mice were infected and outcomes were assessed in early adulthood as described in Fig. 1A. Early life infection with SP and/or IAV did not have any long lasting effects on body weight of male and female mice across all groups into adulthood (Fig. 1B,C). To determine the chronicity of SP carriage in the upper and lower airways, a detailed kinetic study was performed. Bacterial load in nasopharyngeal (Fig. 2A) and lung (Fig. 2B) homogenates were assessed at time points of 20, 30 and 40 days of life. In neonatal mice infected with SP alone, transient nasopharyngeal carriage steadily declined to negligible levels by day 40 of life. This is in contrast to co-infected neonatal mice, where levels of SP upper airway carriage persisted over the kinetic study leading to a significant 1-log increase in load at Day 40 relative to mice infected with SP alone (Fig. 2A).

In this model, there was no evidence for significant invasive dissemination of SP into the lower airway (Fig. 2B), suggesting that the bacteria localised predominately to the upper airways. In addition, the number of inflammatory leukocytes in the BAL compartment was assessed in adult mice following infection during neonatal phase of life. The data is indicative of the inflammatory process being fully resolved in response to IAV and/or SP as total, neutrophil and macrophages numbers were not altered (Fig. 3A–C). There was a small but significant increase in lymphocyte counts in co-infected mice compared to vehicle control (Fig. 3D). To determine whether the increase in BAL lymphocyte numbers were associated with an increase \( T_{H2} \) cytokines in the lung, QPCR was performed on IL-5 and IL-13, where no increase in expression was observed (Fig. 3E,F).
In mice co-infected with SP and IAV as neonates, baseline lung function responses were not different to that of vehicle treated mice. However newtonian resistance ($R_n$), which is equivalent to central airway resistance, increased at the higher doses of MCh, peaking at 30 mg/mL (Fig. 4A). MCh responses at 30 mg/mL were compared across all four groups and presented as a percentage change above nebulised saline response. The data demonstrates a significant increase ($p < 0.05$) in central airway resistance ($R_n$) only in co-infected mice that was 2-fold higher than mice treated with vehicle as neonates (Fig. 4B). Baseline $G$ (tissue damping), $H$ (tissue elastance) and hysteresivity ($\eta = G/H$) were also assessed in adult mice treated with SP and IAV or vehicle in infancy. These additional measures of lung function also showed no difference at baseline (Fig. 5A,C,E). Infection in infancy did not significantly affect MCh-induced increases in $G$ (Fig. 5A,B). The significant increase in MCh-induced $H$ detected in vehicle and co-infected mice was absent in mice treated with either SP or IAV alone (Fig. 5C,D). Hysteresivity ($\eta = G/H$), which has been used to characterise tissue mechanics was shown to increase with increasing concentration of MCh in co-infected mice above vehicle treated mice (Fig. 5E). Maximal MCh responses across all four groups revealed a significant increase in $\eta$ in both IAV and SP/IAV treated mice (Fig. 5F).

To assess whether altered airway responsiveness was due to remodelling processes occurring in the lung, mucus and smooth muscle was quantified histologically and via gene expression analysis of lung tissue. AB-PAS staining revealed no observable increase in goblet cell numbers across all groups (Fig. 6A–D), and the lack of goblet cell expansion was consistent with unchanged Muc5ac mucin gene expression (Fig. 6I). Immunohistochemical assessment of $\alpha$-smooth muscle actin positive area surrounding airways expressed relative to basement membrane area revealed no significant difference across the treatment groups (Fig. 6J). In addition, in vitro airway reactivity was investigated in precision cut lung slices. Consistent with the absence of increased area for $\alpha$-smooth muscle in vivo, contractile responses of size-matched intrapulmonary airways to MCh in vitro were unaltered between all treatment groups, where MCh caused a ~60% reduction in airway lumen area in all mice tested (Fig. 7A–C). Since mucin or smooth muscle abnormalities, were not detected, we considered the potential for epithelial disruption.
contributing to increased airway responsiveness in vivo. Histologically, the epithelium appeared normal in all treatment groups and when quantified, epithelial thickness measures were comparable (Fig. 8A–E). Taqman PCR analysis for gene expression of TLR2 cytokines (E) interleukin-5 and (F) interleukin-13 in lung tissue is expressed as fold-change compared to vehicle-treated control and normalised to GAPDH housekeeping gene (n = 6). *p < 0.05 compared to vehicle lymphocytes. BALF, bronchoalveolar lavage fluid.

