Investigation of the mechanisms of respiratory infection-induced lung disease

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Statement of Originality

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Richard Yong Hoon Kim

September 2014
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Synopsis

Asthma is a chronic allergic inflammatory disease of the airways that affects over 300 million people worldwide. The disease is driven predominantly by aberrant immune responses to normally harmless environmental stimuli. Upon encountering these stimuli, numerous immune and structural cells within the airways of the asthmatic lung release a wide range of inflammatory mediators that cause injury to the airway mucosa and surrounding tissues and leads to mucosal swelling, mucous secreting cell (MSC) hyperplasia and metaplasia and oedema. These inflammatory processes are also accompanied by an increase in bronchial smooth muscle tone in response to non-specific stimuli, a key pathological feature of asthma referred to as airways hyper-responsiveness (AHR), which results in bronchoconstriction. Together, these processes result in widespread but variable airflow obstruction in the asthmatic lung that give rise to the characteristic features of the disease, including difficulty breathing, wheezing, chest tightness and cough. In severe cases, the airflow obstruction can be so extreme that it can result in death via asphyxiation.

The majority of asthmatics can effectively control their disease through the use of bronchodilators (β2 adrenergic receptor agonists) and inhaled corticosteroids (ICS). However, these treatments do not cure disease and, importantly, a significant proportion of moderate to severe asthmatics exhibit persistent airflow obstruction and frequent exacerbations of disease despite high dose long-acting β2 agonist (LABAs) and ICS treatment. These treatment-refractory asthmatics represent a significant health burden and urgently require improved therapeutic options. An increased understanding of the mechanisms that underpin the development of asthma, particularly the pathogenesis of severe, treatment-refractory forms of the disease, may
inform novel targets for the development of improved therapeutic strategies for preventing the development of asthma and/or improving treatment outcomes.

Numerous clinical studies have demonstrated a link between certain respiratory infections and the development of asthma. Significantly, increasing clinical and experimental evidence has shown an association between a number of respiratory infections and the development of more severe, steroid-insensitive forms of asthma. In particular, a large body of evidence associates *Chlamydia* respiratory infection with the development and exacerbation of asthma, particularly severe forms of disease, in both children and adults. However, the mechanisms that underpin the association remain unknown. Our laboratory has developed a research program to investigate the link between *Chlamydia* infection and asthma using murine models of disease. We have shown that a prior neonatal and infant, but not adult, *Chlamydia* respiratory infection results in persistent AHR, emphysema-like alveolar enlargement and increased severity of allergic airways disease (AAD) in later life. We have also shown that ongoing adult *Chlamydia* respiratory infection during AAD suppresses Type 2 T helper (T\(_{H2}\)) lymphocyte and eosinophilic responses but drives a T\(_{H1}/T_{H17}\) and neutrophil-dominated form of disease that recapitulates many of the features of severe forms of asthma in humans. In this Thesis I have extended these findings by identifying key factors and signalling pathways that play important roles in neonatal *Chlamydia* respiratory infection-induced chronic lung disease and severe asthma in later life, and adult *Chlamydia* respiratory infection-induced, severe, steroid-insensitive asthma. The studies described hereafter have made important and novel observations that demonstrate roles of key microRNAs (miRNAs) and immune factors and signalling pathways that underpin *Chlamydia*-induced, severe asthma.
Initially, I used microarray analyses as a discovery tool to identify key miRNAs and genes that are altered by early life and adult *Chlamydia* respiratory infections (Chapter 2). I then conceived and designed novel studies to identify the functional roles of combinations of these factors in neonatal *Chlamydia* respiratory infection-induced severe lung disease in later life (Chapter 3) and adult respiratory infection-induced, severe, steroid-insensitive asthma (Chapters 4 and 5).

I demonstrate that 5 miRNAs (miR-155, miR-21, miR-223, miR-146b and miR-203) induced during neonatal *Chlamydia* respiratory infection promote infection-induced lung inflammation and histopathology, and drive reduced lung function, emphysema-like alveolar enlargement and increased severity of asthma in later life.

I also demonstrate that *Chlamydia* respiratory infection in established AAD induces: 1) a miR-21/phosphoinositide-3-kinase (PI3K)/phosphorylated Akt (pAkt)/histone deacetylase (HDAC)2 signalling axis, and 2) a NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome/Caspase-1/interleukin (IL)-1β signalling axis, to promote severe, neutrophilic, steroid-insensitive AAD. Additionally, I show that miR-21 and the NLRP3 inflammasome/IL-1β signalling axis also drive *Haemophilus* respiratory infection-induced, severe, neutrophilic, steroid-insensitive AAD in order to demonstrate that these factors/signalling pathways may be broadly applicable to infection-induced severe asthma.

These studies have identified potential mechanisms that drive respiratory infection-induced severe asthma. Importantly, these studies demonstrate that therapeutically targeting key respiratory infection-induced factors in the lung, including miRNAs and factors involved in key immune signalling pathways, may be effective for the prevention and/or treatment of severe forms of asthma.
Publications arising from this Thesis

Refereed publications

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Kim RY, Pinkerton JW, Essilfie AT, Robertson AA, Mayall JR, Starkey MR, Cooper MA, Horvat JC, Hansbro PM. NLRP3 inflammasome activation by bacterial respiratory infections promotes steroid insensitivity in experimental asthma. In preparation for submission as an original research article.

Publications that I have contributed to during my PhD:


* denotes equal contribution to manuscript (i.e. co-first author)
Conference publications, presentations and awards

Conference publications:

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Schilter HC, Shum B, Shim D, Maslowski K, Tsai L, Kim RY, Hansbro NG, Hansbro PM, Sewell WA, Mackay CR. Fatty acid binding proteins: A link between metabolism and airway inflammation? Respirology, Darwin, NT 2009 [E3]


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Australian Post Graduate Award (APA) PhD Scholarship. February 2007

Cooperative Research Centre for Asthma and Airways (CRCAA) Travel Award. January 2008

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Abbreviations

AAD: Allergic airways disease
adi: Adult
AHR: Airways hyper-responsiveness
AIM2: Absent in melanoma 2 (also termed PYHIN4)
Akt: Protein kinase B
Alum: aluminium hydroxide
AP-1: Activator protein
APC: Antigen presenting cell
ASC: Apoptosis-associated speck-like protein containing a CARD
ATP: Adenosine triphosphate
α-IL-1β: Anti-IL-1β neutralising antibody
BAL: Bronchoalveolar lavage
BALF: BAL fluid
BCG: Bacillus Calmette-Guerin
BSA: Bovine serum albumin
cAMP: Cyclic adenosine monophosphate
CARD: Caspase-recruitment domain
CBP: cAMP response element-binding (CREB)-binding protein
CCL: Chemokine (C-C motif) ligand
CEBPB: CCAAT/enhancer-binding protein-β
COPD: Chronic obstructive pulmonary disease
COX-2: Cyclooxygenase-2
Cmu: C. muridarum
cRNA: Complementary RNA
CXCL: Chemokine (C-X-C motif) ligand
C. muridarum: Chlamydia muridarum
C. pneumoniae: Chlamydia pneumoniae
C. trachomatis: Chlamydia trachomatis
DAMP: Damage-associated molecular pattern
DAVID: Database for Annotation, Visualization and Integrated Discovery
DC: Dendritic cell
DC-SIGN: DC-specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DGCR8: DiGeorge syndrome critical region gene 8
DEX: Dexamethasone
DMSO: Dimethyl sulfoxide
dpi: Days post infection
DR: Death receptor
dsDNA: Double-stranded DNA
EAE: Experimental autoimmune encephalomyelitis
EB: Elementary body
ELISA: Enzyme linked immunosorbent assay
FADD: Fas-associated death domain protein
FcεRI: High affinity IgE receptors
FEV₁: Forced expiratory volume in one second
FGF: Fibroblast growth factor
F. novicida: Francisella novicida
geomean: Geometric mean
GR: Glucocorticoid receptor
GRE: Glucocorticoid response element
HDAC: Histone deacetylase
HDM: House dust mite
hASMC: Human airway smooth muscle cell
Hinf: H. influenzae
HPRT: Hypoxanthine-guanine phosphoribosyl transferase
H. influenzae: Haemophilus influenzae
H. pylori: Helicobacter pylori
H&E: Hematoxylin and eosin
HRP: Horseradish peroxidase
IBD: Inflammatory bowel disease
IC: Inspiratory Capacity
ICS: Inhaled corticosteroids
IFN: Interferon
IFU: Inclusion-forming units
Ig: Immunoglobulin
IL-1R: IL-1 receptor
inf: Infant
Iso: Isotype control antibodies
i.n.: Intranasally
i.p.: Intraperitoneally
i.t.: Intratracheally
IKKe: IkappaB kinase epsilon
IL-13R: IL-13 receptor
iNOS: Inducible nitric oxide synthase
IL: Interleukin
**IRAK:** Interleukin-1 receptor-associated kinase

**JNK:** c-Jun N-terminal kinase

**KGF:** Keratinocyte growth factor

**LABA:** Long-acting β2 agonist

**LM:** Mean linear intercept

**LPS:** Lipopolysaccharide

**LRR:** Leucine-rich repeat

**LY29:** LY294002

**L. monocytogenes:** *Listeria monocytogenes*

**MAPK:** Mitogen-activated protein kinase

**MCC950:** Novel NLRP3 inhibitor

**Mech:** Methacholine

**MDDC:** Monocyte-derived DC

**miRNA:** MicroRNA

**MSC:** Mucous secreting cell

**MyD88:** Myeloid differentiation primary response gene 88

**NBD:** NACHT nucleotide-binding domain

**ncRNA:** Non-coding RNA

**neo:** Neonatal

**NF-κB:** Nuclear factor κB

**NLR:** NOD leucine-rich repeat-containing receptor

**NLRP:** NOD-like receptor family, pyrin domain containing

**NOD:** nucleotide-binding oligomerisation domain

**nt:** nucleotide

**NTHI:** Non-typeable *H. influenzae*

**Ova:** Ovalbumin

**pAkt:** Phosphorylated Akt

**PAMP:** Pathogen-associated molecular pattern

**PB:** Persistent body

**PBMC:** Peripheral blood mononuclear cell

**PBS:** Phosphate-buffered saline

**PBS-T:** PBS and Tween 20

**PDK:** Phosphoinositide-dependent kinase

**PGE2:** Prostaglandin E2

**PH:** Pleckstrin-homology domain

**PIP2:** Phosphatidylinositol 4,5-bisphosphate

**PIP3:** Phosphatidylinositol 3,4,5-bisphosphate
<table>
<thead>
<tr>
<th>Acronym</th>
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$T_H$: T helper lymphocyte  

TLR: Toll-like receptor  

TNF: Tumour necrosis factor  

TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand  

TRAF: TNF receptor-associated factor  

TRBP: Trans-activating response (TAR) RNA-binding protein  

UTR: Untranslated region  

WT: Wild-type  

YVAD: Ac-YVAD-cho  

ZVAD: z-VAD-fmk
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Chapter 1:

Introduction

In this chapter, I review the literature that supports an association between *Chlamydia* respiratory infection at different ages in the pathogenesis of respiratory disease, particularly severe forms of steroid-insensitive asthma. I also provide important background information that relates to key immune factors and processes that may underpin the association between *Chlamydia* respiratory infection at different ages and the development of severe forms of asthma. Lastly, I introduce the rationale of my PhD, which aimed to identify key mechanisms that drive *Chlamydia* infection-induced severe asthma.
1.1 Asthma

1.1.1 Epidemiology

Asthma is a common, chronic respiratory disease that affects people of all ages and ethnic backgrounds and is estimated to account for 1 in every 250 deaths globally. Conservative estimates suggest that around 300 million people in the world currently have asthma. The global prevalence of both childhood and adult asthma has increased in recent decades and this is linked with the adoption of western lifestyles and urbanisation of communities. Based on current trajectories it is predicted that an additional 100 million people will be diagnosed with asthma by 2025 (Masoli et al., 2004). Australia has a particularly high prevalence of asthma by international standards (ISAAC, 1998), with around one quarter of Australian children and 10-12% of adults affected (Woolcock et al., 2001). Furthermore, the economic cost of asthma is considerable and in the years 2004/05 this disease placed a $606 million burden on the Australian healthcare system (Mogasale and Vos, 2013). Importantly, however, the development of more effective therapies and strategies to reduce the prevalence of asthma are dependent on increasing our understanding of the underlying pathogenic mechanisms.

1.1.2 Asthma pathophysiology

Asthma is predominantly an allergic inflammatory disease of the airways that is characterised by recurrent symptoms of wheeze, dyspnoea, chest tightness and cough (Maddox and Schwartz, 2002; Cohn et al., 2004). The induction and perpetuation of allergic asthma is believed to be driven primarily through the actions of activated mast cells, eosinophils and Type 2 T helper (T\(_{H2}\)) lymphocytes in the asthmatic lung. These inflammatory cells and the mediators they release cause injury
to the airway mucosa and surrounding tissues and result in mucosal swelling, mucous secreting cell (MSC) hyperplasia and metaplasia and oedema (Pare et al., 1997; Homer and Elias, 2000; Maddox and Schwartz, 2002; Cohn et al., 2004). Repeated cycles of tissue damage and repair result in structural remodelling and thickening of the airway wall through deposition of collagen, fibronectin and tenasin in the *lamina reticularis* and reduced diameter of the airway lumen (Roche et al., 1989; Homer and Elias, 2000). These effects are accompanied by increased smooth muscle mass (Armour et al., 1997) and an increased bronchoconstrictor response to non-specific stimuli referred to as airways hyper-responsiveness (AHR). The magnitude of AHR is linked to multiple factors, including increased smooth muscle contractility, vascular permeability and the level of airway inflammation (Cockcroft and Davis, 2006). However, the precise mechanisms that underpin the association between airway inflammation and AHR are not well defined and it is widely accepted that these features may also be independent (Crimi et al., 1998). Therefore, a combined analysis of airway inflammation and lung function in terms of AHR may be the most informative diagnostic strategy for assessing asthma severity. In asthma, the combined effects of airway remodelling, mucous hypersecretion, oedema and AHR result in widespread but variable airflow obstruction that, in severe cases, can lead to death from asphyxiation.

1.1.3 Immunology of allergic asthma

Asthma is characterised by aberrant acquired immune responses to a range of stimuli including pollens, common household allergens, such as house dust mite (HDM) and cockroach antigens, air pollution and respiratory infections (Hansbro et al., 2004; Marks, 2006). In asthma, the immune response to allergen is typically
comprised of two distinct phases. The ‘early phase’ is induced by the release of pre-formed immune mediators from activated mast cells. This results in the development of the ‘late phase’, which is characterised by recruitment and activation of eosinophils and T_{H2} cells. Increasing clinical and experimental evidence also links innate immune responses with asthma, independent of acquired immune responses. Activation of germline-encoded pattern recognition receptors (PRRs) by pathogen-associated molecular pattern molecules (PAMPs) has been shown to be important in the exacerbation of key features, and severity, of asthma (Michel et al., 1989; Rylander et al., 1989; Michel et al., 1992; Michel et al., 1996; Eldridge and Peden, 2000; Tulic et al., 2000; Maddox and Schwartz, 2002).

1.1.3.1 Early Phase

In sensitised asthmatics the early phase response is typically initiated within 20 minutes of allergen exposure. This early asthmatic response is mediated by allergen-specific immunoglobulin (Ig)E antibodies and mast cells and is usually resolved within an hour (Busse and Lemanske, 2001; Maddox and Schwartz, 2002). Production of allergen-specific IgE requires an initial interaction between inhaled aeroallergens and resident antigen presenting cells (APCs) of the airways, such as dendritic cells (DCs). Activated DCs then migrate to the lung-draining lymph nodes and present antigen to naïve T cells, which results in their stimulation and subsequent differentiation (Busse and Lemanske, 2001; Maddox and Schwartz, 2002). In predisposed individuals, the conditions that accompany stimulation result in a greater potential for the differentiation of naïve T cells into T_{H2} cells. Importantly, the presence of the T_{H2} cytokines interleukin (IL)-4 and IL-13 will encourage B cells to switch from IgG to IgE antibody production (Wills-Karp et al., 1998; Busse and
A secondary signal provided by interaction of the T and B cell surface CD40 ligands further promotes B cell antibody class switching (Gould et al., 2000; Busse and Lemanske, 2001; Platts-Mills, 2001). Synthesised allergen-specific IgE antibodies are circulated in the blood and bind to high affinity IgE receptors (FceRI) on the surface of mast cells (Busse and Lemanske, 2001; Platts-Mills, 2001). In the early phase response, re-exposure to allergen causes crosslinking of the FceRI:allergen-specific IgE complex. This results in mast cell activation and the exocytosis of granules containing mediators such as histamine, eicosanoids and the T\(_H\)2 cytokines, IL-4 and IL-13 (Busse and Lemanske, 2001; Maddox and Schwartz, 2002). These mediators induce smooth muscle contraction and vascular permeability that results in infiltration of inflammatory cells, and further promote the differentiation of naïve T cells into T\(_H\)2 cells (Busse and Lemanske, 2001; Maddox and Schwartz, 2002).

1.1.3.2 Late Phase

The late phase response is initiated by the release of cysteinyi leukotrienes and inflammatory cytokines and typically occurs 4-6 hours after the early phase response (Busse and Lemanske, 2001; Maddox and Schwartz, 2002). This late asthmatic response is characterised by heavy cellular infiltration of the airways and predominantly involves the recruitment and activation of T\(_H\)2 cells and eosinophils (Rothenberg, 1998; Busse and Lemanske, 2001; Maddox and Schwartz, 2002). Importantly, recruited T\(_H\)2 cells secrete the T\(_H\)2-associated cytokines IL-4, IL-5 and IL-13 that play key roles in the pathogenesis of asthma. IL-4 promotes the differentiation of naïve T cells into T\(_H\)2 cells whilst suppressing development of T\(_H\)1 cells (Kopf et al., 1993; Chung and Barnes, 1999; Mazzarella et al., 2000; Busse and
IL-5 is a potent chemoattractant that recruits and activates eosinophils. Once activated, eosinophils degranulate and secrete preformed mediators such as major basic protein, eosinophil cationic protein and cysteinyl leukotrienes that are cytotoxic to respiratory epithelium, induce smooth muscle contraction and increase vascular permeability (Rothenberg, 1998; Bousquet et al., 2000; Gleich, 2000; Maddox and Schwartz, 2002; Wills-Karp and Karp, 2004). Finally, IL-13 is important for initiating T_{H}2 responses, airway mucous production and AHR (Chomarat and Banchereau, 1998; Doucet et al., 1998; McKenzie et al., 1998; Wills-Karp et al., 1998; Zhu et al., 1999; Maddox and Schwartz, 2002).

1.2 Severe asthma

1.2.1 Heterogeneity of asthma: Differentiating endotypes of disease

The majority of patients with asthma can achieve control of their disease with the use of combinations of bronchodilators (β2 adrenergic receptor agonists) and inhaled corticosteroids (ICS). However, some asthmatics have persistent airflow obstruction, more frequent exacerbations of disease and remain symptomatic despite treatment with high doses of long-acting β2 agonists (LABAs) and ICS (Bell and Busse, 2013). This group of patients is more likely to have severe asthma. Recent estimates suggest that ~15% of asthmatic patients are severe asthmatics (Bateman et al., 2004; Jarjour et al., 2012). However, despite its overall prevalence, severe asthma applies a disproportionately high burden on asthma-associated health care expenditure (Weiss et al., 1992).

Recent clinical evidence suggests that asthma is a heterogeneous disease that is not exclusively a T_{H}2-dependent, eosinophilic inflammatory disease. In fact, a
number of clinical studies have found evidence for increased $T_h1$- and/or $T_h17$-responses in moderate-to-severe asthma (Liu et al., 2004b; Kumar et al., 2006; Truyen et al., 2006; Bullens, 2007) and have described non-eosinophilic endotypes of disease, that are characterised by predominantly monocytic or neutrophilic, rather than eosinophilic, airway inflammation (Ordonez et al., 2000; Gibson et al., 2001). Importantly, severe asthma is associated with non-eosinophilic endotypes of asthma, especially neutrophilic asthma that is characterised by sputum neutrophil counts exceeding 61% (Green et al., 2002; Simpson et al., 2006; Hansbro et al., 2011). Patients with neutrophilic asthma represent ~20% of all non-eosinophilic asthmatics (Pavord et al., 1999; Simpson et al., 2006) and Simpson et al. found that neutrophilic asthmatics have evidence of increased innate immune activation, including increased expression of toll-like receptor (TLR)2, TLR4, CD14, IL-8 and IL-1β, when compared to eosinophilic and paucigranulocytic asthmatics. Subjects with neutrophilic asthma also have elevated levels of endotoxin in their induced sputum (Simpson et al., 2007).