Figure 3. Neonatal co-infection does not elicit Th2 immune response in adult lung. Bronchoalveolar lavage fluid from adult mice exposed to infant co-infection was assessed for (A) total and differential (B) macrophage, (C) neutrophil and (D) lymphocyte cell counts (n = 5–11). Taqman PCR analysis for gene expression of TLR2 cytokines (E) interleukin-5 and (F) interleukin-13 in lung tissue is expressed as fold-change compared to vehicle-treated control and normalised to GAPDH housekeeping gene (n = 6). *p < 0.05 compared to vehicle lymphocytes. BALF, bronchoalveolar lavage fluid.
Discussion
In this study, a significant increase in central airways resistance in response to MCh challenge was only observed in adult mice co-infected with SP and IAV during infancy, in contrast to mice infected with a single respiratory pathogen. In a previous study, residual lung function defects in adulthood were observed in mice that were challenged with IAV in infancy. The differential response to IAV alone may reflect mouse and viral strain differences, where Balb/c mice inoculated with Mem/71 were previously been used, in contrast to this study where C57BL/6 mice infected with HKx31 were assessed. Histologically, there was no evidence for remodelling of the airways, with no increase in goblet cells or Muc5ac expression or increase in smooth muscle bulk. Functionally, airway smooth muscle appears to be normal as assessment of MCh constrictor responses in isolated lung slices were not altered in co-infected mice.
Since IAV-induced disruption of the airway-epithelial barrier has previously been reported to promote AHR due to increased MCh accessibility to smooth muscle, we also assessed epithelial integrity. Co-infection with virulent strains of IAV and SP has been shown to lead to greater loss of basal epithelial cells and poor re-establishment of the normal airway epithelium during reparative processes. However, histologically there was no evidence for disruption of lower airway epithelial barrier surrounding airways, and transcript expression of the epithelial integrity markers (ZO-1, occludin-1 and E-cadherin) was not altered in co-infected mice. In addition, the relative abundance of AT2 cells in adult mice did not differ between the treatment groups following co-infection during infancy. The strain of virus used in this study is considered to be moderately virulent, hence may not necessarily promote long term damage as seen with more virulent pandemic strains. Alternately, upper airway

Figure 4. Increased airway resistance in vivo following neonatal co-infection. (A) Newtonian resistance (Rn), which is representative of central airways resistance was assessed via SCIREQ® Flexivent small-animal ventilator. Nebulised doses of methacholine (MCh) in injectable saline were delivered via tracheal cannula. (B) Maximal Rn response to MCh is expressed as % saline control response (n = 5–12). *p < 0.05 compared to vehicle and to IAV treated mice.
colonisation requires adhesion to the epithelial lining of the respiratory tract via binding to cell-surface carbohydrates. Increased upper airway carriage in co-infected mice may disrupt the barrier function of polarized upper airway epithelial cells to facilitate greater transmigration of allergens. SP displays significant genetic diversity with over 90 serotypes and commonly inhabits the upper airways where it can lead to persistent carriage, particularly in infants where colonisation rates in excess of 60% have been reported. Although there are multiple risk factors for impaired SP clearance, co-existence of respiratory viruses markedly increase pneumococcal load during the acute phase of infection and also facilitates transmission. Here, we show in an infant model of co-infection, that IAV facilitates long term nasopharyngeal colonisation into adulthood, in contrast to mice inoculated with SP alone, where the pneumococcus was cleared by day 40. Pneumococcal infection is associated with a robust acute inflammatory response, and clearance of SP