Collectively, these studies show that asthma is a complex condition that is likely to be underpinned by several cell types and immune responses. This may explain why broad-acting medications, such as ICS, are not universally effective for the treatment of asthma and highlights the need to develop more targeted therapies. The development of such therapies is best achieved by increasing our understanding of the mechanisms that underpin the central features of asthma, particularly severe endotypes of disease that currently have no effective therapeutic options.
1.2.2 Steroid-based therapy in asthma

Steroid therapy is integral to the clinical management of a number of inflammatory diseases of the lungs, including asthma and chronic obstructive pulmonary disease (COPD). Despite recent progress in the development and clinical application of disease-modifying biologic therapeutics, treatment regimes that incorporate anti-inflammatory corticosteroids are fundamental to the success of managing inflammatory immune diseases and for controlling the frequency and extent of disease exacerbations (Rhen and Cidlowski, 2005; Bucala, 2012). Glucocorticoids are a class of steroid hormones that have potent anti-inflammatory activity and are widely used to treat patients with asthma (Barnes, 1995; Barnes, 2014). ICS therapy uses synthetic, highly lipophilic glucocorticoids that rapidly diffuse into airway cells and exert their effects by binding and activating the cytosolic glucocorticoid receptor (GR) (Ito et al., 2006b; Barnes, 2014). This triggers the formation of GR homodimers that are transported into the nucleus by importin-α and importin-13 (Barnes, 1995; Tao et al., 2006; Barnes, 2014). In the nucleus GR homodimers bind to glucocorticoid response elements (GREs) contained in promoter regions of glucocorticoid response genes to promote or suppress gene transcription. Interaction with transcriptional co-activators, such as cyclic adenosine monophosphate (cAMP) response element-binding (CREB)-binding protein (CBP), results in acetylation of core histones associated with anti-inflammatory glucocorticoid response genes and allows RNA polymerase II-mediated gene transcription. Alternatively, activated GR can interact with CBP proteins that are already complexed with promoter regions of pro-inflammatory genes, including nuclear factor (NF)-κB and activator protein (AP)-1. This results in suppression of pro-inflammatory gene transcription by inhibiting histone acetylation and disallowing access to DNA for RNA polymerase II (Barnes,
Importantly, the anti-inflammatory activity of GRs is mediated, at least in part, by recruitment of the enzyme histone deacetylase (HDAC)2, which is a key transcriptional co-repressor that deacetylates core histone proteins (Ito et al., 2001; de Ruijter et al., 2003).

Although the majority of asthmatics respond well to steroid therapies that effectively control their symptoms, a sub-population of 5-10% of asthmatics, typically with more severe disease, has poor sensitivity to treatment even with high doses of steroids and fails to achieve control of their asthma (Rhen and Cidlowski, 2005; Barnes, 2010). This group has been described as having severe, steroid-insensitive asthma (Barnes and Woolcock, 1998; Chung et al., 1999; Currie et al., 2005; Ito et al., 2006c; Barnes and Adcock, 2009; Fanta, 2009). Significantly, severe, steroid-insensitive asthma is associated with non-eosinophilic endotypes of asthma, including neutrophilic asthma (Green et al., 2002; Hansbro et al., 2011).

Steroid-insensitive asthma was first described by Schwartz et al. who found a small sub-group of asthmatics with poorly controlled asthma that did not respond to treatment with high-doses of orally administered steroid (Schwartz et al., 1968). Today, through refinement of the diagnostic criteria, steroid-insensitive asthma is defined as an inability to achieve >15% improvement in forced expiratory volume in one second (FEV₁) following a 14 day course of oral steroid therapy (prednisolone; 40mg/day) (Adcock et al., 2008; Barnes and Adcock, 2009).

1.2.3 Mechanisms of steroid insensitivity in asthma

Multiple mechanisms have been implicated in promoting the pathogenesis of steroid-insensitive asthma. However, insensitivity to the anti-inflammatory activity of glucocorticoids is likely to be induced by numerous molecular mechanisms. Many
studies have linked steroid insensitivity in asthma to defects in GR expression and activity, including reduced GR expression, reduced GR binding affinity to glucocorticoids and/or GREs, and elevated expression of pro-inflammatory transcription factors (Ito et al., 2006c; Adcock et al., 2008). Increased expression of IL-2, IL-4 and IL-13 in the airways of asthmatics can induce local steroid insensitivity by reducing GR binding affinity in T cells (Ito et al., 2006c; Adcock et al., 2008). Alternatively, Goleva et al., found that silencing of the dominant negative GR-β isoform in bronchoalveolar lavage (BAL) macrophages from patients with steroid-insensitive asthma improved activity of the functional GR-α isoform (Goleva et al., 2006). In another study, Irusen et al. found that activation of the p38 mitogen-activated protein kinase (MAPK) resulted in GR phosphorylation and reduced GR function (Irusen et al., 2002). Patients with steroid-insensitive asthma also have defective nuclear translocation of GR that leads to reduced GR:GRE interactions (Ito et al., 2006c; Adcock et al., 2008). In the nucleus, excessive activation of NF-κB and AP-1, as well as increased expression of c-Fos and c-Jun N-terminal kinase (JNK), can interfere with GR:GRE binding affinity (Adcock and Lane, 2003; Ito et al., 2006c; Loke et al., 2006; Adcock et al., 2008).

Interestingly, many patients with steroid-insensitive asthma have normal nuclear translocation of GR and do not show reduced GR:GRE binding affinity (Barnes, 2004; Adcock et al., 2008). Thus, it is likely that steroid-insensitivity in asthma is also induced by dysfunction of a different mechanism. Importantly, reduced HDAC2 expression and activity is associated with steroid insensitivity and more severe disease in both asthma and COPD (Ito et al., 2005; Barnes, 2009; Marwick et al., 2009). This suggests that deficiencies in transcriptional co-repressor expression and activity may play a role in the pathogenesis of steroid-insensitive asthma.
Patients with steroid-insensitive asthma also present with increased thickness of the airway epithelium and basement membrane compared to steroid-sensitive asthmatics (Bourdin et al., 2007; Cohen et al., 2007). This highlights the role of the airway epithelium in steroid-insensitive asthma and suggests a potential mechanistic link to airway remodelling.

Significantly, there are no treatments for patients with severe, steroid-insensitive asthma and, thus, there is an unmet need for new therapies for these patients. The development of such therapies is most effectively achieved by applying strategies to increase our understanding of the mechanisms that underpin the pathogenesis of steroid insensitivity and identifying and therapeutically targeting the factors identified. Importantly, many of the factors associated with severe asthma are induced by respiratory infections. Significantly, increasing clinical and experimental evidence highlights a number of respiratory infections as playing important roles in the development and exacerbation of asthma, particularly more severe forms of disease, in children and adults.

1.3 *Chlamydia* respiratory infections

1.3.1 Developmental cycle of *Chlamydiae*

*Chlamydia pneumoniae* and *C. trachomatis* are atypical obligate intracellular, Gram-negative bacteria that commonly cause bronchitis and atypical pneumonia in humans (Beem and Saxon, 1977; Harrison et al., 1982; Alexander and Harrison, 1983; Numazaki et al., 1986; Hammerschlag, 2003; Sutherland and Martin, 2007). *Chlamydiae* display an *in vivo* tropism for a variety of host cell types including alveolar- and monocyte-derived macrophages, monocytes, polymorphonuclear neutrophils, vascular endothelia, arterial smooth muscle cells, and respiratory tract
epithelia (Shemer-Avni and Lieberman, 1995; Gaydos et al., 1996; Jahn et al., 2000; Belland et al., 2004; Blasi, 2004; van Zandbergen et al., 2004). Importantly, *Chlamydiae* requires host cell viability for survival and replication and can elicit or inhibit apoptosis under different circumstances (Figure 1.1) (Byrne and Ojcius, 2004). The host wholly provides the energy for chlamydial replication, which involves regular changes between two distinct forms; a spore-like infectious form known as elementary bodies (EBs) and a metabolically active non-infectious form termed reticulate bodies (RBs). Infectious EBs can survive in the extracellular environment for brief intervals, which facilitates the continuation of infection and replication in neighbouring cells (Haranaga et al., 2003). Following infection internalised EBs differentiate into RBs and replicate in membrane-bound vesicles or intracellular inclusions within the cytosol that expand to accommodate *Chlamydiae* progeny. At the completion of the replication phase RBs differentiate back into infectious EBs and induce host cell lysis for release into the extracellular environment (Falsey and Walsh, 1993; Theunissen et al., 1993; von, 2002; Byrne and Ojcius, 2004). Under certain conditions *Chlamydiae* RBs can differentiate into non-replicating persistent bodies (PBs) that cause chronic/persistent infections (Falsey and Walsh, 1993; Theunissen et al., 1993; von, 2002; Byrne and Ojcius, 2004).
**Figure 1.1: The developmental cycle of Chlamydia.** Figure adapted from (Byrne and Ojcius, 2004). (1, 2) Spore-like infectious but metabolically inactive elementary bodies (EBs) bind to host cells through electrostatic attraction and are internalised and form intracellular inclusions within the cytosol. (3) The EBs then differentiate into non-infectious but metabolically active reticulate bodies (RBs) and undergo replication. (4) At the completion of the replication phase RBs differentiate back into EBs and (5) induce cell lysis for release into the extracellular environment. EBs can survive in the extracellular environment for brief intervals, which facilitates infection of surrounding cells. (6) Under certain conditions RBs can differentiate into non-replicating persistent bodies (PBs) that cause chronic and/or persistent infections.
1.3.2 Immune responses to *Chlamydia* respiratory infection

*Chlamydia* respiratory infections induce robust T\_H1-mediated immune responses that are characterised by the production of interferon (IFN)-\(\gamma\), and tumour necrosis factor (TNF)-\(\alpha\). Several studies have shown that T\_H1-mediated immune responses are required to effectively clear *Chlamydia* respiratory infection, whilst T\_H2-mediated responses are associated with increased bacterial load, decreased clearance and increased pathology (Williams et al., 1993; Yang et al., 1996; Yang et al., 1999). Yang *et al.*, found that *Chlamydia* respiratory infection of BALB/c mice, compared to infection of C57BL/6 mice, resulted in a higher mortality rate and delayed clearance from the lungs (Yang et al., 1996). These outcomes in BALB/c mice were associated with increased production of the T\_H2-associated cytokine IL-10 and decreased IFN-\(\gamma\) production. Neutralisation of IL-10 *in vivo* using anti-IL-10 monoclonal antibodies significantly improved clearance of *Chlamydia*. In a separate study, Yang *et al.*, also showed that *Chlamydia* infection of wild-type (WT), but not IL-10 knockout, mice resulted in the formation of granulomas and correlated with higher levels of the T\_H2 cytokine IL-5 (Yang et al., 1999). Importantly, IL-10 inhibits the production of T\_H1 cytokines (Fiorentino *et al.*, 1991; Trinchieri, 2007). Furthermore, Wang *et al.*, found that IFN-\(\gamma\) knockout mice had defective immune responses to *Chlamydia* infection and were unable to effectively clear the infection (Wang et al., 1999). Therefore, T\_H1-mediated immune responses are required to effectively resolve *Chlamydia* respiratory infection, and a T\_H2 immune bias may increase susceptibility to *Chlamydia* infection.

Additionally, others have shown that IL-17 and IL-1\(\beta\) also play important roles in the resolution of *Chlamydia* respiratory infection (Shimada *et al.*, 2011; Zhang *et al.*, 2012). Zhang *et al.* demonstrated that IL-17 synergistically enhances
IFN-γ-mediated inducible nitric oxide synthase (iNOS) expression in *Chlamydia*-infected mouse lung epithelial cells and that inhibition of iNOS promotes chlamydial growth (Zhang et al., 2012). The authors also demonstrated that neutralisation of IL-17 in *Chlamydia*-infected mice increases *Chlamydia* numbers. In a different study, Shimada *et al.* demonstrated that *Chlamydia* infection induces IL-1β production and secretion by macrophages *in vitro* (Shimada et al., 2011). They also showed that inhibition of the IL-1 receptor in *Chlamydia* respiratory infection in mice interferes with the clearance of *Chlamydia* from the lungs. Therefore, IL-17- and IL-1β-mediated immune responses are also important for the resolution of *Chlamydia* respiratory infection.

1.4 *Chlamydia* respiratory infections and asthma

1.4.1 Early-life respiratory infections and asthma

The rising global prevalence of both childhood and adult asthma has been linked with alterations in environmental conditions including, increasing urbanisation as communities adopt more western lifestyles (Masoli et al., 2004). Several hypotheses have been generated to reflect and potentially explain these international patterns of asthma prevalence and have implicated several factors, including air pollution in developed cities and increased atopic sensitisation as a result of increased exposure to indoor allergens. However, none have received as much attention as the ‘hygiene hypothesis’. Despite its simplicity, this hypothesis provided a rational interpretation of the then current epidemiological data surrounding hay fever risk in families and correlated the worsening hay fever epidemic with the industrial revolution of the 19th century (Emanuel, 1988; Aberg, 1989; Wuthrich, 1989; Strachan, 2000). The hygiene hypothesis states that exposure to T<sub>H</sub>1-inducing
infections in early life is required to dampen the T_{H2}-biased immune system of neonates (Adkins and Hamilton, 1992; Garcia et al., 2000; Rowe et al., 2000) and promote a balanced T_{H1}/T_{H2} immune phenotype (Strachan, 1989; Strachan, 2000). This hypothesis suggests that, in western societies, improved hygiene levels and sanitation, increased use of vaccination and antibiotics as well as decreased family size may reduce overall exposure to T_{H1}-inducing infections and result in a persistent T_{H2}-biased immune phenotype (Strachan, 2000; Hansbro et al., 2004; George et al., 2006).

It has been suggested that the age and nature of first infection is critical in shaping the immune responses that predominate in later life and that may predispose to, or protect against, the development of asthma (Bager et al., 2002; Hansbro et al., 2004; Openshaw et al., 2004; Horvat et al., 2007). Importantly, several studies have shown that neonatal exposure to infections has polarised effects on the development of asthma in later life.

Several studies suggest that certain infections in early life can induce responses that may protect against the development of asthma in later life. Arnold et al. found that gastric infection of mice with _Helicobacter pylori_, a T_{H1}-inducing infection (Smythies et al., 2000), protected against the hallmark features of allergic airways disease (AAD), including MSC metaplasia, eosinophilic airway inflammation and AHR (Arnold et al., 2011). Also, Marchant et al. showed that newborn humans develop T_{H1} immune responses following vaccination with _Bacillus Calmette-Guerin_ (BCG) vaccine (Marchant et al., 1999), and in a separate study, Li et al. found that vaccination of neonatal mice with BCG protected against eosinophilic airway inflammation and AHR in AAD (Li et al., 2006). By contrast, other studies suggest that early life infections may promote or exacerbate features of asthma in later life.
Clinical studies have shown that viral respiratory infections, such as respiratory syncytial virus (RSV) infection, can predispose to the development of asthma. Infection with RSV is a leading cause of acute bronchiolitis requiring hospitalisation (Leader and Kohlhase, 2002; Vicente et al., 2003) and a meta-analysis of 12 original articles by Pérez-Yarza et al. revealed that early life RSV respiratory infection is associated with increased risk for the development of asthma (Perez-Yarza et al., 2007). In murine models of RSV infection, neonatal infections lead to the exacerbation of features of AAD in later life (Lukacs et al., 2001; You et al., 2006). One study used a mouse model of neonatal RSV infection followed by reinfection in later life to show that RSV promoted AHR and T\textsubscript{H}2-mediated immune responses, including increased IL-13 production and mucous hypersecretion (Dakhama et al., 2005).

Collectively, these studies suggest that some T\textsubscript{H}1-inducing infections in early life may not afford protection against the development of asthma in later life. In fact, certain infections may enhance T\textsubscript{H}2 immune responses and predispose individuals to worsened disease. There is a need for studies that examine the immunological mechanisms that underpin the association between certain early life respiratory infections and the development of asthma in later life. Significantly, several studies implicate \textit{Chlamydia} respiratory infection in the development of asthma and severe asthma.

\subsection*{1.4.1.1 Early-life \textit{Chlamydia} respiratory infections and asthma}

Increasing evidence supports a role for early life \textit{Chlamydia} respiratory infection in the development and exacerbation of asthma. However, it is unclear how T\textsubscript{H}1-inducing \textit{Chlamydia} infections are linked to T\textsubscript{H}2-mediated asthma.
Respiratory infections with *Chlamydia* are a common cause of pneumonia in both neonates and children. Importantly, however, *Chlamydia*-induced neonatal and childhood pneumonia are likely to have different aetiologies and be caused by different strains. *C. trachomatis* is a common cause of neonatal pneumonia (Beem and Saxon, 1977; Alexander and Harrison, 1983; Numazaki et al., 1986) and is also the most common sexually transmitted disease in the world (Darville, 2005). Significantly, between 2-24% of pregnant women have evidence of cervical *C. trachomatis* infection and several studies provide evidence to suggest that intrauterine *C. trachomatis* infection is transferred to neonates during childbirth (Beem and Saxon, 1977; Mardh et al., 1980; Alexander and Harrison, 1983; Numazaki et al., 1986). Indeed, 15-20% of infants born to *C. trachomatis* culture-positive mothers had evidence of nasopharyngeal infection and 3-18% developed pneumonia (Alexander and Harrison, 1983).

Numerous clinical studies have demonstrated a correlation between *C. pneumoniae* respiratory infection and the development and exacerbation of asthma in children. *C. pneumoniae* is a common cause of community acquired pneumonia in children (Hammerschlag, 2003) and infection in early life is associated with abnormal lung function and chronic respiratory sequelae (Harrison et al., 1982). In one study, 23.6% of children with stable chronic lung disease (including 26 children with asthma and 12 children with chronic bronchitis), but none of the healthy controls, were PCR-positive for *C. pneumoniae* (Teig et al., 2005). In a different study, Webley *et al.* examined the blood and BAL of 70 paediatric patients with severe respiratory disease (including 42 with clinically diagnosed asthma) for evidence of viable *C. pneumoniae* (Webley et al., 2005). They report that 54% of the BAL samples collected were PCR-positive for *C. pneumoniae* and 31% of the PCR-positive samples were also culture-
positive. Significantly, 28 of the PCR-positive and 14 of the culture-positive BAL samples, respectively, were from asthmatics. The authors also found that 24 (34.3%; including 17 from asthmatics) of the 70 blood samples tested were culture-positive for *C. pneumoniae* and, in a separate analysis, report that elevated total IgE was associated with culture-positivity for *C. pneumoniae* in the BAL. Furthermore, a recent study demonstrated that wheezing infants with sero-positivity for *C. pneumoniae* more frequently progressed to asthma when compared to non-infected wheezing infants (Zaitsu, 2007). *C. pneumoniae* respiratory infection may also be linked with the exacerbation of asthma in children. Cunningham *et al.* performed a longitudinal study on 108 school-age children with asthma symptoms and found that children who reported multiple episodes of asthma symptoms over a 13-month period tended to have evidence of chronic *C. pneumoniae* infection (PCR-positive nasal aspirates) (Cunningham *et al.*, 1998). Furthermore, children who reported ≥4 exacerbations during the course of the study had more than 7-fold higher levels of *C. pneumoniae*-specific secretory IgA antibodies compared to those who reported one exacerbation. Taken together, these are important studies that demonstrate an association between early life *C. pneumoniae* respiratory infection and the development and severity of asthma.

Importantly, these clinical data are supported by experimental evidence from our group and others (Horvat *et al.*, 2007; Horvat *et al.*, 2010b; Jupelli *et al.*, 2011; Starkey *et al.*, 2012; Starkey *et al.*, 2013a; Starkey *et al.*, 2014). We demonstrated that neonatal *Chlamydia* respiratory infection in mice, with the natural mouse chlamydial pathogen *C. muridarum*, results in chronic respiratory pathology, including persistent AHR and emphysema-like alveolar enlargement (Horvat *et al.*, 2010b; Starkey *et al.*, 2014) and this has since been confirmed by others (Jupelli *et al.*, 2011). We then
superimposed our mouse model of neonatal *Chlamydia* respiratory infection with the established model of ovalbumin (Ova)-induced AAD. *Chlamydia* infection of neonatal mice increased the severity of Ova-induced AAD in later life by increasing airway-associated MSC numbers and lung IL-13 expression, and further increased Ova-induced AHR. Additionally, neonatal *Chlamydia* infection suppressed Ova-induced, $T_H2$-associated responses in AAD, including airway-associated eosinophilic inflammation, increased IL-5 and IL-13 production from Ova-restimulated mediastinal lymph node cells, and numbers of recruited and activated DCs in the lungs. Moreover, neonatal *Chlamydia* infection resulted in mixed $T_H1/T_H2$ responses to antigen (Horvat et al., 2007). In a more recent study we demonstrated that neonatal *Chlamydia* respiratory infections induce emphysema-like alveolar enlargement and persistent AHR through a tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated mechanism (Starkey et al., 2014).

### 1.4.2 *Chlamydia* respiratory infections and severe asthma in adults

*C. pneumoniae* respiratory infections cause between 5-20% of all cases of community-acquired pneumonia in both children and adults (Hammerschlag, 2000; Hansbro et al., 2004). Furthermore, 50-80% of adults possess antibodies against *C. pneumoniae*, indicating that the majority of individuals are infected with this pathogen at some stage in their lives (Grayston, 1992b). Importantly, a large body of evidence has associated *C. pneumoniae* respiratory infection with acute and chronic asthma in adults, however, its exact contribution to the development and/or exacerbation of asthma remains to be determined.

A seminal study by Hahn *et al.* demonstrated an association between increased anti-*C. pneumoniae* antibody levels and wheezing in adults with pre-existing
respiratory illness (Hahn et al., 1991). Furthermore, the authors found that 4 infected patients developed asthma for the first time. In a separate study, Hahn et al. also showed that 72% of adults with recently diagnosed asthma (compared to 44% of controls) had increased titres of C. pneumoniae-specific IgA, suggesting that these asthmatics had chronic C. pneumoniae infection (Hahn et al., 1996). In a later study, the authors showed that of a cohort of 20 patients with diagnosed C. pneumoniae infection, 10 patients developed wheeze for the first time and 6 patients developed chronic asthma, indicating that infection with C. pneumoniae can result in the development of asthma (Hahn and McDonald, 1998). In a more recent study, Martin et al. examined a cohort of 55 chronic stable asthmatics and found that 6 patients (11%) were PCR-positive for Chlamydia species (Martin et al., 2001). Additionally, others have demonstrated that in a cohort of adult-onset non-atopic asthmatics, sero-positivity for C. pneumoniae is associated with the development of persistent airflow limitation, suggesting that ongoing C. pneumoniae infection may cause a decline in lung function (ten Brinke et al., 2001).