Figure 5. Increased airway hysteresitivity following neonatal co-infection. Tissue damping (G; (A,B)), tissue elastance (H; (C,D)) and tissue hysteresivity (η; (E,F)) were also assessed with SCIREQ® Flexivent in response to nebulised doses of MCh (n = 5−12). *p < 0.05 compared to vehicle treated mice.
Figure 6. Neonatal co-infection does not induce mucus hyper-secretion or increased airway smooth muscle bulk. Representative images of immunohistochemistry shows Alcian Blue-PAS (A–D) and α-smooth muscle actin (α-SMA; (E–H) staining of lung tissue from adult mice exposed to infection in early life. (I) Taqman PCR analysis of mucin marker Muc5ac gene expression in lung tissue is expressed as fold-increase compared to vehicle-treated control and normalised to GAPDH housekeeping gene (n = 6). (J) Quantification of airway α-SMA staining (brown) is expressed as area of stain/basement membrane (BM) length (n = 5–11).
is dependent on recruitment of leukocytes to the airways and engagement of humoral-mediated immunity. It has been established that during co-infection with IAV and SP, interferon-dependent anti-viral responses can alter acute cell-mediated immunity to SP leading to a marked increase in pneumococcal load. Here, we show that infant IAV infection has long lasting consequences on pneumococcal colonisation of the upper airways into adulthood.

Acute inflammatory responses to IAV are also known to transiently alter lung function. Interestingly, mice deficient in neutrophils display impaired lung function associated with an increase in airway resistance, tissue damping and tissue elastance as a consequence of impaired viral clearance and more severe histopathology. Hence, an appropriate acute inflammatory response can prevent significant structural changes underlying respiratory functional abnormalities. Infection of neutropenic mice was also characterized by impaired lung function associated with an increase in IL-13 expression; hence co-infection did not induce a classic TH2 response. SP is known to induce expansion of multiple T lymphocyte subsets including mucosal innate T cells that contribute to control of pneumococcus, and our data suggest that co-infection with IAV suppresses lymphocyte accumulation. It has been previously shown that bacterial components of SP can initiate the accumulation of T regulatory cells, which suppress allergic airways disease in response to ovalbumin sensitisation and challenge. In our model, the presence of SP did not confer protection to AHR, possibly as an allergic adjuvant was not used to initiate lung function abnormalities.

Prospective childhood cohorts have now identified viral respiratory infections as a major risk factor for developing persistent wheeze and asthma in individuals with co-existing aeroallergen sensitization. Post natal lung growth represents an important susceptibility period where the combination of viral infections and aeroallergen sensitisation in children can lead to persistent pathological changes and respiratory dysfunction.
co-infection in this context is less clear; however there is emerging data to suggest that nasopharyngeal colonisation may increase risk of developing asthma. In a recent study, the presence of bacterial pathogens including SP in the nasopharyngeal compartment during infancy was identified as significant risk determinant for febrile lower respiratory infections and later asthma development29. Hence, it is plausible that prevention of bacterial colonisation in infancy can contribute to the prevention of lower respiratory tract infections and persistent wheeze. This remains a challenging proposition using current strategies as a potential causal link between the use of antibiotics and development of asthma was identified, where disruption of the upper airway microbiome selects for resistant strains that are potentially more pathogenic29. The pneumococcal vaccine can reduce risk; however, the niche created by eliminating vaccine-targeted serotypes can be replaced by other serotypes and pathogenic bacteria.

In summary, co-infection caused a significant increase in central airway resistance that was not associated with conventional airway remodelling that underlies AHR to nebulised MChs such as mucus overproduction, smooth muscle thickening or epithelial leakage. An increase in hysteresivity was observed in both IAV and co-infected
mice. Hence, co-infection in infancy may be causing distinct structural changes that contribute to ventilation heterogeneity, which persists into adulthood. Lung hysteresis is a term used to reflect the mechanical coupling between energy dissipative forces and tissue-elastic properties in the parenchyma. Remodelling of the tissue increases hysteresivity in both emphysema and fibrosis in a way that correlates with the volume proportion of collagen, however the physical basis of this phenomenon is not well understood\(^1\). Our data suggests that there may be microstructural changes contributing to ventilation heterogeneity. Future studies should investigate the impact of co-infection on extracellular matrix components that contribute to the parenchymal architecture and also assess the consequences of combining infant co-infection with aeroallergen sensitisation.

References


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Author Contributions

M.F. and S.R. conceived and conducted the experimental design, analysis of data and wrote the manuscript. O.W., P.F., L., J.B., J.M., G.A., A.S. and S.R. assisted with experimental design, interpretation and analysis of data and preparation of manuscript. All authors reviewed the manuscript.
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