Significantly, increasing evidence associates C. pneumoniae respiratory infection with severe forms of asthma. In a large case-controlled study, Von Hertzen et al. recruited 430 patients with asthma-like symptoms to examine the association between sero-positivity for C. pneumoniae and asthma in adults and found a strong correlation between increased C. pneumoniae-specific IgG and/or IgA antibody titres and chronic severe asthma (Von Hertzen et al., 1999). In another large study Black et al. investigated the association between anti- C. pneumoniae IgG and IgA antibody titres and the severity of asthma (Black et al., 2000). The authors demonstrated that the usage of high-dose ICS was associated with marked increases in the titres of C. pneumoniae IgG (74.1% increase) and IgA (70.6% increase) when compared to
subjects on lose-dose ICS. Furthermore, increased titres of *C. pneumoniae* antibodies were associated with reduced FEV$_1$ and higher daytime symptom scores. The authors conclude that higher titres of *C. pneumoniae* antibodies, and, potentially, ongoing infection with *C. pneumoniae*, are associated with increased asthma severity. More recently, Wark *et al.* demonstrated that in a cohort of 54 acute severe asthmatics, subjects with increased levels of *C. pneumoniae*-specific antibodies in their serum exhibited significantly higher neutrophil numbers and elevated concentrations of eosinophil cationic protein in their sputum when compared to controls (Wark *et al.*, 2002). In a different study, Patel *et al.* analysed BAL fluid from 2 asthma patient populations (Bronx, NY and Massachusetts) for evidence of *C. pneumoniae* infection (using PCR) and IL-8 production (using enzyme linked immunosorbent assay [ELISA]) (Patel *et al.*, 2010). 33% of Bronx samples and 50% of Massachusetts samples analysed were PCR-positive for *C. pneumoniae* and exhibited significantly higher (10.5-fold) concentrations of IL-8 compared to *C. pneumoniae*-negative controls. Importantly, BAL neutrophil numbers predicted the presence of *C. pneumoniae*. The authors conclude that *C. pneumoniae* infection is associated with increased IL-8 production and BAL neutrophilia in asthma and suggest that *C. pneumoniae* may contribute to poor asthma control. Additionally, Cho *et al.* have demonstrated that *C. pneumoniae* infection results in steroid insensitivity of immune and inflammatory cells from asthmatic patients (Cho *et al.*, 2005). Collectively, these studies implicate *C. pneumoniae* respiratory infection in the pathogenesis of severe, non-eosinophilic endotypes of asthma, such as neutrophilic asthma, that are associated with steroid insensitivity (Green *et al.*, 2002; Hansbro *et al.*, 2011).

Importantly, these clinical data are supported by recent experimental evidence. We have demonstrated that *Chlamydia* respiratory infections induce neutrophilic, $T_{H1}$
and/or T<sub>H</sub>17 immune responses in an experimental model of asthma (Horvat et al., 2010a). We demonstrated that ongoing, but not resolved, Chlamydia respiratory infection during sensitisation to Ova reduced eosinophilic airway inflammation, IL-5 production, MSC hyperplasia and AHR in Ova-induced AAD. Furthermore, ongoing Chlamydia infection during sensitisation induced robust neutrophilic airway inflammation and IFN-γ production from re-stimulated T cells. Significantly, this phenotype is reminiscent of neutrophilic asthma in humans.

1.5 MicroRNAs (miRNAs)

1.5.1 miRNA biogenesis

miRNAs are highly conserved, single-stranded non-coding RNAs (~22 nucleotides [nt] in length) that regulate gene expression at the post-transcriptional level either through inhibition of mRNA translation or degradation of mRNA transcripts. Importantly, recent evidence suggests that each miRNA can target as many as 200 gene transcripts (Bartel, 2009; Winter et al., 2009).

The current model of miRNA biogenesis proposes that RNA polymerase II (pol II) initiates the transcription of miRNA genes contained within introns of protein-coding, and both introns and exons of non-coding RNA (ncRNA), genes (Figure 1.2). These transcripts can be a few hundred nts in length and are identified as primary miRNA (Pri-miRNA) genes (Bartel, 2004; Rodriguez et al., 2004; Baskerville and Bartel, 2005; Bartel, 2009). Next, a microprocessor complex formed by Drosha (an RNase III endonuclease) and DiGeorge syndrome critical region gene 8 (DGCR8) processes Pri-miRNA into hairpin loop Precursor-miRNA (Pre-miRNA) that are ~70nts in length with 2nt overhangs on both 3’ ends (Lee et al., 2003; Denli et al., 2004; Han et al., 2004). These overhangs facilitate active transport of Pre-miRNA out
of the nucleus through a Ran-GTP/Exportin 5-mediated mechanism, as well as cleavage in the cytoplasm by Dicer (an RNase III endonuclease like Drosha) (Lee et al., 2003; Bohnsack et al., 2004; Denli et al., 2004; Lund et al., 2004). Dicer, in conjunction with human immunodeficiency virus trans-activating response (TAR) RNA-binding protein (TRBP), cleave the Pre-miRNA loop and terminal base pairs to create short, mature miRNA duplexes ~22nts in length. Notably, these duplexes possess imperfect complementarity and are degraded into miRNA ‘passenger’ (non-functional) and ‘guide’ (functional) strands (Lee et al., 2002; Bohnsack et al., 2004; Lund et al., 2004). This process is governed by the thermodynamic properties of the mature miRNA duplex. Unwinding commences at the terminal exhibiting the least thermodynamic stability and the strand with a 5’ terminus at this end becomes the functional guide strand (Hutvagner, 2005).
Figure 1.2: MicroRNA (miRNA) biogenesis. Figure adapted from (Bartel, 2004; Bartel, 2009). RNA polymerase II- (pol II) mediated transcription (1) generates a primary-miRNA (Pri-miRNA) (2) that in turn interacts with Drosha and DGCR8 to form a microprocessor complex. This initiates Pri-miRNA cleavage (3) and produces a hairpin loop structure called precursor-miRNA (Pre-miRNA) (4). Ran-GTP/Exportin 5-mediated active transport out of the nucleus (5) is followed by Dicer processing (in conjunction with TRBP), which cleaves off the loop and terminal base pairs of the Pre-miRNA (6) and generates a miRNA duplex (7).

1.5.2 miRNA mechanism of action

Post-transcriptional gene silencing is facilitated by both miRNA and short-interfering RNA (siRNA) duplexes through either RNA interference (RNAi) or translational repression. However, only functional single-stranded RNA strands are incorporated into the RNA-induced-silencing-complex (RISC), which is a multi-protein complex comprised of Dicer, TRBP and Argonaute2. Recent evidence also suggests that Pre-miRNA can be loaded directly into the RISC and achieve both
unwinding and greater levels of target mRNA cleavage concurrently (Bartel, 2004; Gregory et al., 2005; Bartel, 2009).

Formation of the miRNA-RISC ribonucleoprotein complex facilitates the recognition and binding of corresponding miRNA motifs in the 3’ untranslated region (UTR) of target mRNA (Lai, 2002; Bartel, 2004; Bartel, 2009). Importantly, the degree of binding complementarity determines whether degradation of mRNA transcripts or inhibition of translation occurs. Near-perfect to perfect complementarity will initiate mRNA cleavage whereas partial complementarity will inhibit translation (Bartel, 2004; Denli et al., 2004; Bartel, 2009) (Figure 1.3). mRNA targets are cleaved by RNAi through the endonuclease activity of the Piwi domain of Argonaute2 (Hammond et al., 2001; Liu et al., 2004a), however, this process exists primarily in plants and is important in plant miRNA-mediated gene regulation (Tang et al., 2003). In mammalian cells miRNA:mRNA interactions largely exhibit partial complementarity and result in the inhibition of translation (Bartel, 2004; Bartel, 2009). The miRNA-RISC complex is capable of inhibiting translation by immobilising polyribosomes bound to target mRNAs (Olsen and Ambros, 1999). Additionally, others have shown that miRNA-RISC complexes can promote target mRNA degradation by cleaving a 5’-7-methylguanosine segment to initiate degradation in processing-bodies (P-bodies) (Liu et al., 2005).
**Figure 1.3: MicroRNA (miRNA)-mediated post-transcriptional gene silencing.** Figure adapted from (He and Hannon, 2004). Both double-stranded miRNA and short-interfering RNA (siRNA) molecules are involved in post-transcriptional gene silencing. Importantly, only a single-strand is incorporated into the RNA-induced-silencing-complex (RISC), a protein complex that facilitates silencing activity. Once bound, miRNA-RISC ribonucleoprotein complexes initiate gene silencing through the recognition and binding of corresponding miRNA motifs present in target mRNA. Near-perfect to perfect complementarity initiates mRNA cleavage whereas partial complementarity is associated with the immobilisation of polyribosomes and interferes with peptide formation.

### 1.5.3 Role of miRNAs in immune function

A large body of evidence implicates miRNAs in a multitude of biological processes, including immune responses. However, defining their precise functional role is often challenging and can be complicated by many factors. Given that miRNAs can potentially regulate up to 30% of all protein-coding genes it is essential to
decipher and compare their contributions to both innate and acquired immune function (Bartel, 2004).

In a seminal study, Taganov et al. profiled the expression of 200 miRNAs in a lipopolysaccharide (LPS)-treated human monocytic cell line (THP-1) and found that the expression of miR-146a/b, miR-132 and miR-155 is LPS-responsive (Taganov et al., 2006). Further characterisation revealed that the expression of miR-146 is increased following stimulation of cell-surface TLR2, TLR4 and TLR5. The authors demonstrate that miR-146 directly targets TNF receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1), which are key mediators of the TLR and IL-1β pathways, and conclude that miR-146 functions as part of a negative feedback mechanism. Others have shown that miR-146, as well as miR-203, are associated with psoriasis. Sonkoly et al. demonstrated that miR-146 is highly expressed in immune cells (CD4+CD25high regulatory T cells, monocyte-derived DCs [MDDCs] and mast cells) in psoriatic inflamed skin but not in keratinocytes and fibroblasts (Sonkoly et al., 2007). In contrast, the authors found that miR-203 is enriched in keratinocytes and that miR-203 overexpression in psoriatic plaques is associated with down-regulation of suppressor of cytokine signalling 3 (SOCS-3). A recent study by O’Connell et al. showed that the expression of miR-155 was markedly increased in primary murine macrophages stimulated with polyriboinosinic:polyribocytidylic acid (poly[I:C]), an established synthetic TLR3 ligand, as well as with IFN-β (O’Connell et al., 2007). MiR-155 was also induced by stimulation with IFN-γ through a mechanism involving TNF-α autocrine signalling. Additionally, Tili et al. demonstrated that LPS-stimulation of mouse macrophages increased the expression of miR-155 and decreased the expression of miR-125b (Tili et al., 2007). They then repeated these observations in LPS-treated C57BL/6 mice.
The authors also found that LPS-exposed miR-155 transgenic mice had higher levels of TNF-α, and identified the LPS signalling factors Fas-associated death domain protein (FADD), IkappaB kinase epsilon (IKKε), and the receptor (TNFR superfamily)-interacting serine-threonine kinase 1 (RIPK1) as direct targets of miR-155. Further characterisation showed that miR-125b directly targets the 3’-UTR of TNF-α. Collectively, these data suggest that innate TNF-α production, in response to LPS stimulation, is regulated by miR-155 and miR-125b. Importantly, these data indicate that miRNAs are induced or suppressed as part of the innate immune response to a range of inflammatory mediators as well as to ligands that mimic the stimulatory activity of bacterial and viral infections.

Increasing evidence also implicates miR-155 in normal immune function. Rodriguez et al. examined the role of miR-155 in the development of protective immunity to oral challenge with Salmonella typhimurium using mice deficient for bic/miR-155 (Rodriguez et al., 2007). The authors initially showed that bic/miR-155-deficient mice exhibited spontaneous lung airway remodelling with evidence of increased collagen deposition and increased smooth muscle cross-sectional area as well as enteric inflammation, suggesting that bic/miR-155 is important for immune homeostasis. WT and bic/miR-155-deficient mice were vaccinated intravenously (i.v.) with live attenuated S. typhimurium (aroA) and subjected to oral challenge with virulent S. typhimurium. The authors found that bic/miR-155-deficient mice were immunodeficient, even with vaccination, and most of them succumbed to infection compared to WT controls. Further characterisation revealed that bic/miR-155-deficient mice exhibited defects in DC-mediated T cell activation and that CD4+ T cells from bic/miR-155-deficient mice produced significantly less IFN-γ following ex vivo stimulation. Interestingly these CD4+ T cells were biased toward Th2
differentiation. In a concurrently performed but independent study, Thai et al. demonstrated that bic/miR-155-deficient T-cells cultured in non-polarising conditions exhibited a similar bias toward Th2 differentiation (Thai et al., 2007). The authors found that these T-cells produced more of the Th2 cytokine IL-4, less of the Th1 cytokine IFN-γ, and more IL-10. Collectively, these studies provide strong evidence for miR-155 in normal immune function and demonstrate that a single miRNA can play a critical role in immunological decision-making.

1.5.4 Role of miRNAs in asthma and respiratory infections

Increasing evidence implicates miRNAs in asthma pathogenesis (Oglesby et al., 2010; Foster et al., 2013). Importantly, certain respiratory infections, including those caused by Chlamydia, are linked with the development and exacerbation of asthma. Significantly, there is strong evidence for miRNA involvement in responses that are common to asthma and immunity against pathogens. This suggests that respiratory infection-induced miRNA responses are important in the development and exacerbation of asthma. However, this is likely to be a complex relationship and there is very limited evidence that miRNA expression and function directly drive pathogen clearance in animals, let alone in the lung environment. Herein, I describe the potential contribution of miRNAs in these processes with a specific focus on miR-155 as it has emerging roles in this context.

Recent evidence highlights a role for miR-155 in hyporesponsiveness to β2 agonists, which is a clinical feature of severe asthma (Bell and Busse, 2013). Comer et al. demonstrated that human airway smooth muscle cells (hASMCs) from patients with asthma, following treatment with a cocktail of proinflammatory mediators (IL-1β, TNF-α and IFN-γ), exhibit enhanced miR-155 expression compared to hASMCs.
from non-asthmatics (Comer et al., 2014). The authors also found that the levels of miR-155 positively correlated with the expression and secretion of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2), respectively. Importantly, COX-2 expression and PGE2 secretion is linked with hyporesponsiveness to β2 agonists (Barnes and Pride, 1983; Hakonarson et al., 1996; Laporte et al., 1998). These data show that elevated expression of miR-155 can induce responses that are associated with severe asthma and poor asthma control. Additionally, this highlights the possibility that inhaled stimuli such as respiratory infections may promote exacerbations and/or severe forms of asthma.

As mentioned earlier mice deficient for bic/miR-155 exhibit increased remodelling of the airways that is characterised by increased collagen deposition, increased smooth muscle area and predominantly monocyte and macrophage inflammation, as determined in the BAL fluid (BALF) (Rodriguez et al., 2007). These features are reminiscent of airway remodelling events in the asthmatic lung, which suggests that miR-155 is required to control airway remodelling in asthma. Furthermore, and in contrast, increased expression of miR-155 may induce alterations in lung epithelial integrity, including vascular leakage and epithelial damage, which is an important feature in the pathogenesis of asthma (Chung et al., 1990). Increasing evidence shows that fibroblast growth factor 7 (FGF-7; also known as keratinocyte growth factor [KGF]) induces the proliferation of epithelial cells, maintains epithelial tight junctions, and suppresses neutrophilic inflammation to protect against acute lung injury (Wu et al., 1998; Atabai et al., 2002; Chapman et al., 2002; Nemzek et al., 2002; Ware and Matthay, 2002). Significantly, Pottier et al. demonstrated that miR-155 directly inhibited the expression of FGF-7 (Pottier et al., 2009). Furthermore, Tillie-Leblond et al. demonstrated that treatment with recombinant FGF-7 before Ova
aerosol challenge in sensitised rats suppressed allergen-induced neutrophilic inflammation (in BALF), decreased vascular leakiness and prevented epithelial damage in the bronchial epithelium (Tillie-Leblond et al., 2007). Taken together, these studies highlight the potential importance of miR-155 in immune responses that are associated with airway remodelling in chronic asthma. Thus, it is possible that respiratory infections, or other inhaled stimuli, alter the baseline expression of miR-155 in key lung compartments to promote features of severe asthma, including neutrophilic airway inflammation, vascular leakiness, bronchial epithelial damage, and collagen deposition and smooth muscle hypertrophy (i.e. airway remodelling).

Several studies have shown that the expression of miR-155 (as well as miR-21 and miR-146) is increased during infection with a range of bacterial pathogens, including *Helicobacter pylori*, *S. typhimurium*, *Listeria monocytogenes* and *Francisella novicida* (Cremer et al., 2009; Xiao et al., 2009; Schulte et al., 2011; Izar et al., 2012). Furthermore, Wang *et al.* demonstrated that miR-155 expression is induced in a TLR/Myeloid differentiation primary response gene 88 (MyD88)-independent manner in virus-infected macrophages and enhances innate antiviral immunity (Wang et al., 2010a). These data demonstrate that miRNAs, such as miR-155, are induced in response to bacterial and viral infections and may be important for immunity. However, based on current evidence it is unclear whether infection-induced miR-155 responses are protective or detrimental to the development and/or exacerbation of asthma. As described earlier, *S. typhimurium*-challenged bic/miR-155-deficient mice were immunodeficient, and bic/miR-155-deficient CD4⁺ T cells exhibited defects in IFN-γ production (Rodriguez et al., 2007). Indeed, bic/miR-155-deficient T cells are naturally biased toward Th2 differentiation in culture (Rodriguez et al., 2007; Thai et al., 2007). These data highlight the possibility that miR-155 plays
a central role in the immune response to bacterial infection, and that miR-155 deficiency may predispose to the development of Th2-mediated allergic asthma. In a more recent study, Martinez-Nunez et al. demonstrated that miR-155 regulates IL-13-dependent responses in human macrophages through direct targeting of IL-13 receptor (IL-13R)α1 mRNA, which results in reduced levels of IL-13Rα1 protein and suppressed activation of signal transducer and activator of transcription (STAT)6 (Martinez-Nunez et al., 2011). The authors also showed that miR-155 affected the regulation of several IL-13-dependent genes, including SOCS-1, DC-specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), chemokine (C-C motif) ligand 18 (CCL18), CD23 and serpin peptidase inhibitor, clade E (SERPINE), that have roles in the establishment of the M2 (pro-Th2) macrophage phenotype. These data suggest that miR-155 is an important factor in promoting M1 versus M2 macrophage phenotypes and that the level of its expression in macrophages can potentially influence Th1/Th2-type inflammatory responses in the lung.

These studies highlight the potential role of miRNAs in modulating pathways associated with asthma. Furthermore, it is possible that respiratory infections induce immune responses that alter baseline miRNA expression and function. Hence, it is tempting to speculate that certain respiratory infections, including those of Chlamydia, induce miRNA responses that promote the development of asthma as well as severe forms of asthma. However, based on current evidence, it is unclear whether these factors are connected. There is a need for studies that rigorously examine this potential association in order to rule in, or rule out, the importance of respiratory infection-induced miRNAs in the pathogenesis of asthma, and possibly other chronic
inflammatory diseases of the lung, including COPD, cystic fibrosis, and idiopathic pulmonary fibrosis.

1.6 Emerging factors in severe, steroid-insensitive asthma

Chronic inflammation is a feature associated with asthma and several other respiratory diseases, including COPD, cystic fibrosis, and idiopathic pulmonary fibrosis (Barnes, 2008b; Barnes, 2008a; Nichols et al., 2008). While treatment with anti-inflammatory glucocorticoids is effective in some diseases, their efficacy is strongly reduced in others, including severe asthma (Adcock and Lane, 2003; Adcock and Ito, 2004; Rhen and Cidlowski, 2005; Bucala, 2012). Importantly, there are no treatments for these patients and this results in significant morbidity. Significantly, increasing clinical and experimental evidence now implicates phosphoinositide-3-kinase (PI3K) and the nucleotide-binding oligomerisation domain (NOD)-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in the pathogenesis of severe asthma that is steroid-insensitive.

1.6.1 PI3K

The PI3K family of intracellular protein kinases are involved in a multitude of processes, including cell growth, differentiation, metabolism, and inflammatory responses (Cantrell, 2001; Engelman et al., 2006; Gunn and Hailes, 2008; Marwick et al., 2010). The PI3Ks are heterodimers comprised of catalytic and regulatory subunits that phosphorylate phosphoinositol lipids (Vanhaesebroeck et al., 2001) and are categorised into three classes (I, II and III) based on structural composition, regulation and lipid substrate specificity. Class I PI3Ks are the best characterised and are further divided into two sub-classes (IA and IB) based on associated catalytic subunits. Class
IA PI3Ks consist of p110α, p110β and p110δ catalytic subunits that dimerise with an Src-homology 2 (SH2) domain-containing regulatory subunit (p85α, p85β, p55α, p55γ or p50α) to form three distinct isoforms (PI3Kα, PI3Kβ, PI3Kδ). Furthermore, class IA PI3Ks are activated by cell surface receptor tyrosine kinases (Geering et al., 2007; Marwick et al., 2010). In contrast, the class IB PI3K is a single catalytic subunit (p110γ) that interacts with either p101 or p84/p87 adaptor proteins to form PI3Kγ and is activated by G-protein coupled receptors. The PI3Kα and PI3Kβ isoforms are ubiquitously expressed while the PI3Kδ and PI3Kγ isoforms are mostly restricted to immune cells (Wymann and Marone, 2005). Importantly, PI3Kδ and PI3Kγ (and to a lesser degree PI3Kα and PI3Kβ) are implicated in the induction of immune responses in diseases characterised by chronic inflammation including COPD and asthma (Marwick et al., 2010; Fung-Leung, 2011).

1.6.1.1 Class I PI3K signalling

Activation of PI3K receptors leads to receptor auto-phosphorylation and generates a docking site for p85 regulatory subunits or, in some cases, an adaptor protein (Cantrell, 2001; Engelman et al., 2006; Marwick et al., 2010). At the plasma membrane, class I PI3Ks convert (by phosphorylation) phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). Importantly, under normal conditions this process is negatively regulated by phosphatase and tensin homologue (PTEN) and/or SH2 domain containing inositol phosphatase (SHIP) that convert PIP3 back to PIP2 (Cantrell, 2001; Engelman et al., 2006; Marwick et al., 2010). Un-reverted PIP3 promotes the localisation of phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane, which in turn recruits and activates (by phosphorylation) proteins with pleckstrin-homology (PH) domains, including protein
kinase B (Akt) (Cantrell, 2001; Engelman et al., 2006; Marwick et al., 2010). Increased levels of phosphorylated Akt (pAkt) are a well-established indicator of PI3K activity (Lee et al., 2006; Newcomb et al., 2008).

1.6.1.2 PI3K, asthma and steroid insensitivity

Several studies implicate PI3K signalling in the pathogenesis of asthma. In a recent study Duan et al. examined the role of PI3K in allergic airway inflammation in an Ova-induced model of asthma in mice (Duan et al., 2005). The authors demonstrated that treatment with LY294002 (a non-selective PI3K inhibitor) two hours before aerosol challenge suppressed the levels of pAkt in the lungs in AAD compared to sham-treated, allergic controls. Furthermore, treatment with LY294002 decreased IL-5, IL-13 and eotaxin levels in the BALF and suppressed eosinophilic airway inflammation, tissue eosinophilia, mucous production and AHR in AAD. In a different study, Kwak et al. demonstrated that the levels and activity of PTEN are decreased in mice in Ova-induced AAD (Kwak et al., 2003). The authors also reported that the epithelial layers around the bronchioles in control mice were enriched for PTEN and that these levels were markedly decreased following Ova aerosol challenge. Treatment with wortmannin (another non-selective PI3K inhibitor), LY294002 or adenovirus-mediated overexpression of PTEN during the challenge phase suppressed Ova-induced increases in the levels of IL-4 and IL-5 in the BALF and suppressed AHR in AAD. These results are supported by a different study performed by Ezeamuzie et al. that demonstrated a role for PI3K in allergen-induced increases of eosinophil peroxidase activity (assessed in BALF) and AHR in guinea pigs (Ezeamuzie et al., 2001). Together, these studies show that targeting PI3K directly in the lungs with non-specific pharmacological inhibitors can suppress
allergic inflammation. However, more recent studies have advanced upon these findings by investigating the roles of specific PI3K isoforms. In one study, Lee et al. treated Ova-sensitised mice with IC87114 (a selective PI3Kδ inhibitor) and demonstrated that PI3Kδ activity promotes allergic airway inflammation (eosinophils, neutrophils and lymphocytes) and AHR as well as increases in the levels of total IgE in the serum and Ova-specific IgE in the BALF (Lee et al., 2006). Additionally, two independent studies have demonstrated that Ova-sensitised, PI3Kγ-deficient mice have diminished allergen-induced eosinophilic airway inflammation and decreased airway remodelling (Lim et al., 2009; Takeda et al., 2009). Collectively, these studies highlight the potential importance of the PI3Kδ and PI3Kγ isoforms in the pathogenesis of asthma. However, there is little direct evidence of the contribution of PI3K in the pathogenesis of severe asthma.

Significantly, several studies implicate PI3K signalling in the manifestation of steroid insensitivity, which is an important feature of severe asthma and is associated with poor asthma control. In a seminal study Marwick et al. demonstrated that the inhibition of PI3Kδ restored steroid sensitivity in a mouse model of cigarette smoke-induced COPD (Marwick et al., 2009). The authors showed that cigarette smoke exposure in mice reduced the activity of HDAC2 in the lung and also reduced glucocorticoid function. Significantly, PI3Kδ kinase dead knock-in transgenic mice exhibited normal HDAC2 activity and glucocorticoid function as well as reduced tyrosine nitration of HDAC2. These results are supported by a study performed by To et al. that also examined the role of PI3Kδ in the development of steroid insensitivity in COPD (To et al., 2010). The authors demonstrated that treatment of cigarette smoke-exposed mice with low-dose theophylline (another non-selective PI3K inhibitor) increased the anti-inflammatory effect of steroids (dexamethasone; DEX)
and restored HDAC2 activity. Similar effects were observed following treatment with IC87114. Furthermore, theophylline treatment of peripheral blood mononuclear cells (PBMCs) from patients with COPD restored steroid sensitivity as determined by decreased IC$_{50}$-DEX values. The authors also show that PBMCs pre-treated with HDAC2 siRNA exhibited increased IC$_{50}$-DEX values. Taken together, these studies provide strong evidence for the role of PI3K signalling in promoting the development of steroid insensitivity. Furthermore, these findings highlight the potential importance of therapeutic strategies that target PI3K signalling in order to reinstate HDAC2 activity and sensitivity to steroids.

Significantly, PI3K signalling is activated by factors that are associated with severe, steroid-insensitive asthma, such as binding of the Th17-inducing cytokines IL-6 and IL-1β to their receptors (Hideshima et al., 2001; Neumann et al., 2002). Furthermore, Haylock-Jacobs et al. demonstrated that mice with a catalytically inactive PI3Kδ subunit exhibited defects in Th17 responses in experimental autoimmune encephalomyelitis (EAE) (Haylock-Jacobs et al., 2011). This suggests that PI3K activity can also promote Th17 immune responses and highlights the potential for PI3K activity to self-perpetuate in the lungs of severe asthmatics.

1.6.1.3 PI3K, respiratory infections and miRNAs

Importantly, we, and others, have shown that respiratory infections with Chlamydia and Haemophilus induce neutrophilic, Th1 and/or Th17 immune responses in mouse models of asthma (Horvat et al., 2010a; Essilfie et al., 2011; Essilfie et al., 2012) and increasing clinical evidence links Chlamydia and Haemophilus respiratory infections with severe, non-eosinophilic endotypes of asthma, such as neutrophilic asthma, that are more likely to be steroid-insensitive (Green et al., 2002; Hansbro et
al., 2011). Additionally, studies have shown that PI3K activity plays an important role in facilitating *Chlamydia* and *Haemophilus* entry into host cells (Coombes and Mahony, 2002; Marti-Lliteras et al., 2009). Significantly, there is also the possibility that respiratory infection-induced miRNAs can enhance PI3K activity in the lung microenvironment. Collectively, these studies suggest that increased activation of PI3K may promote steroid insensitivity. Furthermore, respiratory infections may induce a range of factors that promote PI3K activity and drive steroid insensitivity in the asthmatic lung. Thus, this highlights the potential importance of increased PI3K activity in the pathogenesis of infection-induced, severe, steroid-insensitive asthma.

As discussed earlier, several studies have shown that the expressions of miR-21 and miR-155 are increased during infection by a range of bacterial pathogens (Cremer et al., 2009; Xiao et al., 2009; Schulte et al., 2011; Izar et al., 2012). Given that these miRNAs are central regulators of immune responses it is highly likely that certain respiratory infections will augment their expressions also. Significantly, miR-21 and miR-155 can potentiate PI3K activity by inhibiting the expression and activity of PTEN and SHIP, respectively (Meng et al., 2007; Yamanaka et al., 2009), suggesting that targeting infection-induced miR-21 and miR-155 may be a more specific therapeutic strategy than targeting PI3K directly.

1.6.2 NLR proteins and the inflammasome

The innate immune system relies on the activation of germline-encoded PRRs to mount immediate responses to a range of infectious or noxious stimuli (Stutz et al., 2009; Schroder and Tschopp, 2010). Several classes of PRRs have been identified and their individual roles in innate immunity are reflected by their subcellular localisation.
NOD leucine-rich repeat (LRR)-containing receptors (NLRs) are a recently identified family of intracellular PRRs that are able to detect not only PAMPs but also damage-associated molecular pattern (DAMP) molecules, which are released by cells following tissue injury (Stutz et al., 2009; Schroder and Tschopp, 2010). Members of the NLR family are characterised by their C-terminal LRR domains that detect ligands and are important for autoregulation, and central NACHT nucleotide-binding domains (NBD). NLRs are further categorised based on their N-terminal domains and the largest group (containing 14 members) possess N-terminal pyrin domains (PYD) (termed NLRP1-14). Alternatively, some NLRs possess a common N-terminal caspase-recruitment domain (CARD) and variations of nucleotide-binding oligomerisation domains (NOD; NOD1[NLRC1], NOD2[NLRC2]). There is also a distinct sub-family called NLRC4 (also called IPAF) (Martinon et al., 2002; Stutz et al., 2009; Schroder and Tschopp, 2010).

1.6.2.1 Inflammasomes

Several NLRs (NLRP1, NLRP3 and NLRC4) can assemble multi-protein complexes termed ‘inflammasomes’ that serve as platforms for the recruitment and activation of inflammatory caspases such as Caspase-1. Inflammasome-activated Caspase-1 in turn activates the IL-1 family cytokines IL-1β and IL-18, and inactivates IL-33 (Martinon et al., 2002; Martinon et al., 2009; Stutz et al., 2009; Schroder and Tschopp, 2010). Studies have also reported that the non-NLR protein absent in melanoma 2 (AIM2; also called PYHIN4) can assemble an inflammasome that recruits and activates Caspase-1 (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). Of the inflammasomes, the NLRP3 inflammasome (also called NALP3, PYPAF1 or cryopyrin) is the best characterised and has been
widely implicated in a number of pathogenic inflammatory diseases. The NLRP3 inflammasome is structurally comprised of NLRP3 and an apoptosis-associated speck-like protein containing a CARD (ASC) adaptor protein (Martinon et al., 2002; Stutz et al., 2009; Schröder and Tschopp, 2010). Upon activation the NLRP3 inflammasome interacts with Caspase-1 through ASC, which results in autocatalysis and activation of Caspase-1 (Hornung and Latz, 2010; Schröder and Tschopp, 2010). Most significantly, augmented NLRP3 inflammasome activation and exaggerated production of IL-1β are strongly implicated in the pathogenesis of severe, steroid-insensitive asthma (Konno et al., 1996; Wanderer, 2009; Hastie et al., 2010; Baines et al., 2011; Besnard et al., 2012; Kim et al., 2014; Simpson et al., 2014).

1.6.2.2 The NLRP3 inflammasome

The precise mechanisms that lead to NLRP3 inflammasome activation remain poorly defined. However, the prevailing concept is that this process consists of two distinct events, the first of which involves the expression and assembly of inflammasome components (Stutz et al., 2009; Schröder and Tschopp, 2010). Increasing evidence shows that PAMP-induced TLR ligation and pro-inflammatory cytokines such as TNF-α are important in initiating NLRP3 inflammasome expression and assembly as well as inducing IL-1β expression through NF-κB activation (Schindler et al., 1990a; Schindler et al., 1990b; Hiscott et al., 1993; Martinon et al., 2002; Martinon et al., 2009; He et al., 2013). Interestingly, one study by Ikejima et al. demonstrated that rabbits injected with recombinant IL-1β produced endogenous IL-1β (Ikejima et al., 1990). The authors also showed that human PBMCs expressed TNF-α in response to treatment with IL-1β. This may suggest that IL-1β responses can self-perpetuate. Importantly, however, IL-1β is not constitutively expressed and,
unlike the vast majority of cytokines, IL-1β is produced as an inactive 31kDa pro-form that requires proteolytic activation by the IL-1-converting enzyme, Caspase-1 (Thornberry et al., 1992), which, under normal conditions, is also present in an inactive pro-form (pro-Caspase-1) (Thornberry et al., 1992). Therefore, in NLRP3 inflammasome signalling, the induction of pro-IL-1β constitutes a form of ‘priming’ that then requires a secondary round of activation of assembled inflammasome components to drive a proteolytic pathway that leads to the activation and release of IL-1β (Hornung and Latz, 2010). Many studies mimic this priming effect in experiments by pre-treating with TLR agonists and/or pro-inflammatory cytokines, such as LPS and TNF-α, respectively (Martinon et al., 2002; Stutz et al., 2009; Schroder and Tschopp, 2010). Additionally, this may represent an important form of regulation given that IL-1β and other IL-1 family members are potent pro-inflammatory factors that can have destructive consequences if activation is uncontrolled (Dinarello, 2009).

A range of factors can activate primed NLRP3 inflammasomes, including endogenous stress signals in the form of DAMPs (Stutz et al., 2009; Hornung and Latz, 2010; Schroder and Tschopp, 2010). Importantly, the first DAMP described was extracellular adenosine triphosphate (ATP) and is thus the best studied for its role in the activation of the NLRP3 inflammasome (Mariathasan et al., 2006; Hornung and Latz, 2010). In a seminal study Mariathasan et al. stimulated NLRP3-deficient macrophages with ATP in conjunction with TLR agonists and demonstrated that NLRP3 was required to activate Caspase-1 (Mariathasan et al., 2006). The authors also demonstrated that treatment with a range of inflammasome activators, including nigericin and maitotoxin, failed to induce IL-1β in the absence of NLRP3. Additionally, ASC-deficient macrophages were unable to induce Caspase-1 activation
and produce IL-1β when infected with *S. typhimurium* or *Francisella tularensis*. Therefore, this study highlights that DAMPs such as extracellular ATP and infection with whole pathogens are important inducers of the NLRP3 inflammasome. ATP is of special interest because it can bind to, and activate, the ligand-gated cation channel purinergic receptor P2X, ligand-gated ion channel, 7 (P2X7R) to induce potassium (K⁺) efflux (Perregaux and Gabel, 1994; Walev et al., 1995; Colomar et al., 2003). Importantly, K⁺ efflux is also likely to be important for other known activators of the NLRP3 inflammasome, including pore-forming toxins (such as the potassium ionophore nigericin) and crystals (such as silica crystals, uric acid crystals and aluminium salts) (Mariathasan et al., 2006; Hornung et al., 2008; Stutz et al., 2009; Kool et al., 2011; De Nardo et al., 2014).

### 1.6.2.3 Role of the NLRP3 inflammasome in asthma and respiratory infection

There is a growing body of evidence that implicates the NLRP3 inflammasome in the pathogenesis of allergic asthma, however its role remains controversial. Recent studies have shown that the levels of ATP and P2X7R are increased in asthma and that the inhibition of ATP-mediated P2X7R signalling suppressed key disease features in experimental asthma (Idzko et al., 2007; Muller et al., 2011). This suggests that ATP-mediated NLRP3 inflammasome activation is involved in the pathogenesis of asthma. Importantly, mouse models of asthma typically involve sensitisation to protein allergens (such as Ova) in the presence of the Th2-inducing adjuvant aluminium hydroxide (alum), which is a known activator of the NLRP3 inflammasome (Hornung et al., 2008). One study performed by Eisenbarth *et al.* used NLRP3-deficient mice to demonstrate that NLRP3 is required for adjuvanticity of alum in allergic antibody responses to antigen (Eisenbarth et al.,
Additionally, a different study demonstrated the importance of NLRP3 in allergic airway inflammation using an adjuvant(alum)-free Ova model. Besnard et al. used mice deficient in NLRP3, IL-1 receptor (IL-1R)1, IL-1β or IL-1α to demonstrate a critical role for NLRP3-mediated IL-1β responses in Ova-induced allergic airway inflammation. They also reported that all factor-deficient mice exhibited marked decreases in the production of Ova-induced, Th2-associated cytokines (Besnard et al., 2011). In contrast, a different study performed by Kool et al. demonstrated that uric acid potently induces Th2 cell immunity in an NLRP3-independent manner. The authors also showed that uric acid promoted Th2 cell immunity through PI3Kδ (Kool et al., 2011). These findings are supported by a different study performed by Allen et al., which showed that WT and NLRP3-deficient mice exhibited no differences in the key features of acute or chronic Ova-induced allergic airway disease, including eosinophilic airway inflammation, mucous hypersecretion and AHR (Allen et al., 2012). Taken together, the role of the NLRP3 inflammasome in the pathogenesis of allergic asthma is controversial and remains undefined.

Significantly, increasing clinical evidence links innate immune activation and the NLRP3 inflammasome with non-eosinophilic endotypes of asthma that are associated with severe asthma (Green et al., 2002; Simpson et al., 2006; Hansbro et al., 2011). A study by Simpson et al. showed that neutrophilic asthmatics had increased expression of TLR2, TLR4, IL-1β and IL-8 and elevated levels of LPS in their sputum compared to other asthma phenotypes and healthy controls (Simpson et al., 2007). These data are supported by a different study by Baines et al. who performed gene expression profiling analyses on induced sputum from different subtypes of asthmatics and showed that the overexpression of factors involved in the IL-1 and TNF-α/NF-κB signalling pathways correlated with neutrophilic airway
inflammation (Baines et al., 2011). In a very recent study Simpson et al. showed that the sputum macrophages of neutrophilic asthmatics exhibited elevated expression of NLRP3, Caspase-1, Caspase-4, Caspase-5 and IL-1β compared to other groups. The authors also showed that neutrophilic asthmatics have increased protein levels of IL-1β in the sputum that correlated with IL-8 levels (Simpson et al., 2014). Furthermore, immunocytochemical analyses revealed that sputum neutrophils from patients with neutrophilic asthma, but not other asthma phenotypes, exhibited strong immunoreactivity for NLRP3 and Caspase-1. Collectively, these studies strongly implicate roles for innate immune activation and NLRP3 inflammasome signalling in the pathogenesis of severe asthma. Importantly, and as described earlier in detail, substantial clinical evidence links Chlamydia and Haemophilus respiratory infections with severe, steroid-insensitive asthma. This highlights the possibility that patients with asthma may encounter stimuli, such as respiratory infections, that induce innate immune responses that prime for NLRP3 inflammasome activity. Significantly, Darville et al. have demonstrated that ATP-mediated P2X7R signalling is important for immunity against Chlamydia infection (Darville et al., 2007), which suggests that infection induces DAMP signalling. Furthermore, both Chlamydia and Haemophilus respiratory infections can induce the release of active IL-1β in an NLRP3 inflammasome-dependent, Caspase-1-mediated manner (He et al., 2010; Rotta Detto Loria et al., 2013). These data highlight the possibility that Chlamydia and Haemophilus respiratory infections can induce immune responses in the asthmatic lung that can prime for, and activate, NLRP3 inflammasome activity.
1.7 Study rationale and hypothesis

Increasing clinical and experimental evidence supports a role for early life *Chlamydia* respiratory infection in the development and exacerbation of asthma. However, it is unknown how Th1-inducing *Chlamydia* infection may trigger or exacerbate Th2-mediated asthma in later life. Recent studies from our laboratory demonstrate that neonatal *Chlamydia* respiratory infection in mice induces persistent AHR, emphysema-like alveolar enlargement and enhanced severity of AAD in later life. Increasing evidence also strongly implicates *Chlamydia* respiratory infection in the pathogenesis of severe, non-eosinophilic endotypes of asthma, such as neutrophilic asthma, that are associated with steroid insensitivity. The studies described hereafter were designed to identify key miRNAs, genes and signalling pathways that are induced in the lung microenvironment by *Chlamydia* respiratory infection at different ages to promote infection-induced lung disease. I investigated the mechanisms that underpin infection-induced disease *in vivo* using appropriate murine models of *Chlamydia* respiratory infection and AAD. I used the natural mouse chlamydial pathogen *C. muridarum* to induce respiratory infections as it is the most appropriate strain to examine host:pathogen interactions in mice. Furthermore, I used BALB/c mice in these studies, as this strain is more susceptible to *Chlamydia* infection and mounts Th2-biased immune responses. Two different murine models of Ova-induced AAD were employed, the first of which is the classical model of acute Ova-induced AAD. This model is comprised of systemic Ova sensitisation followed by intranasal (i.n.) Ova challenge. The second model of Ova-induced AAD is a novel model developed in our laboratory that replicates established asthma in humans that is followed by re-exposure to allergen in later life. This model also incorporates a steroid treatment regime during the re-challenge phase to facilitate examination of
steroid sensitivity. Importantly, both of these models recapitulate the hallmark features of human asthma. Using these models I performed the following novel studies during my PhD:

1. miRNA and gene (mRNA) expression profiling of lungs during neonatal, infant and adult *Chlamydia* respiratory infections to identify key, age-specific *Chlamydia*-induced factors.

2. Investigation of the roles of five miRNAs (miR-155, miR-21, miR-223, miR-146b and miR-203) in the pathogenesis of neonatal *Chlamydia* respiratory infection-induced chronic lung disease using novel miRNA-specific inhibitors (antagomirs) *in vivo*.

3. Investigation of the role of a miR-21/PI3K/pAkt/HDAC2 signalling axis in the pathogenesis of *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, neutrophilic, steroid-insensitive AAD using miR-21-specific antagomirs and LY294002 (PI3K inhibitor) and the model corticosteroid DEX *in vivo*.

4. Investigation of the role of an NLRP3 inflammasome/Caspase-1/IL-1β signalling axis in the pathogenesis of *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, neutrophilic, steroid-insensitive AAD using anti-IL-1β neutralising antibodies, z-VAD-fmk (caspase inhibitor), Ac-YVAD-cho (Caspase-1-specific inhibitor), MCC950 (NLRP3 inflammasome inhibitor) and DEX *in vivo*.
Chapter 2:

Microarray-based miRNA and gene (mRNA) expression profiling in *Chlamydia* respiratory infection at different ages

In this chapter, I show that neonatal, infant and adult *Chlamydia* respiratory infections alter miRNA and gene (mRNA) expression in the lung during infection. Using microarray-based profiling of lungs from *Chlamydia*-infected mice as a discovery tool, I identified and validated the increased expression of 7 miRNAs (miR-223, miR-146b, miR-142-5p, miR-203, miR-1896, miR-155 and miR-21) in neonatal *Chlamydia* respiratory infection. I also show that two of these miRNAs (miR-155 and miR-21) and a group of 15 genes are up-regulated by *Chlamydia* respiratory infection irrespective of the age of infection. These data were used as a platform to conceive and design the studies described in Chapters 3-5 of this Thesis.
2.1 Introduction

Numerous studies associate *Chlamydia* respiratory infection with the development of asthma. Recent studies have shown that wheezing infants with serological evidence of *C. pneumoniae* infection were more likely to develop asthma (Hansbro et al., 2004; Zaitsu, 2009). Others have shown that nasal brushings and induced sputum from 23.6% of children with chronic lung disease, including asthma, were PCR-positive for *C. pneumoniae* (Teig et al., 2005) and BALF from 46% of paediatric asthma patients was culture-positive for *Chlamydia* (Webley et al., 2005; Webley et al., 2009). These studies indicate that *Chlamydia* respiratory infection is clinically associated with the development of asthma. Importantly, increasing experimental evidence supports these clinical data. We, and others, have shown that *Chlamydia* respiratory infection of neonatal and/or infant, but not adult, mice promotes features of chronic lung disease, including persistent AHR and emphysema-like alveolar enlargement, and increased severity of features of AAD, including increased MSC numbers, IL-13 responses and AHR in later life (i.e. approximately 9 weeks post infection) (Figure 2.1) (Horvat et al., 2010b; Jupelli et al., 2011; Starkey et al., 2013a; Starkey et al., 2014).

Substantial clinical evidence also links *Chlamydia* respiratory infections to severe, steroid-insensitive asthma (Metz and Kraft, 2010). Recent studies have shown that asthmatics with acute anti-*Chlamydia* antibody responses had increased sputum neutrophil numbers (Wark et al., 2002; Cho et al., 2005; Patel et al., 2010) and BAL neutrophilia predicted the presence of *Chlamydia* in severe, steroid-insensitive asthma (Patel et al., 2010). We, and others, have shown that *Chlamydia* respiratory infection promotes increased neutrophilic airway inflammation and T\textsubscript{H}1 and T\textsubscript{H}17, rather than T\textsubscript{H}2, responses in experimental asthma (Zhou et al., 2009; Horvat et al., 2010a).
Collectively, these studies indicate that *Chlamydia* respiratory infections may be important in the development of asthma as well as the induction of severe, steroid-insensitive forms of asthma. Investigations that provide mechanistic insight into how *Chlamydia* respiratory infections may promote these outcomes could identify key factors that can be targeted to prevent and treat disease. Thus, we sought to examine the impact of *Chlamydia* respiratory infections at different ages on the expression of lung miRNAs and genes (mRNAs) during infection, in order identify immune responses and signalling pathways that are induced by *Chlamydia* respiratory infection that may be responsible for infection-induced asthma. It was important to carefully consider the time points at which miRNA and gene expression profiling analyses were to be performed. Hence, we sought to develop the most appropriate strategy to facilitate detection of *Chlamydia* respiratory infection-induced responses rather than those that are enriched by resultant increases in inflammatory leukocytes in the lung. We chose appropriate time points to assess miRNA (5 days post infection [dpi]) and gene expression (10dpi) based upon the following. In our models, *Chlamydia* respiratory infection peaks at 10dpi and infection-induced pulmonary inflammation and histopathology in the lung peak between 14-15dpi (Hansbro et al., 2004; Horvat et al., 2007; Horvat et al., 2010b). This indicates that the peak of infection occurs 4-5 days before the peak of infection-induced lung inflammation. Importantly, however, *Chlamydia*-induced lung disease (i.e. increased *Chlamydia* numbers and histopathology in the lung) is observed from 10dpi. Thus, the miRNA and gene expression profiles after 10dpi will be strongly influenced by inflammatory leukocytes and will interfere with the detection of *Chlamydia*-induced responses that drive disease processes. We aimed to characterise the innate miRNA responses that are induced by resident lung cells following neonatal, infant and adult *Chlamydia*
respiratory infections at 5dpi, which is prior to significant immune cell infiltration and when *Chlamydia* growth enters the exponential phase in our models. We then aimed to characterise age-matched, *Chlamydia* infection-induced gene expression profiles at 10dpi in order to examine and identify the factors and signalling pathways that are induced by infection and potentially under the regulation of *Chlamydia* infection-induced miRNAs.

In this chapter, I describe the outcomes of microarray-based profiling analyses performed on total RNA isolated from the lungs of mice that were infected with *Chlamydia* as neonates, infants or adults. I developed this strategy to facilitate cross-comparisons of the miRNA (at 5dpi) and gene expression (at 10dpi) profiles that are induced by *Chlamydia* infection at different ages and to identify responses that are 1) commonly induced by *Chlamydia* infection irrespective of the age of infection, 2) common between groups of two different ages of infection, and 3) unique to specific ages of infection.
2.2 Methods

2.2.1 Ethics statement

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

2.2.2 Neonatal, infant and adult C. muridarum respiratory infection

Neonatal (< 24 hours old; neo), infant (3 week old weanlings; inf) and adult (6 weeks old; adu) WT BALB/c mice were infected i.n. with the natural mouse pathogen C. muridarum. Neonatal mice received 400 inclusion-forming units (IFU) ATCC VR-123 in 5µL sucrose phosphate glutamate (SPG) buffer; Cmu(neo). Infant and adult mice received 100 IFU ATCC VR-123 in 30µL SPG; Cmu(inf) and Cmu(adu), respectively. Sham-infected, age-matched controls received the equivalent volumes of SPG i.n. (Horvat et al., 2007; Horvat et al., 2010b; Starkey et al., 2014).

2.2.3 Lung sample collection, storage and total RNA extraction

Lungs were harvested from Chlamydia-infected mice at 5dpi and 10dpi (Figure 2.1) and immediately submerged in RNAlater® (Ambion, Austin, TX, USA) to inactivate tissue-derived ribonucleases. Samples were initially stored at 4°C overnight and then at -20°C for further analysis. Total RNA was isolated from homogenised lungs with TRIzol® Reagent (Invitrogen, Life Technologies, Australia) according to the manufacturer’s protocol.
Figure 2.1: Experimental protocol. Investigation of microRNA (miRNA) and gene (mRNA) expression profiles in neonatal, infant and adult *Chlamydia* respiratory infections. Microarray-based miRNA and gene expression profiling (highlighted in red) was performed on lung total RNA isolates from wild-type (WT) BALB/c mice that were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(neo)), infants (3 week old weanlings; Cmu(inf)) or adults (6 weeks old; Cmu(adu)) at 5 days post infection (d5; miRNA expression profiling) and d10 (gene expression profiling). Age-matched controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. Additional information pertaining to these models is illustrated for reference (highlighted in blue) and is described previously (Horvat et al., 2007; Horvat et al., 2010b; Starkey et al., 2013a; Starkey et al., 2014). In our models the historic peaks of Cmu respiratory infection and infection-induced lung inflammation are at d10 and d14-15, respectively. Neonatal Cmu infection results in persistent AHR and emphysema-like alveolar enlargement at d63. Early life (neonatal and infant), but not adult, Cmu infection increases the severity of features of ovalbumin (Ova)-induced allergic airways disease (AAD; intraperitoneal sensitisation to Ova [d47] followed by i.n. Ova challenges [d59-62]) at d63.

2.2.4 Bioanalysis of whole lung RNA samples

The RNA integrity of each sample was assessed using an Agilent 2100 Bioanalyser system (Agilent Technologies, Santa Clara, CA, USA) and Agilent RNA 6000 Nano LabChip Kits (Agilent Technologies, Santa Clara, CA, USA) according to
the manufacturer’s protocol. Immediately prior to analysis the electrodes on the Agilent 2100 Bioanalyser were decontaminated with a RNaseZap Electrode Cleaner chip containing 350µL RNaseZap and then nuclease-free deionised water. Next, 1µL of dye concentrate was added to 65µL of column-filtered gel matrix, then 9µL of the gel-dye mix was sequentially added to each of two purpose-built wells on the chip to seal and prime the chip. 5µL of nano marker (buffer) was then added to the sample and ladder wells, then 1µL each of heat-denatured RNA samples (70°C for 2 minutes) and ladder were added to the appropriate wells. Loaded chips were vortexed (240rpm for 1 minute) before starting the run. Samples were considered for microarray-based miRNA and gene expression profiling based on their RNA integrity number (RIN; >8) as well as the presence of a small RNA population.

2.2.5 Microarray-based miRNA expression profiling

miRNAs in total RNA samples were fluorescently labelled using an Agilent miRNA Complete Labelling and Hyb Kit (Agilent Technologies, Santa Clara, CA, USA), purified, and hybridised onto Agilent unrestricted Mouse miRNA (8 x 15k arrays per slide, AMADID Number: 021828, Sanger Version 12) microarrays (Agilent Technologies, Santa Clara, CA, USA) to generate miRNA expression profiles. Briefly, 100ng of total RNA (in 2µL nuclease-free water) was incubated with 2µL of calf intestinal alkaline phosphatase (CIP) master mix (0.4µL 10X CIP buffer, 1.1µL nuclease-free water, 0.5µL CIP; 37°C for 30 minutes) then supplemented with 2.8µL of dimethyl sulfoxide (DMSO) prior to heat-denaturation (100°C for 5 minutes). Next, the dephosphorylated and denatured RNA samples were 3’ labelled by incubating with 4.5µL of ligation master mix (1µL 10X T4 RNA ligase buffer, 3µL Cyanine3-pCp, 0.5µL T4 RNA ligase; 16°C for 2 hours). Labelled samples were
supplemented with nuclease-free water (total of 50µL) and purified of DMSO and free Cyanine3-pCp by elution through Micro Bio-Spin 6 columns (1000xg for 2 minutes). Purified samples were dried in a vacuum concentrator (50°C for 1 hour) then reconstituted with 18µL of nuclease-free water and 4.5µL of kit-supplied 10X gene expression blocking agent and 22.5µL of Hi-RPM hybridisation buffer. Samples were heated (100°C for 5 minutes), cooled on ice (5 minutes), then hybridised with the microarray chips (55°C for 20 hours). Hybridised microarrays were washed with kit-supplied wash buffers according to the manufacturer’s protocol and then scanned with an Agilent microarray scanner. Raw microarray data was extracted using the scanner software’s feature extraction function in a format compatible for analysis using GeneSpring GX 11.3 software (Agilent Technologies, Santa Clara, CA, USA).

2.2.6 Microarray-based gene (mRNA) expression profiling

Lung total RNA was initially amplified using an Illumina® TotalPrep RNA Amplification kit (Ambion, Austin, TX, USA). Briefly, total RNA (50ng; 11µL final volume in nuclease-free water) was combined with reverse transcription master mix (1µL T7 Oligo[dT] primer, 2µL 10X First Strand buffer, 4µL dNTPs, 1µL RNase inhibitor, 1µL ArrayScript) and incubated (42°C for 2 hours) to synthesise First Strand cDNA, which was then combined with Second Strand master mix (63µL nuclease-free water, 10µL 10X Second Strand buffer, 4µL dNTPs, 2µL DNA polymerase, 1µL RNase H) and incubated (16°C for 2 hours) to generate double-stranded (ds)DNA transcription templates. cDNA was supplemented with 250µL cDNA binding buffer, passed through a kit-supplied filter cartridge, washed (500µL wash buffer) and eluted (20µL pre-heated [55°C] nuclease-free water). Purified cDNA was combined with IVT master mix (2.5µL T7 10X reaction buffer, 2.5µL T7
enzyme mix, 2.5μL biotin-NTP mix) and incubated (37°C, overnight) to synthesise biotin-labelled complementary RNA (cRNA), which was then diluted (75μL nuclease-free water) and mixed with 350μL cRNA binding buffer and 250μL 100% ethanol. Samples were run through kit-supplied cRNA filter cartridges, washed (650μL wash buffer) and eluted (200μL pre-heated [55°C] nuclease-free water).

Purified biotin-labelled cRNA was quantitated using a Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen, Mulgrave, Victoria, Australia) according to the manufacturer’s protocol. cRNA was then hybridised onto Illumina® Mouse-WG-6 v2 Expression BeadChips (>45,000 probes based on RefSeq release 22, supplemented with MEEBO and RIKEN FANTOM2 content; Illumina® [BD-201-0202], Scoresby, Victoria, Australia) according to instructions outlined in Illumina’s Whole-Genome Gene Expression Direct Hybridisation user card. Briefly, 1.5μg of pre-heated (65°C for 5 minutes) cRNA was combined with 10μL nuclease-free water and 20μL pre-heated (58°C for 10 minutes) kit-supplied HYB mix in preparation for hybridisation. BeadChips were placed in pre-assembled Illumina® Hyb chambers (pre-loaded with 200μL kit-supplied HCB mix) then loaded with prepared samples (30μL each) and incubated (58°C, overnight). Hybridised BeadChips were run through a series of wash steps: 1) Kit-supplied High Temperature Wash buffer at 55°C, 2) kit-supplied Wash E1BC buffer, 3) 100% ethanol, and 4) in Wash E1BC buffer again. BeadChips were then blocked (kit-supplied Block E1 buffer), stained (Cy3-Streptavidin, 1:1000 dilution in Block E1 buffer; 10 minute incubation), washed (Wash E1BC buffer) and dried by centrifugation. BeadChips were scanned using an Illumina® BeadArray Reader and unprocessed raw microarray fluorescence intensity data was packaged and exported using Illumina® GenomeStudio Data Analysis software in a format
compatible for analysis using GeneSpring GX 11.3 software (Agilent Technologies, Santa Clara, CA, USA).

2.2.7 Quantification of lung miRNAs by custom miRNA real-time quantitative PCR (qPCR)

Expression of miRNAs was assessed by real-time qPCR, as described previously (Beveridge et al., 2008; Beveridge et al., 2010; Santarelli et al., 2011). Briefly, multiplex reverse transcriptions were performed on DNase I-treated total RNA using a combination of reverse primers specific for mature miRNAs and the endogenous controls U6 small nuclear RNA (snRNA) and U49 small nucleolar RNA (snoRNA), to a final concentration of 40nM each. The relative abundance of each miRNA was calculated against the geometric mean of the expression of U6 and U49 in each sample. For primer sequences refer to Table 2.1. All reactions were performed using BioScript™ reverse transcriptase in one times first-strand buffer according to manufacturer’s instructions (Bioline Pty. Ltd., NSW, Australia). Real-time qPCR assays were performed with SYBR Green Supermix (KAPA Biosystems, Inc., MA, USA) and a Mastercycler® ep realplex2 system (Eppendorf South Pacific Pty. Ltd., NSW, Australia).
Table 2.1: Oligonucleotide sequences used to validate microRNA (miRNA) microarray data by real-time quantitative PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6-F339</td>
<td>5'-CGGCAGCACATATACTAAAAATTGG-3'</td>
<td>U6 snRNA</td>
</tr>
<tr>
<td>U6-probe</td>
<td>5'-GCCATGCTAATCTCCTCCTGTATC-3'</td>
<td>U6 snRNA</td>
</tr>
<tr>
<td>U49 forward</td>
<td>5'-ATCACAATAGGAGTCGCGCT-3'</td>
<td>U49 snoRNA</td>
</tr>
<tr>
<td>U49 reverse</td>
<td>5'-ACAGGAAGTGCTTTTCGTAGT-3'</td>
<td>U49 snoRNA</td>
</tr>
<tr>
<td>miR-21 forward</td>
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<td>mmu-miR-21</td>
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<tr>
<td>miR-21 reverse</td>
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<td>mmu-miR-21</td>
</tr>
<tr>
<td>miR-142-5p forward</td>
<td>5'-CATAA+AGT+AGAAAGC-3'</td>
<td>mmu-miR-142-5p</td>
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<td>miR-142-5p reverse</td>
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<td>miR-146b forward</td>
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<td>miR-1896 forward</td>
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<td>mmu-miR-1896</td>
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</tbody>
</table>

1 LNA™-modified bases are preceded by a [+] symbol

2.2.8 Statistics

Comparisons between two groups were made using unpaired t-Tests or a non-parametric equivalent where appropriate. Comparisons between multiple groups were made using a One-way ANOVA and an appropriate Post Test or a non-parametric equivalent where appropriate. Analyses were performed using GraphPad Prism Software (San Diego, California).
2.3 Results

2.3.1 Chlamydia respiratory infection alters the expression of miRNAs in the lung that are unique and common to neonatal, infant and adult infections

To determine the lung miRNA expression patterns that are induced by Chlamydia respiratory infection at different ages, we performed microarray-based miRNA expression profiling analyses on total RNA isolated from the lungs of mice infected as neonates, infants or adults versus sham-infected, age-matched controls at 5dpi (Figure 2.1). Importantly, 5dpi represents a time point 4-5 days before the historic peak of infection in this model and is, therefore, the most appropriate stage to profile innate miRNA responses that are induced by Chlamydia infection in the absence of signals derived from infiltrating cells.

Of the 627 mouse miRNAs examined, 45 miRNAs displayed altered expression following Chlamydia respiratory infection at different ages (i.e. Cmu(neo), Cmu(inf) and Cmu(adu)) at 5dpi compared to sham-infected, age-matched (SPG(neo), SPG(inf) and SPG(adu), respectively) controls (Figure 2.2). Of these, neonatal Chlamydia infection uniquely altered the expression of 11 miRNAs (8 up-regulated and 3 down-regulated) compared to sham-infected, age-matched controls (Table 2.2). Chlamydia infection at all ages (Cmu(neo), Cmu(inf) and Cmu(adu)) commonly induced the up-regulation of 7 miRNAs compared to sham-infected, age-matched (SPG(neo), SPG(inf) and SPG(adu), respectively) controls (Table 2.3). The remaining 27 miRNAs with altered expression were divided as follows: 12 miRNAs were uniquely induced by infant Chlamydia infection and 4 miRNAs were uniquely induced by adult infection. Infant and adult infections commonly induced 10 miRNAs, and the expression of 1 miRNA was commonly altered by neonatal and
adult infections (Figure 2.2, Table 2.4). No miRNAs were commonly induced by neonatal and infant infections.
Figure 2.2: MicroRNA (miRNA) expression profiles that are induced by neonatal, infant and adult *Chlamydia* respiratory infections. Microarray-based miRNA expression profiling was performed on lung total RNA isolates from wild-type (WT) BALB/c mice that were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old), infants (3 week old weanlings) or adults (6 weeks old) at 5 days post infection (see study protocol; Figure 2.1). Age-matched controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. Cmu-induced responses were initially compared to sham-infected, age-matched controls. The resulting data was filtered using a volcano plot (P-value <0.05; absolute fold-change >2) and cross-comparisons of the miRNA responses performed between neonatal, infant and adult Cmu infections (one experiment; n=4).
Table 2.2: MicroRNAs (miRNAs) with altered expression that are uniquely induced by neonatal *Chlamydia* respiratory infection

<table>
<thead>
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<th>Absolute FC</th>
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<th>P-value</th>
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<td>mmu-miR-223</td>
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<td>mmu-miR-106b*</td>
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<td>4.94E-02</td>
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<td>mmu-miR-496</td>
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Table 2.3: MicroRNAs (miRNAs) with altered expression that are commonly induced by neonatal, infant and adult *Chlamydia* respiratory infections

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Neonatal infection</th>
<th>Infant infection</th>
<th>Adult infection</th>
</tr>
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<td></td>
<td>Absolute FC</td>
<td>Regulation</td>
<td>P-value</td>
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<td>mmu-miR-21*</td>
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<td>mmu-miR-155</td>
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<td>mmu-miR-1196</td>
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<td>mmu-miR-468</td>
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Table 2.4: MicroRNAs (miRNAs) with altered expression that are commonly induced by combinations of neonatal, infant and adult *Chlamydia* respiratory infections

<table>
<thead>
<tr>
<th>Unique to Infants microRNA</th>
<th>Unique to Adults microRNA</th>
<th>Common to Adults + Infants microRNA</th>
<th>Common to Adults + Neonates microRNA</th>
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<tr>
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<td>mmu-miR-188-5p</td>
<td>mmu-miR-669b</td>
<td>mmu-miR-1187</td>
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<td>mmu-miR-669c</td>
<td>mmu-miR-7a</td>
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<tr>
<td>mmu-miR-207</td>
<td>mmu-miR-466f-3p</td>
<td>mmu-miR-449c</td>
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<tr>
<td>mmu-miR-291b-5p</td>
<td>mmu-miR-469c</td>
<td>mmu-miR-466h</td>
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</tr>
<tr>
<td>mmu-miR-322*</td>
<td>mmu-miR-669c</td>
<td>mmu-miR-376c</td>
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</tr>
<tr>
<td>mmu-miR-31*</td>
<td>mmu-miR-489</td>
<td>mmu-miR-489</td>
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<tr>
<td>mmu-miR-877</td>
<td>mmu-miR-721</td>
<td>mmu-miR-466f-5p</td>
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<td>mmu-miR-680</td>
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<td>mmu-miR-7b</td>
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<tr>
<td>mmu-miR-466g</td>
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<tr>
<td>mmu-miR-466j</td>
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<td></td>
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</tbody>
</table>
2.3.2 Validation of miRNAs with altered expressions that are unique to neonatal *Chlamydia* infection and common across all ages of infection

Microarray analyses are widely used to simultaneously profile the expression of large sets of genes and also miRNAs (Davison et al., 2006; Yin et al., 2008; Li and Ruan, 2009). However, like other high-throughput profiling technologies, microarrays can be affected by technical reproducibility. Importantly, and compared to gene (mRNA) expression microarrays, miRNA microarray analyses also have increased potential for non-specific cross-hybridisation due to the short and highly similar sequences of miRNAs (Chuaqui et al., 2002; Yin et al., 2008; Zhao et al., 2011). Thus, we sought to validate the altered expressions of miRNAs that are unique to neonatal *Chlamydia* respiratory infection and common across all ages of infection by qPCR (Figures 2.3 and 2.4).

We show, through qPCR validations, that neonatal *Chlamydia* respiratory infection (Cmu(neo)) increases the lung expression of miR-223, miR-146b, miR-142-5p, miR-203 and miR-1896 at 5dpi compared to sham-infected (SPG(neo)) controls (Figure 2.3). Both the regulation and relative increases in the expression of these miRNAs were consistent with our microarray analyses (Table 2.2). *Chlamydia* infection of neonates had no effect on the expression of miR-10b*, miR-29b, miR-142-3p, miR-106b*, miR-133a* and miR-496 at 5dpi (data not shown).
Figure 2.3: qPCR-validated microRNAs (miRNAs) that are uniquely altered by neonatal *Chlamydia respiratory infection*. Microarray-based miRNA expression profiling was performed on lung total RNA isolates from wild-type (WT) BALB/c mice that were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(neo)), infants (3 week old weanlings; Cmu(inf)) or adults (6 weeks old; Cmu(adu)) at 5 days post infection (see study protocol; Figure 2.1). Cmu-induced responses were initially compared to age-matched controls that were sham-infected i.n. with equivalent volumes of sucrose phosphate glutamate (SPG) buffer. Further analysis identified 11 miRNAs that are uniquely induced by neonatal infection (Figure 2.2, Table 2.2). Cmu-induced lung expression of miRNAs was quantified by qPCR at 5dpi, normalised against the geometric mean (geomean) of the endogenous controls U6 small nuclear RNA and U49 small nucleolar RNA and then compared to sham-infected controls (two experiments; n=5-8). All data are presented as mean±s.e.m. *P<0.05; **P<0.01.
We also show that *Chlamydia* respiratory infection of neonates, infants and adults (Cmu(neo), Cmu(inf) and Cmu(adu), respectively) commonly induces the up-regulation of miR-155 and miR-21 at 5dpi compared to sham-infected, age-matched (SPG(neo), SPG(neo) and SPG(adu), respectively) controls (Figure 2.4). In contrast, the expressions of miR-21*, miR-1196, miR-135b, miR-135a* and miR-468 were not commonly altered by *Chlamydia* infection at all ages (data not shown).
Figure 2.4: qPCR-validated microRNAs (miRNAs) that are commonly altered by neonatal, infant and adult *Chlamydia* respiratory infections. Microarray-based miRNA expression profiling was performed on lung total RNA isolates from wild-type (WT) BALB/c mice that were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(neo)), infants (3 week old weanlings; Cmu(inf)) or adults (6 weeks old; Cmu(adu)) at 5 days post infection (see study protocol; Figure 2.1). Cmu-induced responses were initially compared to age-matched controls that were sham-infected i.n. with equivalent volumes of sucrose phosphate glutamate (SPG) buffer. Further analysis identified 7 miRNAs that are commonly induced at all ages of infection (Figure 2.2, Table 2.3). Cmu-induced lung expression of miRNAs was quantified by qPCR at 5dpi, normalised against the geometric mean (geomean) of the endogenous controls U6 small nuclear RNA and U49 small nucleolar RNA and expressed as fold change compared to sham-infected, age-matched controls (≥two experiments; n=5-8). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001.
2.3.3 *Chlamydia* respiratory infection alters the expression of genes in the lung that are unique and common to neonatal, infant and adult infections

To determine the impact of *Chlamydia* respiratory infection at different ages on the lung gene expression profile, we performed microarray-based gene expression profiling analyses on total RNA isolated from the lungs of mice infected as neonates, infants or adults versus sham-infected, age-matched controls at 10dpi (Figure 2.1). Importantly, 10dpi is a time point 5 days after our miRNA microarray analyses and represents the historic peak of infection in this model. Thus, we aimed to characterise age-matched, *Chlamydia* infection-induced gene expression profiles at 10dpi to examine and identify factors and signalling pathways that are induced by infection and potentially under the regulation of *Chlamydia* infection-induced miRNAs.

Of the >45,000 probes examined, 761 genes displayed altered expression following *Chlamydia* respiratory infection (Cmu(neo), Cmu(inf) and Cmu(adu)) at 10dpi compared to sham-infected, age-matched (SPG(neo), SPG(inf) and SPG(adu), respectively) controls (Figure 2.5). Of these genes, *Chlamydia* infection at all ages (Cmu(neo), Cmu(inf) and Cmu(adu)) commonly altered the expression of 185 genes compared to sham-infected, age-matched (SPG(neo), SPG(inf) and SPG(adu), respectively) controls. We hypothesised that this list of commonly induced genes represents the *Chlamydia*-induced immune responses that are not determined by the age of infection and may be generally important in the development of *Chlamydia*-induced lung disease. Thus, in order to identify potentially pathogenic processes that are induced by *Chlamydia* respiratory infection we sought to examine and rationalise this list of 185 commonly induced genes by performing functional annotation using the Database for Annotation, Visualization and Integrated Discovery (DAVID; National Institute of Allergy and Infectious Diseases [NIAID], NIH) functional
annotation tool, as previously described (Huang da et al., 2009b; Huang da et al., 2009a). These analyses identified 15 genes that are commonly induced by neonatal, infant and adult *Chlamydia* respiratory infections for further consideration (Table 2.5). Interestingly, the gene expression microarray data indicated that all of these genes are robustly up-regulated following *Chlamydia* infection at all ages. The remaining genes with altered expression were divided as follows: 26 genes were uniquely induced by neonatal *Chlamydia* infection, 69 genes were uniquely induced by infant infection, and 164 genes were uniquely induced by adult infection. Neonatal and infant *Chlamydia* infections commonly induced 16 genes, and neonatal and adult infections commonly induced 5 genes. Finally, infant and adult infections commonly induced 296 genes.
Figure 2.5: Gene (mRNA) expression profiles that are induced by neonatal, infant and adult *Chlamydia* respiratory infections. Microarray-based gene expression profiling was performed on lung total RNA isolates from wild-type (WT) BALB/c mice that were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old), infants (3 week old weanlings) or adults (6 weeks old) at 10 days post infection (see study protocol; Figure 2.1). Age-matched controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. Cmu-induced responses were initially compared to sham-infected, age-matched controls. The resulting data was filtered using a volcano plot (P-value <0.05; absolute fold-change >2) and cross-comparisons of gene expression profiles performed between neonatal, infant and adult Cmu infections (one experiment; n=4).
Table 2.5: Genes (mRNAs) with altered expression that are commonly induced by neonatal, infant and adult *Chlamydia* respiratory infections

<table>
<thead>
<tr>
<th>Gene</th>
<th>Definition</th>
<th>Neonatal infection</th>
<th>Infant infection</th>
<th>Adult infection</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>Absolute FC</td>
<td>Regulation</td>
<td>P-value</td>
</tr>
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<td>Casp1</td>
<td>Caspase 1</td>
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<tr>
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</table>
2.4 Conception of studies

The microarray-based profiling experiments discussed in Section 2.3 were used as a discovery platform to expand our understanding of the mechanisms that are induced by *Chlamydia* respiratory infection at different ages to promote chronic lung disease. I have shown that neonatal, infant and adult *Chlamydia* respiratory infections alter the expression of miRNAs and genes in the lung during infection. Importantly, the outcomes of these microarray analyses were the foundation upon which I conceived and designed the studies for my PhD, and these novel studies are described hereafter in this Thesis (*Chapters 3, 4 and 5*).

Through qPCR validations of the miRNA microarray data I identified 7 miRNAs (miR-223, miR-146b, miR-142-5p, miR-203, miR-1896, miR-155 and miR-21) that are increased by neonatal *Chlamydia* respiratory infection, two of which (miR-155 and miR-21) are commonly induced by *Chlamydia* infection at all ages. Our lab has previously demonstrated that neonatal *Chlamydia* respiratory infection, but not adult infection, induces emphysema-like alveolar enlargement and persistent AHR in later life (Horvat et al., 2010b). Furthermore, early life (neonatal and infant), but not adult, *Chlamydia* infection increased the severity of features of Ova-induced AAD in later life, including increased MSC numbers, IL-13 responses and AHR. Thus, I sought to expand our understanding of neonatal *Chlamydia* respiratory infection-induced responses by performing a pilot study that investigated the functional role(s) of neonatal *Chlamydia* infection-induced miRNAs in the pathogenesis of chronic lung disease (*Chapter 3*).

Additionally, we have previously shown that *Chlamydia* respiratory infection of adult mice induces neutrophilic, T_{H1} and/or T_{H17} immune responses in an experimental model of asthma (Horvat et al., 2010a). We demonstrated that ongoing,
but not resolved, *Chlamydia* respiratory infection during Ova sensitisation resulted in reduced eosinophilic airway inflammation, IL-5 production, MSC hyperplasia and AHR in Ova-induced AAD. Furthermore, ongoing *Chlamydia* infection during sensitisation led to robust neutrophilic airway inflammation and IFN-γ production from re-stimulated T cells. Significantly, this phenotype is reminiscent of neutrophilic asthma in humans that is more likely to be steroid-insensitive (Green et al., 2002; Hansbro et al., 2011). Importantly, substantial clinical evidence also links *Chlamydia* respiratory infections with neutrophilic asthma (Wark et al., 2002; Patel et al., 2010).

In my PhD, I proposed that it is respiratory infection in patients with established asthma that drives the development of severe, neutrophilic, steroid-insensitive asthma. Thus, I sought to advance our earlier findings by initially developing a novel model that more accurately reflects the human scenario. In this model, I established Ova-induced AAD in mice and then induced a *Chlamydia* respiratory infection. I then recapitulated AAD with a second set of Ova challenges to model a scenario where patients with established asthma are exposed to respiratory infection and allergens (Chapter 4). Significantly, *Chlamydia* respiratory infection induced steroid-insensitive neutrophilic airway inflammation and AHR in AAD, which is reminiscent of severe, steroid-insensitive neutrophilic asthma in humans. Importantly, others have demonstrated that miR-21 can potentiate PI3K signalling (Meng et al., 2007; Yamanaka et al., 2009) and that PI3K signalling reduces HDAC2 activity in the lung and reduces glucocorticoid function (Marwick et al., 2009). Thus, based on the outcomes of my miRNA microarray profiling experiment, I hypothesised that *Chlamydia* infection-induced increases in miR-21 expression, which is common to all ages of infection, plays a role in the pathogenesis of *Chlamydia* infection-induced, severe, steroid-insensitive AAD. Specifically, I sought to examine the role of a
Chlamydia respiratory infection-induced miR-21/PI3K/pAkt/HDAC2 signalling axis in the pathogenesis of severe, steroid-insensitive AAD (Chapter 4).

Finally, through microarray-based gene expression profiling, and bioinformatics-based functional annotation of genes that are induced by Chlamydia respiratory infection irrespective of the age of infection, I identified 5 genes (Caspase-1, IL-1β, serum amyloid A (SAA)3, TLR2, TNF-α) that are associated with innate immune activation and NLRP3 inflammasome signalling (Stutz et al., 2009; Schroder and Tschopp, 2010). Significantly, increasing clinical evidence links these genes, innate immune activation and the NLRP3 inflammasome with severe, non-eosinophilic endotypes of asthma, such as neutrophilic asthma (Green et al., 2002; Simpson et al., 2006; Simpson et al., 2007; Baines et al., 2011; Hansbro et al., 2011; Simpson et al., 2014). In separate studies, Simpson et al. showed that neutrophilic asthmatics have increased expression of TLR2 and IL-1β (Simpson et al., 2007) and Baines et al. showed that the overexpression of factors involved in IL-1 and TNF-α/NF-κB signalling pathways correlated with neutrophilic airway inflammation in a cohort of asthmatics (Baines et al., 2011). Furthermore, another study by Simpson et al. showed that the sputum macrophages of neutrophilic asthmatics have increased expression of NLRP3, Caspase-1 and IL-1β, and that sputum neutrophils from neutrophilic asthmatics, but not other asthma phenotypes, exhibit strong immunoreactivity for NLRP3 and Caspase-1 (Simpson et al., 2014). Collectively, these studies implicate NLRP3 inflammasome signalling in the pathogenesis of severe asthma. Furthermore, Chlamydia respiratory infections can induce the release of active IL-1β in an NLRP3 inflammasome-dependent, Caspase-1-mediated manner (He et al., 2010; Rotta Detto Loria et al., 2013). Additionally, we have shown that Chlamydia respiratory infection induces neutrophilic, T\textsubscript{H}1 and/or T\textsubscript{H}17 immune
responses in mouse models of asthma (Horvat et al., 2010a). Taken together, these data highlight the possibility that *Chlamydia* respiratory infection can increase NLRP3 inflammasome signalling in the asthmatic lung. Thus, based on the outcomes of my gene expression profiling experiment, and to expand our understanding of the mechanisms that underpin *Chlamydia* respiratory infection-induced severe lung disease, I sought to examine the role of a *Chlamydia* respiratory infection-induced NLRP3 inflammasome/Caspase-1/IL-1β signalling axis in the pathogenesis of severe, steroid-insensitive AAD (Chapter 5).

In future studies the miRNA and gene expression microarray data (Chapter 2) will be re-analysed using more rigorous *in silico* methodologies that will cross-examine the two datasets in order to investigate the potential interactions between the miRNAs and genes that are affected by *Chlamydia* respiratory infection. This approach will offer mechanistic insight into the pathogenic processes that are induced by *Chlamydia* respiratory infection and, potentially, identify novel therapeutic targets.
Chapter 3:

Neonatal *Chlamydia* respiratory infection induces five miRNAs that drive severe lung disease in later life.

In this chapter, I performed a pilot study to show that neonatal *Chlamydia* respiratory infection induces a subset of five miRNAs that have differential effects on infection-induced lung inflammation and histopathology, as well as impaired lung function, emphysema-like alveolar enlargement and infection-induced increases in the severity of AAD in later life. These findings highlight the potential importance of infection-induced miRNAs in the lung in driving the pathogenesis of severe respiratory disease associated with early life respiratory infection in humans and suggest that therapeutically targeting these miRNAs during early life respiratory infection may protect against the development of chronic lung disease.

This chapter is currently in preparation for submission as an original research article.
3.1 Abstract

Respiratory infections in early life are commonly linked with the development of chronic respiratory disease. *Chlamydia* respiratory infections are a common cause of neonatal pneumonia and early life *Chlamydia* infections have been associated with permanent deficits in lung function and the development of asthma, particularly severe asthma in later life. In this study, we identify novel roles for a subset of 5 miRNAs (miR-155, miR-21, miR-223, miR-146b and miR-203) in promoting acute and chronic sequelae of neonatal *Chlamydia* respiratory infection. By infecting neonatal mice with *Chlamydia* and using miRNA-specific inhibitors (antagomirs) *in vivo*, we demonstrate that 5 miRNAs have differential effects on airway inflammation and/or lung histopathology and/or reduced weight gain during infection. We also demonstrate that these miRNAs differentially promote persistent AHR, emphysema-like alveolar enlargement and/or increased severity of AAD in later life. Our data suggest that the inhibition of specific miRNAs during a neonatal *Chlamydia* respiratory infection can protect against the development of several key features of neonatal *Chlamydia* respiratory infection-induced diseases that are induced either during infection and/or in later life.
3.2 Introduction

Asthma is a chronic inflammatory disease of the airways that is underpinned by aberrant T\textsubscript{H}2-mediated responses to normally innocuous environmental antigens. The T\textsubscript{H}2 cytokines IL-4, IL-5 and IL-13 drive the hallmark features of asthma including eosinophilic airway inflammation, mucous hypersecretion and AHR (Foster et al., 2002a; Hansbro et al., 2011). Importantly, asthma is most common in children and adult asthma has paediatric origins (von Mutius, 2001; Sears et al., 2003; Piippo-Savolainen et al., 2006) suggesting that early life events play an important role in the development of asthma.

*Chlamydia* respiratory infections in early life are common (Beem and Saxon, 1977; Harrison et al., 1982; Alexander and Harrison, 1983; Numazaki et al., 1986; Hammerschlag, 2003) and 50% of young adults possess antibodies against *C. pneumoniae* (Grayston, 1992a; Kuo et al., 1995). *Chlamydia* infections are often mild or asymptomatic but are responsible for a significant proportion of cases of community-acquired pneumonia that require hospitalisation (Blasi, 2004; Vila-Corcoles et al., 2009), and early life infection is associated with reduced lung function and chronic respiratory pathology (Harrison et al., 1982). *Chlamydia* respiratory infections induce strong T\textsubscript{H}1 immune responses that are required for resolution (Yang et al., 1996; Yang et al., 1999; Hansbro et al., 2004) but these protective responses may indirectly cause respiratory tract pathology. Significantly, *Chlamydia* respiratory infections are associated with wheezing in infants and the development of asthma (Hansbro et al., 2004; Zaitu, 2009) and *Chlamydia* has been detected in the BALF of 46% (59 out of 128) of paediatric asthma patients within a subset of patients with severe respiratory diseases (Webley et al., 2005; Webley et al., 2009). However, the mechanisms of how T\textsubscript{H}1-inducing *Chlamydia* respiratory infections are associated
with T_h2-mediated asthma are unknown. We have previously shown that neonatal *Chlamydia* respiratory infection in mice, with *C. muridarum*, promotes chronic respiratory pathology, including reduced lung function in terms of persistent AHR and emphysema-like alveolar enlargement (Horvat et al., 2007; Horvat et al., 2010b). We also showed that neonatal *Chlamydia* infection increased the severity of Ova-induced AAD in later life (Horvat et al., 2010b) and these findings have since been confirmed by others (Jupelli et al., 2011).

MiRNAs are highly conserved, single-stranded RNA molecules that regulate gene expression at the post-transcriptional level (Bartel, 2009; Winter et al., 2009) and have been shown to be dysregulated in several human diseases, including asthma (Oglesby et al., 2010; Foster et al., 2013). Significantly, miRNAs play important roles in immune function and bacterial infections have been shown to alter miRNA expression profiles suggesting that the infection-induced dysregulation of miRNAs could shape the phenotype of the immune response (Bartel, 2004; Cremer et al., 2009; Xiao et al., 2009; Xiao and Rajewsky, 2009; Schulte et al., 2011; Izar et al., 2012).

In this study we show for the first time that neonatal *Chlamydia* respiratory infection induces the overexpression of a subset of 5 miRNAs (miR-155, miR-21, miR-223, miR-146b and miR-203) in the lungs. These miRNAs differentially promote key disease features induced by neonatal *Chlamydia* respiratory infection, including infection-induced airway inflammation and histopathology at 14dpi (miR-155, miR-21, miR-223, miR-146b, and miR-203), reduced rate of weight gain (miR-155 and miR-223), persistent AHR in later life (miR-155, miR-21 and miR-203), emphysema-like alveolar enlargement (miR-155), and increased severity of AAD (miR-155, miR-21, and miR-203). These data identify infection-induced miRNAs as
novel therapeutic targets for the prevention of neonatal *Chlamydia* respiratory infection-induced chronic respiratory sequelae, both during infection and in later life.
3.3 Methods

3.3.1 Ethics statement

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

3.3.2 Neonatal *C. muridarum* respiratory infection

Neonatal (< 24 hours old; neo) WT BALB/c mice were infected i.n. with the natural mouse pathogen *C. muridarum*. Neonatal mice received 400 IFU ATCC VR-123 in 5µL SPG buffer (Cmu(neo)). Sham-infected controls received the equivalent volume of SPG (5µL) i.n. (Horvat et al., 2007; Horvat et al., 2010b; Starkey et al., 2014). Mice were sacrificed at 5, 10, 14 or 63dpi to assess the impact of infection on pathology and lung function. In other groups, *Chlamydia*-infected neonates were intraperitoneally (i.p.) sensitised to Ova (47dpi) and AAD induced by i.n. Ova challenge (12-15 days post i.p. sensitisation) (Figure 3.1), as we have shown before (Horvat et al., 2010b). Mice were sacrificed at 63dpi (16 days post i.p. sensitisation) and hallmark features of AAD assessed.

3.3.3 In vivo administration of miRNA inhibitors (antagomir)

We inhibited miRNAs *in vivo* during neonatal *Chlamydia* respiratory infection using miRNA-specific antagomirs. The sequences of mmu-miR-155/miR-21/miR-223/miR-146b/miR-203 were downloaded from miRBase University of Manchester, UK (http://www.mirbase.org/) and complementary miRNA antagomirs were
designed. Ant-155/Ant-21/Ant-223/Ant-146b/Ant-203 and scrambled antagonir control (Ant-Scram; nonspecific RNA VIII, BLAST searched against the mouse genome) were synthesised by Sigma-Aldrich (refer to Table 3.1 for details). Lyophilised antagonirs were reconstituted in sterile nuclease-free water and the concentrations quantified by spectrophotometry prior to final stock dilution.

WT BALB/c neonates (< 24 hours old; neo) were treated i.n. with miRNA-specific antagonirs (Ant-155, Ant-21, Ant-223, Ant-146b and Ant-203; 2mg/kg; in sterile nuclease-free water) on day 0 (6 hours before Chlamydia respiratory infection) and every 5 days post infection up until, and including, 15dpi (Figure 3.1), as described previously (Krutzfeldt et al., 2005; Mattes et al., 2009; Collison et al., 2011). Antagonirs were delivered in gradually increasing volumes i.n. as the neonatal mice gained weight and size. The i.n. volumes were: 5µL (day 0 and 5dpi), 10µL (10dpi) and 15µL (15dpi). Antagonirs were administered without isoflurane anaesthesia until the neonatal mice were 15 days of age. Infected and sham-infected (SPG) controls received the equivalent concentrations and volumes of scrambled antagonir (Ant-Scram) i.n. on the same days. The effects of treatment with miRNA antagonirs during neonatal Chlamydia respiratory infection were assessed at 10dpi (miRNA expression), 14dpi (airway inflammation and lung histopathology) and 63dpi (lung function and structure).

In other groups, Chlamydia-infected neonates were i.p. sensitised to Ova at 47dpi and AAD induced by i.n. Ova challenge (12-15 days post i.p. sensitisation) (Figure 3.1), as previously described (Horvat et al., 2010b). Mice were sacrificed at 63dpi (16 days post i.p. sensitisation) and hallmark features of AAD assessed.
3.3.4 Quantification of miRNA and mRNA expression by real-time qPCR

Total RNA was isolated from homogenised lungs with TRIzol® Reagent (Invitrogen, Life Technologies, Australia). Expression of miRNAs was assessed by real-time qPCR at 5, 10 and 14dpi, as described previously (Beveridge et al., 2008; Beveridge et al., 2010; Santarelli et al., 2011). Briefly, multiplex reverse transcriptions were performed on DNase I-treated total RNA using a combination of reverse primers specific for mature miRNAs and the endogenous controls U6 snRNA and U49 snoRNA, to a final concentration of 40nM each. The relative abundance of each miRNA was calculated against the geometric mean of the expression of U6 and U49 in each sample. All reactions were performed using BioScript™ reverse transcriptase in one times first-strand buffer according to manufacturer’s instructions (Bioline Pty. Ltd., NSW, Australia). Real-time qPCR assays were performed with SYBR Green Supermix (KAPA Biosystems, Inc., MA, USA) and a Mastercycler® ep realplex2 system (Eppendorf South Pacific Pty. Ltd., NSW, Australia). Random-primed reverse transcriptions were performed on total RNA for mRNA real-time qPCRs. Gene expression was normalised to the transcript of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Horvat et al., 2010b). For primer sequences refer to Table 3.2.
Table 3.2: Oligonucleotide sequences used for qPCR analyses

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6-F339</td>
<td>5’-CGGCAGCAGCATACTAAATTTGG-3’</td>
<td>U6 snRNA</td>
</tr>
<tr>
<td>U6-probe</td>
<td>5’-GCCATGCTAATCTTCTCTGTATC-3’</td>
<td>U6 snRNA</td>
</tr>
<tr>
<td>U49 forward</td>
<td>5’-ATCACAATAGGAAGTGCCGTC-3’</td>
<td>U49 snoRNA</td>
</tr>
<tr>
<td>U49 reverse</td>
<td>5’-ACAGGAGTAGTCCTTCGTCAG-3’</td>
<td>U49 snoRNA</td>
</tr>
<tr>
<td>miR-21 forward</td>
<td>5’-T+AGCTTATCAGACTG-3’</td>
<td>mmu-miR-21</td>
</tr>
<tr>
<td>miR-21 reverse</td>
<td>5’-GTAAACGACGCGCAGTTCAACAT-3’</td>
<td>mmu-miR-21</td>
</tr>
<tr>
<td>miR-142-5p forward</td>
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<td>miR-146b forward</td>
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<td>mmu-miR-146b</td>
</tr>
<tr>
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<td>mmu-miR-146b</td>
</tr>
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<tr>
<td>miR-155 reverse</td>
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<td>mmu-miR-155</td>
</tr>
<tr>
<td>miR-203 forward</td>
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<td>5’-GTAAACGACGCGCAGTAGCTAGTG-3’</td>
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</tr>
<tr>
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<td>Fgf-7</td>
</tr>
<tr>
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<td>5’-CGGGTTTTGCTCCCTTTGA-3’</td>
<td>Fgf-7</td>
</tr>
<tr>
<td>HPRT forward</td>
<td>5’-AGGCCAGAATTTTGATTTGGA-3’</td>
<td>HPRT</td>
</tr>
<tr>
<td>HPRT reverse</td>
<td>5’-CAACTTGCCTCATCTAGGCTT-3’</td>
<td>HPRT</td>
</tr>
</tbody>
</table>

1 LNA™-modified bases are preceded by a [+] symbol
Figure 3.1: MicroRNA (miRNA) antagonir administration regime during neonatal Chlamydia respiratory infection. Wild-type (WT) BALB/c mice were infected intranasally (i.n.) with C. muridarum (Cmu) as neonates (<24 hours old; Cmu(neo)) on day 0 (d0). Controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. MiRNA-depleting antagonirs were administered i.n. on d0 (6 hours before infection), d5, d10 and d15 to suppress Cmu-induced miRNA expression. Infected and sham-infected control groups received scrambled antagonir in equivalent concentrations and volumes on the same days. Mice were sacrificed on d10, d14 and d63 to assess antagonir-mediated effects on Cmu-induced miRNA expression, airway inflammation and lung histopathology, and lung function and structure in later life, respectively. In other groups, infected, antagonir-treated mice were intraperitoneally (i.p.) sensitised to ovalbumin (Ova) (d47), allergic airways disease (AAD) induced by i.n. Ova challenge (d59-62) and sacrificed on d63 to assess the effect of Cmu infection on the key features of AAD. Infected and sham-infected control groups received scrambled antagonir.
3.3.5 Airway inflammation, histopathology and alveolar enlargement

Airway inflammation was assessed in cytospin preparations of cells from BALF collected at 14dpi (Figure 3.1) (Horvat et al., 2007; Horvat et al., 2010a). BALF was collected by two 0.5mL washes with Hank’s Balanced Salt Solution (Life Technologies, Australia) via a cannula inserted into the trachea, centrifuged (300xg, 10 min, 4°C), treated with red blood cell lysis buffer (100µL, Tris-buffered NH₄Cl) and pelleted before total leukocyte numbers were determined using a haemocytometer. Cells were cytocentrifuged and stained with May-Grunwald-Giemsa. Differential leukocyte counts were determined using morphological criteria (≈175 cells by light microscopy [x40 magnification]) (Horvat et al., 2007; Horvat et al., 2010a). Airway inflammation was also assessed in infected, antagomir-treated mice in AAD at 63dpi (two 1mL washes with Hank’s Balanced Salt Solution). All samples were coded and counts were performed in a blinded fashion.

Formalin-fixed, paraffin-embedded lung sections (4-6µm thickness) were stained with hematoxylin and eosin (H&E) stain and gross histopathology was assessed at 14dpi by light microscopy (x40 magnification) (Horvat et al., 2007; Horvat et al., 2010a). The average alveolar diameter was evaluated at 63dpi by determination of the mean linear intercept (Lₘ), as previously described (Robbesom et al., 2003; Horvat et al., 2010b).

3.3.6 Lung function

Mice were anaesthetised with ketamine (100mg/kg) and xylazine (10mg/kg, Troy Laboratories, Smithfield, Australia) and their tracheas were cannulated (tracheostomy with ligation) (Horvat et al., 2007; Horvat et al., 2010a; Horvat et al., 2010b; Starkey et al., 2012; Starkey et al., 2013a; Starkey et al., 2013b). Work of
breathing and Inspiratory Capacity (IC) were measured using a forced pulmonary manoeuvre system (Buxco, Wilmington, NC) using an average respiratory rate of 150 breaths/min, as previously described (Beckett et al., 2013). Work of breathing and IC were determined using the quasistatic pressure-volume loop. Each manoeuvre was performed a minimum of three times and the mean calculated. FlexiVent apparatus (FX1 System; SCIREQ, Montreal, Canada) was used to assess airway-specific resistance (Rn; tidal volume of 8 mL/kg at a respiratory rate of 450 breaths/min) (Beckett et al., 2013) in response to increasing doses of nebulised methacholine (Sigma-Aldrich, Sydney, Australia). This combination of anaesthesia and ventilation is common and recommended by the manufacturer (Horvat et al., 2010a; Li et al., 2010; Beckett et al., 2013). Assessments were performed at least three times per dose of saline/methacholine and the average calculated.

**3.3.7 Statistics**

Comparisons between two groups were made using unpaired t-Tests or a non-parametric equivalent where appropriate. Comparisons between multiple groups were made using a One-way ANOVA and an appropriate Post Test or a non-parametric equivalent where appropriate. Lung function data were assessed using a Two-way ANOVA and an appropriate Post Test or a non-parametric equivalent. Analyses were performed using GraphPad Prism Software (San Diego, California). All data shown are representative of individual mice. No data has been pooled.
3.4 Results

3.4.1 Neonatal *Chlamydia* respiratory infection increases the expression of miR-155, miR-21, miR-223, miR-146b and miR-203 in the lungs during the first 14dpi

To investigate the role of miRNAs in neonatal *Chlamydia* respiratory infection-induced chronic lung disease we first assessed the expression of 7 qPCR-validated miRNA microarray signatures (Section 2.3.2) over a time course at 5, 10 and 14dpi (Figure 3.2). *Chlamydia* respiratory infection of neonates resulted in an increase in the expression of miR-155, miR-21, miR-223, miR-146b and miR-203 during infection, when compared to sham-inoculated controls. In contrast, the expression of miR-1896 and miR-142-5p were transiently increased by infection at 5dpi (time point of miRNA microarray analyses; Chapter 2).
Figure 3.2: Neonatal Chlamydia respiratory infection increases the expression of microRNA (miR)-155, miR-21, miR-223, miR-146b and miR-203 in the lungs during infection. Wild-type (WT) BALB/c mice were infected intranasally (i.n.) with C. muridarum (Cmu) as neonates (<24 hours old; Cmu(neo)). Controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. Whole lungs were harvested at 5, 10 and 14dpi and total RNA isolated. Cmu-induced lung expression of 7 qPCR-validated miRNA microarray signatures (Section 2.3.2) was assessed at each time point by qPCR then normalised against the geometric mean (geomean) of the endogenous controls U6 small nuclear RNA and U49 small nucleolar RNA and expressed as fold change compared to SPG(neo) controls ≥ two
experiments; n=5-10). All data are presented as mean±s.e.m. *P<0.05; **P<0.01;
***P<0.001; ****P<0.0001.
3.4.2 *In vivo* inhibition of miRNAs during neonatal *Chlamydia* respiratory infection reduces infection-induced lung inflammation and histopathology

Initially, we sought to assess the effects of treatment with antagomirs during neonatal infection on *Chlamydia*-induced miRNA expression at 10dpi (historic peak of infection in this model) compared to sham-infected and infected, scrambled antagomir-treated (SPG(neo)/Scram and Cmu(neo)/Scram, respectively) controls (Figure 3.3). Infected mice were treated with miRNA-specific antagomirs as previously described (Krutzfeldt et al., 2005; Mattes et al., 2009; Collison et al., 2011). Infected, scrambled antagomir-treated (Cmu(neo)/Scram) mice had increased lung expression of all 5 miRNAs of interest at 10dpi compared to sham-infected, scrambled antagomir-treated (SPG(neo)/Scram) controls. Importantly, treatment with antagomirs during neonatal *Chlamydia* respiratory infection (Cmu(neo)/Ant-155, Cmu(neo)/Ant-21, Cmu(neo)/Ant-223, Cmu(neo)/Ant-146b and Cmu(neo)/Ant-203, respectively) decreased the expression of each corresponding miRNA at 10dpi when compared to infected, scrambled antagomir-treated (Cmu(neo)/Scram) controls. Indeed, infected mice treated with antagomirs had similar miRNA expression to sham-infected, scrambled antagomir-treated (SPG(neo)/Scram) controls. Treatment of sham-infected and Cmu-infected mice with scrambled antagomir (SPG(neo)/Scram and Cmu(neo)/Scram, respectively) had no effect on miRNA expression when compared to treatment with nuclease-free water (vehicle control; SPG(neo)/Water and Cmu(neo)/Water, respectively) (Figure 3.3).

Neonatal *Chlamydia* infection (Cmu(neo)/Scram) significantly reduced weight gain from 6dpi compared to sham-infected, scrambled antagomir-treated (SPG(neo)/Scram) controls (Figure 3.4). Importantly, treatment with Ant-155 or Ant-223 during infection (Cmu(neo)/Ant-155 and Cmu(neo)/Ant-223, respectively)
improved weight gain compared to infected, scrambled antagonir-treated (Cmu(neo)/Scram) mice. Treatment with Ant-21 or Ant-146b during infection (Cmu(neo)/Ant-21 and Cmu(neo)/Ant-146b, respectively) had no effect on weight gain. In contrast, the administration of Ant-203 (Cmu(neo)/Ant-203) further reduced weight gain compared to infected, scrambled antagonir-treated (Cmu(neo)/Scram) mice.
Figure 3.3: Inhibition of microRNAs (miRNAs) *in vivo* during neonatal *Chlamydia* respiratory infection. Wild-type (WT) BALB/c mice were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(neo)). Controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. Lung expression of miR-155, miR-21, miR-223, miR-146b and miR-203 was determined by qPCR at 10dpi in Cmu- and sham-infected groups in the presence of anti-miR antagonim [Ant-155 [Cmu(neo)/Ant-155], Ant-21 [Cmu(neo)/Ant-21], Ant-223 [Cmu(neo)/Ant-223], Ant-146b [Cmu(neo)/Ant-146b] and Ant-203 [Cmu(neo)/Ant-203], respectively] or scrambled (Scram; [SPG(neo)/Scram and Cmu(neo)/Scram]) antagonim treatment or with vehicle (sterile nuclease-free water, [SPG(neo)/Water and Cmu(neo)/Water]) (≥two experiments; n=8-10). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P< 0.0001.
Figure 3.4: Neonatal *Chlamydia* respiratory infection-induced microRNA (miR)-155 and miR-223 expression promote reduced weight gain. Wild-type (WT) BALB/c mice were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(neo)). Controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. Weight gain was tracked from birth to 21dpi (historic time point of clearance of infection and infection-induced airway inflammation) in the presence of anti-miR antagonir (Ant-155 [Cmu(neo)/Ant-155], Ant-21 [Cmu(neo)/Ant-21], Ant-223 [Cmu(neo)/Ant-223], Ant-146b [Cmu(neo)/Ant-146b] and Ant-203 [Cmu(neo)/Ant-203], respectively) or scrambled (Scram; [SPG(neo)/Scram and Cmu(neo)/Scram]) antagonir treatment ($\geq$two experiments; n=4-12). Weight data for sham-infected and infected, scrambled antagonir-treated (SPG(neo)/Scram
and Cmu(neo)/Scram, respectively) controls is repeated for each comparison (all experiments were performed concurrently). All data are presented as mean±s.e.m. *P<0.05.
Given that miR-155, miR-21, miR-223, miR-146b and miR-203 showed profiles of increased expression during neonatal *Chlamydia* respiratory infection, we next sought to examine the roles of these miRNAs in *Chlamydia*-induced airway inflammation and lung gross histopathology at 14dpi (historic peak of infection-induced airway inflammation). Infected, scrambled antagonir-treated (Cmu(neo)/Scram) mice had increased airway inflammation at 14dpi compared to sham-infected, scrambled antagonir-treated (SPG(neo)/Scram) controls (*Figure 3.5*). Treatment with each miRNA antagonir (Cmu(neo)/Ant-155, Cmu(neo)/Ant-21, Cmu(neo)/Ant-223, Cmu(neo)/Ant-146b and Cmu(neo)/Ant-203, respectively) decreased *Chlamydia*-induced airway inflammation, in particular macrophages, at 14dpi compared to infected, scrambled antagonir-treated (Cmu(neo)/Scram) controls.

Infected, scrambled antagonir-treated (Cmu(neo)/Scram) mice also had increased gross lung histopathology with evidence of interstitial inflammation around small airways and airway-associated vasculature at 14dpi compared to sham-infected, scrambled antagonir-treated (SPG(neo)/Scram) controls (*Figure 3.5*). Treatment with each antagonir (Cmu(neo)/Ant-155, Cmu(neo)/Ant-21, Cmu(neo)/Ant-223, Cmu(neo)/Ant-146b and Cmu(neo)/Ant-203) suppressed *Chlamydia*-induced lung histopathology compared to infected, scrambled antagonir-treated (Cmu(neo)/Scram) controls.
Figure 3.5: Neonatal *Chlamydia* respiratory infection-induced microRNA (miR)-155, miR-21, miR-223, miR-146b and miR-203 expression promotes infection-induced airway inflammation and lung histopathology during the peak of inflammation. Wild-type (WT) BALB/c mice were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(neo)). Controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. (A) Airway inflammation at 14 days post infection assessed in bronchoalveolar lavage fluid following treatment with miRNA antagomirs (Ant-155 [Cmu(neo)/Ant-155], Ant-21 [Cmu(neo)/Ant-21], Ant-223 [Cmu(neo)/Ant-223], Ant-146b [Cmu(neo)/Ant-146b] and Ant-203 [Cmu(neo)/Ant-203], respectively) or scrambled (Scram; [SPG(neo)/Scram and Cmu(neo)/Scram]) antagomir during neonatal Cmu respiratory infection. (B) Representative photomicrographs (x40 magnification) of small airways at 14dpi following treatment with miRNA antagomirs (Cmu(neo)/Ant-155, Cmu(neo)/Ant-21, Cmu(neo)/Ant-223, Cmu(neo)/Ant-146b and Cmu(neo)/Ant-203) or scrambled (SPG(neo)/Scram and Cmu(neo)/Scram) antagomir during neonatal Cmu respiratory infection (two experiments;
n=6-14). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
3.4.3 Neonatal *Chlamydia* respiratory infection-induced increases in the expression of miR-155, miR-21, miR-223, miR-146b and miR-203 have differential effects on several lung function parameters in later life

Given that antagonir treatment of infected neonates (Cmu(neo)/Ant-155, Cmu(neo)/Ant-21, Cmu(neo)/Ant-223, Cmu(neo)/Ant-146b and Cmu(neo)/Ant-203, respectively) markedly reduced infection-induced airway inflammation and lung histopathology at 14dpi, we next sought to determine the effects of treatment on the long-term sequelae of neonatal *Chlamydia* infection, including persistent AHR and emphysema-like alveolar enlargement. We have previously shown that neonatal *Chlamydia* respiratory infection promotes persistent AHR in mice in later life that is characterised by increased transpulmonary resistance and decreased dynamic compliance in response to increasing doses of nebulised methacholine (Horvat et al., 2007; Horvat et al., 2010b). In this study we replicate and advance upon these observations by showing that neonatal *Chlamydia* infection (Cmu(neo)/Scram) induces persistent AHR in later life in terms of airways resistance (Rn) in response to increasing doses of methacholine (Figure 3.6 A). We also show that neonatal *Chlamydia* infection (Cmu(neo)/Scram) is associated with increased work of breathing and IC at 63dpi when compared to sham-infected, scrambled antagonir-treated (SPG(neo)/Scram) controls (Figure 3.6 B). Importantly, the inhibition of miR-155, miR-21 or miR-203 during neonatal *Chlamydia* respiratory infection (Cmu(neo)/Ant-155, Cmu(neo)/Ant-21 and Cmu(neo)/Ant-203, respectively), but not of miR-223 or miR-146b (Cmu(neo)/Ant-223 and Cmu(neo)/Ant-146b, respectively), markedly suppressed infection-induced persistent AHR at 63dpi compared to infected, scrambled antagonir-treated (Cmu(neo)/Scram) controls (Figure 3.6 A). Interestingly, the inhibition of miR-155, miR-223 or miR-146b during neonatal
infection (Cmu(neo)/Ant-155, Cmu(neo)/Ant-223 and Cmu(neo)/Ant-146b, respectively), but not of miR-21 or miR-203 (Cmu(neo)/Ant-21 and Cmu(neo)/Ant-203, respectively), suppressed infection-induced increases in the work of breathing at 63dpi compared to infected, scrambled antagonim-treated (Cmu(neo)/Scram) controls (Figure 3.6 B). Finally, the inhibition of miR-155 or miR-223 during neonatal infection (Cmu(neo)/Ant-155 and Cmu(neo)/Ant-223, respectively), but not of miR-21, miR-146b or miR-203 (Cmu(neo)/Ant-21, Cmu(neo)/Ant-146b and Cmu(neo)/Ant-203, respectively), suppressed infection-induced increases in the IC at 63dpi compared to infected, scrambled antagonim-treated (Cmu(neo)/Scram) controls (Figure 3.6 B).
Figure 3.6: Neonatal *Chlamydia* respiratory infection-induced microRNA (miR)-155, miR-21, miR-223, miR-146b and miR-203 expression have differential effects on several lung function parameters in later life. Wild-type (WT) BALB/c mice were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(neo)). Controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. (A) Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch), and 10mg/mL of Mch (shows statistics for maximal resistance from AHR curves) was determined in all groups at 63 days post infection (dpi) following treatment.
with miRNA antagomirs (Ant-155 [Cmu(neo)/Ant-155], Ant-21 [Cmu(neo)/Ant-21], Ant-223 [Cmu(neo)/Ant-223], Ant-146b [Cmu(neo)/Ant-146b] and Ant-203 [Cmu(neo)/Ant-203], respectively) or scrambled (Scram; [SPG(neo)/Scram and Cmu(neo)/Scram]) antagomir during neonatal Cmu respiratory infection. AHR data for sham-infected and infected, scrambled antagomir-treated (SPG(neo)/Scram and Cmu(neo)/Scram, respectively) controls is repeated for each comparison (all experiments were performed concurrently) (≥two experiments; n=3-9). (B) Work of breathing and Inspiratory Capacity (IC) were assessed in all groups at 63dpi following treatment with miRNA antagomirs (Cmu(neo)/Ant-155, Cmu(neo)/Ant-21, Cmu(neo)/Ant-223, Cmu(neo)/Ant-146b and Cmu(neo)/Ant-203) or scrambled (SPG(neo)/Scram and Cmu(neo)/Scram) antagomir during neonatal Cmu respiratory infection (two experiments; n=5-10). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P< 0.0001.
3.4.4 Neonatal *Chlamydia* respiratory infection-induced miR-155 expression, but not miR-21, miR-223, miR-146b or miR-203 expression, promotes emphysema-like alveolar enlargement in later life

We have previously shown that *Chlamydia* respiratory infection of neonatal WT BALB/c mice results in emphysema-like alveolar enlargement in later life, and others have confirmed this effect (Horvat et al., 2010b; Jupelli et al., 2011). In this study, we initially replicate this observation and show that *Chlamydia* respiratory infection of neonatal WT BALB/c mice results in emphysema-like alveolar enlargement at 63dpi (Cmu(neo)/Scram), compared to sham-infected, scrambled antagonir-treated (SPG(neo)/Scram) controls (Figure 3.7).

Recent studies have shown that lung expression and activity of FGF-7 is important for early lung branching and epithelial proliferation during postnatal alveolarisation (Bottaro et al., 1990; Post et al., 1996; Yano et al., 2000; Padela et al., 2008). Given that FGF-7 possesses a functional binding site for miR-155 (Pottier et al., 2009) and that neonatal *Chlamydia* respiratory infection induces a profile of increased expression of miR-155, we assessed the expression of FGF-7 at the peak of neonatal *Chlamydia* respiratory infection (10dpi) in the absence, and presence, of treatment with Ant-155 (Cmu(neo)/Scram and Cmu(neo)/Ant-155, respectively). *Chlamydia* infection (Cmu(neo)/Scram) decreased the expression of FGF-7 at 10dpi compared to sham-infected, scrambled antagonir-treated (SPG(neo)/Scram) controls (Figure 3.7 B). Importantly, treatment with Ant-155 during neonatal *Chlamydia* infection (Cmu(neo)/Ant-155) increased the expression of FGF-7 at 10dpi compared to infected, scrambled antagonir-treated (Cmu(neo)/Scram) controls. Indeed, the expression of FGF-7 in Ant-155-treated mice (Cmu(neo)/Ant-155) was similar to that observed in sham-infected, scrambled antagonir-treated (SPG(neo)/Scram) mice.
Thus, neonatal *Chlamydia* respiratory infection decreases the expression of FGF-7 in the lungs at 10dpi and treatment with Ant-155 during infection can restore the expression of FGF-7 in the lungs back to baseline levels. Most significantly, inhibition of miR-155 during neonatal *Chlamydia* infection (Cmu(neo)/Ant-155), but not of miR-21, miR-223, miR-146b or miR-203 (Cmu(neo)/Ant-21, Cmu(neo)/Ant-223, Cmu(neo)/Ant-146b and Cmu(neo)/Ant-203, respectively), prevented the development of emphysema-like alveolar enlargement at 63dpi. Indeed, mice treated with Ant-155 during neonatal *Chlamydia* respiratory infection (Cmu(neo)/Ant-155) had similar mean alveolar sizes to sham-infected, scrambled antagomir-treated (SPG(neo)/Scram) mice in later life (Figure 3.7).

Collectively, these data show that the *Chlamydia*-induced increase in miR-155 expression during neonatal infection is associated with a reduction in lung FGF-7 expression and that inhibition of infection-induced miR-155 *in vivo* protects against the development of emphysema-like alveolar enlargement in later life.
Figure 3.7: Neonatal *Chlamydia* respiratory infection-induced microRNA (miR)-155 expression promotes emphysema-like alveolar enlargement. Wild-type (WT) BALB/c mice were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(geo)). Controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. (A) Representative photomicrographs (x40 magnification) of alveoli at 63 days post infection (dpi) following treatment with miRNA antagonirs (Ant-155 [Cmu(geo)/Ant-155], Ant-21 [Cmu(geo)/Ant-21], Ant-223 [Cmu(geo)/Ant-223], Ant-146b [Cmu(geo)/Ant-146b] and Ant-203 [Cmu(geo)/Ant-203], respectively) or scrambled (Scram; [SPG(geo)/Scram and Cmu(geo)/Scram]) antagonir during neonatal Cmu respiratory infection. (B) Alveolar diameter at 63dpi (≥two experiments; n=4) following treatment with miRNA antagonirs (Cmu(geo)/Ant-155, Cmu(geo)/Ant-21, Cmu(geo)/Ant-223, Cmu(geo)/Ant-146b and Cmu(geo)/Ant-203) or scrambled (SPG(geo)/Scram and Cmu(geo)/Scram) antagonir during neonatal Cmu respiratory infection, and fibroblast growth factor (Fgf)7 mRNA expression at
10dpi in the absence, and presence, of treatment with Ant-155. All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
3.4.5 Inhibition of miR-155, miR-21 and miR-203, but not of miR-223 and miR-146b, during neonatal Chlamydia respiratory infection prevents infection-induced increases in the severity of AAD in later life

We have previously shown that neonatal Chlamydia respiratory infection in mice increases the severity of the hallmark features of AAD in later life, including AHR (Horvat et al., 2007; Horvat et al., 2010b). In this study, we sought to assess the potential roles of neonatal Chlamydia infection-induced miRNAs in the infection-induced AAD phenotype. Infected, antagonim-treated mice were exposed to the acute Ova-induced model of AAD at 47dpi and hallmark features of AAD assessed at 63dpi. As expected, neonatal Chlamydia respiratory infection (Cmu(neo)/Scram/Ova) increased AHR compared to sham-infected, allergic, scrambled antagonim-treated (SPG(neo)/Scram/Ova) controls (Figure 3.8 A). Furthermore, neonatal infection amplified the numbers of total leukocytes in the BALF in AAD (Cmu(neo)/Scram/Ova) when compared to sham-infected, allergic, scrambled antagonim-treated (SPG(neo)/Scram/Ova) mice (Figure 3.8 B). Significantly, inhibition of miR-155, miR-21 or miR-203 during neonatal Chlamydia respiratory infection (Cmu(neo)/Ant-155/Ova, Cmu(neo)/Ant-21/Ova and Cmu(neo)/Ant-203/Ova, respectively), but not of miR-223 or miR-146b (Cmu(neo)/Ant-223/Ova and Cmu(neo)/Ant146b/Ova, respectively), suppressed Chlamydia-induced increases in AHR in AAD in later life (Figure 3.8 A). Indeed, treatment with Ant-155, Ant-21 or Ant-203 during neonatal Chlamydia infection (Cmu(neo)/Ant-155/Ova, Cmu(neo)/Ant-21/Ova and Cmu(neo)/Ant-203/Ova, respectively) suppressed infection-enhanced AHR in AAD down to sham-infected, allergic, scrambled antagonim-treated (SPG(neo)/Scram/Ova) levels. Inhibition of miR-155 and to some degree of miR-203 (P=0.07) (Cmu(neo)/Ant-155/Ova and Cmu(neo)/Ant-203/Ova,
respectively), but not of miR-21, miR-223 or miR-146b (Cmu(neo)/Ant-21/Ova, Cmu(neo)/Ant-223/Ova and Cmu(neo)/Ant-146b/Ova, respectively), suppressed the infection-induced increase in the number of leukocytes in the BALF in AAD compared to infected, allergic, scrambled antagomir-treated (Cmu(neo)/Scram/Ova) controls (Figure 3.8 B).
Figure 3.8: Neonatal *Chlamydia* respiratory infection-induced microRNA (miR)-155, miR-21 and miR-203 expression promote the key features of the infection-induced allergic airways disease (AAD) phenotype in later life. Wild-type (WT) BALB/c mice were infected intranasally (i.n.) with *C. muridarum* (Cmu) as neonates (<24 hours old; Cmu(neo)). Controls were sham-infected with sucrose phosphate glutamate (SPG) buffer. 47 days after infection mice were subjected to ovalbumin (Ova)-induced AAD and features of AAD were assessed at 63dpi. (A) Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch), and 10mg/mL of Mch
(shows statistics for maximal resistance from AHR curves) was determined in all groups at 63dpi following treatment with miRNA antagonirs (Ant-155 [Cmu(neo)/Ant-155/Ova], Ant-21 [Cmu(neo)/Ant-21/Ova], Ant-223 [Cmu(neo)/Ant-223/Ova], Ant-146b [Cmu(neo)/Ant-146b/Ova] and Ant-203 [Cmu(neo)/Ant-203/Ova], respectively) or scrambled (Scram; [SPG(neo)/Scram/Ova and Cmu(neo)/Scram/Ova]) antagonist during neonatal Cmu respiratory infection. AHR data for sham-infected and infected, allergic, scrambled antagonist-treated (SPG(neo)/Scram/Ova and Cmu(neo)/Scram/Ova, respectively) controls is repeated for each comparison (all experiments were performed concurrently) (≥two experiments; n=5-14). (B) Number of leukocytes in the bronchoalveolar lavage fluid (BALF) at 63dpi in sham-infected and infected, allergic mice following treatment with miRNA antagonirs (Cmu(neo)/Ant-155/Ova, Cmu(neo)/Ant-21/Ova, Cmu(neo)/Ant-223/Ova, Cmu(neo)/Ant-146b/Ova and Cmu(neo)/Ant-203/Ova) or scrambled (SPG(neo)/Scram/Ova and Cmu(neo)/Scram/Ova) antagonist during neonatal Cmu respiratory infection (≥two experiments; n=5-13). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
3.5 Discussion

In this study we show that neonatal Chlamydia respiratory infection induces the overexpression of 5 miRNAs (miR-155, miR-21, miR-223, miR-146b and miR-203) in the lungs during infection. Using novel miRNA-specific inhibitors (antagomirs) in vivo we demonstrate that these miRNAs differentially promote the key disease features induced by neonatal Chlamydia respiratory infection, including airway inflammation and lung histopathology (miR-155, miR-21, miR-223, miR-146b, and miR-203), reduced rate of weight gain (miR-155 and miR-223), persistent AHR (miR-155, miR-21 and miR-203) and emphysema-like alveolar enlargement (miR-155), as well as infection-enhanced AHR and allergic airway inflammation in AAD (miR-155, miR-21, and miR-203) in later life. This study highlights the potential relevance of targeting miRNAs therapeutically and supports their role in the immune response to respiratory infections.

We used C. muridarum to infect neonatal mice in this study as this is a natural mouse chlamydial pathogen that generates a productive infection, which replicates the immunological and pathophysiological features and time course of human disease, as we have previously described (Hansbro et al., 2004; Horvat et al., 2007; Horvat et al., 2010b).

This pilot study was conceived based on a 7 miRNA qPCR-validated microarray signature that is induced by neonatal Chlamydia respiratory infection at 5dpi (Section 2.3.2) and aimed to identify miRNAs that can be targeted therapeutically during neonatal Chlamydia infection. The mechanisms of action of the identified miRNAs will be investigated further in future studies. Two of these miRNAs were commonly overexpressed during neonatal, infant and adult infections (miR-155 and miR-21) whereas the remaining 5 miRNAs were uniquely increased
during neonatal infection (miR-223, miR-146b, miR-203, miR-142-5p, miR-1896). Further validations identified a subset of 5 miRNAs that exhibit a profile of increased expression during neonatal infection (miR-155, miR-21, miR-223, miR-146b and miR-203) (Figure 3.2). Importantly, these increases overlap with the historic peaks of infection (10dpi) and infection-induced airway inflammation (14-15dpi) in this model (Horvat et al., 2010b), which suggests that these miRNAs play a role in the immune response to neonatal Chlamydia respiratory infection. Infection only induced a transient increase in the expression of miR-142-5p and miR-1896 at 5dpi, suggesting that these miRNAs are unlikely to play a significant role in the immune response to neonatal infection and the development of neonatal Chlamydia infection-induced chronic lung disease.

Increasing evidence links miRNAs with the regulation of immune responses and also a multitude of disease processes. Studies have shown that miR-155, miR-21 and miR-146 are increased during bacterial infection with H. pylori, S. typhimurium, L. monocytogenes and F. novicida (Cremer et al., 2009; Xiao et al., 2009; Schulte et al., 2011; Izar et al., 2012) and that miR-21, miR-223 and miR-146 negatively regulate TLR-dependent signalling, inflammatory responses and granulocyte differentiation and activation (Taganov et al., 2006; Johnnidis et al., 2008; Sheedy et al., 2010). miR-155 in particular has consistently been shown to play an important role in immune function. Mice deficient in bic/miR-155 do not develop protective immunity against oral challenge with S. typhimurium following immunisation with live attenuated S. typhimurium (Rodriguez et al., 2007). Furthermore, T cells from miR-155-deficient mice produce higher levels of T_{H2} cytokines compared to WT controls (Rodriguez et al., 2007; Thai et al., 2007). Thus, given that miRNAs are important in immune function it is possible that the protracted profile of
overexpression of miRNAs during neonatal *Chlamydia* infection may have permanent influences on the developing lung and immune system, which has the potential to induce long-term deleterious effects. We sought to examine the potential pathogenic role of miRNAs in our model of neonatal *Chlamydia* respiratory infection by suppressing infection-induced miRNA expression back to the same levels observed in un-infected controls rather than depleting expression altogether.

Administration of antagonomirs to neonatal mice during infection suppressed *Chlamydia*-induced expression of each miRNA at 10dpi, thus confirming effective inhibition in the lungs (Figure 3.3). Importantly, 10dpi represents a time point 5 days after the most recent administration of antagonomir, which demonstrates that the inhibitory effect of antagonomirs are long-lived in vivo and can overcome robust *Chlamydia*-induced miRNA expression at the peak of infection. Indeed, the potency and longevity of antagonomir-mediated inhibition of miRNAs in our model is consistent with the findings of others (Mattes et al., 2009; Collison et al., 2011), including those of Krutzfeldt et al. (Krutzfeldt et al., 2005) who initially described ‘antagomirs’. Additionally, we have shown in testing prior to this study that miRNA antagonomirs are specific for their intended targets (data not shown - part of a separate manuscript not presented in this Thesis). In the current study we show that scrambled control antagonomirs do not affect infection-induced miRNA expression in the lungs compared to water(vehicle)-treated groups. In contrast, miRNA-specific antagonomirs suppressed infection-induced miRNA expression for each miRNA investigated.

We have previously shown that neonatal mice display reduced weight gain, influx of all major inflammatory leukocytes into the lungs and increased lung histopathology following *Chlamydia* respiratory infection, compared to sham-infected controls (Horvat et al., 2007; Starkey et al., 2014). In this study, we replicate these
observations by showing that *Chlamydia*-infected neonatal mice treated with scrambled antagonim have reduced weight gain, increased airway inflammation and lung histopathology compared to sham-infected, scrambled antagonim-treated controls (Figures 3.4 and 3.5). Interestingly, the administration of Ant-155 or Ant-223 to neonatal mice during *Chlamydia* infection partially improved the rate of weight gain whilst treatment with Ant-21 or Ant-146b had no effect. Surprisingly, the administration of Ant-203 further decreased the rate of weight gain. In contrast, the inhibition of each miRNA suppressed infection-induced airway inflammation, particularly macrophages, and lung histopathology at 14dpi back to baseline, sham-infected levels. Taken together, these data suggest that the miRNAs under examination in this study affect common pathways that promote some of the acute disease features of neonatal *Chlamydia* infection, including lung inflammation and histopathology but also have unique effects on the rate of weight gain. Thus, we next sought to assess the impact of inhibiting neonatal infection-induced miRNA expression on the development of *Chlamydia*-induced disease features in later life.

We have previously shown that neonatal *Chlamydia* respiratory infection in mice promotes the development of persistent AHR in later life (Horvat et al., 2010b), which has been confirmed by others (Jupelli et al., 2011). In this study, we replicate then advance upon these observations by identifying the miRNAs that may be involved. We show that neonatal *Chlamydia* infection-induced miR-155, miR-21 and miR-203 drive the development of persistent AHR in terms of Rn at 63dpi (Figure 3.6). Furthermore, we show that *Chlamydia*-infected neonates acquire other permanent deleterious changes to lung function, including increased work of breathing and inspiratory capacity compared to sham-infected, scrambled antagonim-treated controls. To the best of our knowledge, this is the first study to demonstrate
the functional roles of lung miRNAs that are induced during neonatal *Chlamydia* respiratory infection in promoting reduced lung function in later life.

We have previously shown that neonatal, but not infant or adult, *Chlamydia* respiratory infection results in emphysema-like alveolar enlargement that persists into later life (Horvat et al., 2010b) and this effect was recently confirmed by others (Jupelli et al., 2011). Here, we replicate these observations and demonstrate a potential miRNA-mediated mechanism. At birth the human lung contains up to around 50 million alveoli, which is approximately 15-20% of the adult alveolar complement (Langston et al., 1984; Burri, 2006). This indicates that alveolarisation is largely a postnatal event in humans. In contrast, rodents are born without alveoli but undergo the same postnatal development of the lungs and alveoli as humans (Burri, 2006; Padela et al., 2008). Recent studies have shown that FGF-7 (also known as KGF) is required for early lung organogenesis and postnatal alveolar formation in rodents (Post et al., 1996; Padela et al., 2008). FGF-7 is endogenously expressed in the lung mesenchyme and potently induces the proliferation of type II pneumocytes by binding to a splice variant of FGF receptor 2 on the airway epithelium (Bottaro et al., 1990; Panos et al., 1993; Post et al., 1996; Yano et al., 2000; Padela et al., 2008).

Importantly, FGF-7 mRNA possesses a functional binding site for miR-155 in its 3’ UTR (Pottier et al., 2009) and, in this study, we show that neonatal *Chlamydia* respiratory infection increases lung miR-155 expression and concomitantly decreases FGF-7 mRNA expression (Figure 3.7). Most significantly, the inhibition of miR-155 during neonatal *Chlamydia* respiratory infection restores the expression of FGF-7 at 10dpi and prevents emphysema-like alveolar enlargement. Collectively, these data suggest that *Chlamydia*-induced miR-155 expression interferes with postnatal
alveolarisation in the lungs of infected neonates and that this may occur through the targeted disruption of FGF-7.

We previously showed that neonatal *Chlamydia* respiratory infection enhances the severity of features of AAD in later life (Horvat et al., 2010b). In this study, we further these observations by demonstrating that inhibition of miR-155, miR-21 or miR-203 during neonatal *Chlamydia* respiratory infection also prevents infection-enhanced AHR in AAD in later life (*Figure 3.8*). Our data suggest that neonatal infection-enhanced AHR in AAD in later life may be a summative consequence of infection-induced persistent AHR and Ova-induced AHR and that this effect is dependent on *Chlamydia*-induced miR-155, miR-21 and miR-203. Interestingly, the inhibition of miR-155, and to some extent miR-203, during neonatal *Chlamydia* infection also suppressed airway inflammation in the infection-induced AAD phenotype, suggesting that the early overexpression of these miRNAs in the lungs have common long-term deleterious effects on AHR and airway inflammation. Indeed, others have shown that early life respiratory infections, particularly viral infections, similarly increase the severity of AAD in later life (Lukaes et al., 2001; You et al., 2006). However, to the best of our knowledge this is the first study to identify that targeting miRNAs during a neonatal *Chlamydia* respiratory infection can suppress the short- and long-term consequences of infection, including infection-induced increases in the severity of features of AAD. This highlights the potential importance of the early life respiratory infection-induced lung miRNA expression profile in priming for exaggerated responses in respiratory disease in later life.
Chapter 4:

MicroRNA-21 promotes steroid insensitivity in infection-induced, severe, steroid-insensitive asthma by amplifying PI3K-mediated suppression of HDAC2

In this chapter, I show that Chlamydia respiratory infection induces severe, neutrophilic AAD that is steroid-insensitive. Infection-induced, severe, steroid-insensitive AAD is associated with increased expression of miR-21 that is not suppressed by steroid treatment and drives disease through the amplification of PI3K activity that suppresses HDAC2. Inhibition of miR-21 or PI3K reversed the suppression of PTEN and HDAC2, and restored steroid sensitivity of airway inflammation and AHR. This suggests that miR-21 is pivotal in inducing steroid-insensitive inflammation and AHR in severe AAD and that this occurs through a miR-21/PI3K axis that suppresses HDAC2. I show that inhibition of miR-21 also suppresses Haemophilus-induced, severe, steroid-insensitive AAD. Therefore, these data highlight that therapeutic targeting of miR-21 may be an effective therapeutic strategy for severe, steroid-insensitive asthma.

This chapter was submitted and peer-reviewed in the J Allergy Clin Immunol and is currently under preparation for resubmission to the J Allergy Clin Immunol.
4.1 Abstract

Corticosteroids are the mainstay therapy for numerous inflammatory conditions, however, some patients are steroid-insensitive and are therefore difficult to treat. In asthma, steroid insensitivity is associated with respiratory infections, and is more pronounced in non-eosinophilic disease endotypes. It is a substantial medical problem with more severe steroid-insensitive asthmatics accounting for >50% of asthma-associated health-care-costs. Thus, effective therapies are required for steroid-insensitive patients, however, a lack of understanding of the mechanisms that promote this phenotype has hampered the development of such treatments. Here, we developed a novel mouse model of steroid-insensitive, neutrophilic (i.e. non-eosinophilic) asthma by combining Chlamydia respiratory infection and Ova-induced AAD. We demonstrate that infection induces a miR-21-dependent, PI3K-mediated cell-signalling pathway. This promotes steroid-insensitive neutrophilic inflammation and AHR in AAD through the suppression of nuclear HDAC2. Inhibition of miR-21 or PI3K suppressed nuclear phospho-Akt\textsuperscript{Ser473} levels, which restored HDAC2 levels and sensitivity to steroid treatment. We show that inhibition of miR-21 also suppresses the key features of Haemophilus-induced, severe, steroid-insensitive AAD. Thus, we show that respiratory infections may drive the development of steroid-insensitive asthma by inducing miR-21-dependent, PI3K-mediated suppression of HDAC2. Our findings suggest that targeting miR-21 may be effective in treating steroid-insensitive asthma.
4.2 Introduction

Corticosteroids are broadly active anti-inflammatory agents that are widely used to treat many chronic inflammatory diseases, such as asthma, lupus erythematosus, inflammatory bowel diseases (IBDs) and rheumatoid arthritis (Barnes and Woolcock, 1998; Faubion et al., 2001; Chikanza and Kozaci, 2004; Rhen and Cidlowski, 2005; Bucala, 2012). However, there are cohorts of these patients with poor sensitivity to steroid treatment even with high doses, which can lead to many iatrogenic side effects (Munkholm et al., 1994; Probert et al., 2003; Chikanza and Kozaci, 2004; Rhen and Cidlowski, 2005; Barnes, 2010; Wang et al., 2012). The lack of effective therapeutic alternatives leads to substantial excess morbidity and health care expenditure (Barnes, 2010).

Asthma is an allergic inflammatory condition of the airways archetypally mediated by aberrant Th2 lymphocyte responses to otherwise harmless environmental antigens (Thorburn and Hansbro, 2010). These responses drive eosinophilic airway inflammation, mucous hypersecretion, and AHR (Bochner et al., 1994; Wills-Karp, 1999; Umetsu et al., 2002; Asher et al., 2006; Eder et al., 2006; Kim et al., 2010). Recent clinical evidence, however, shows that asthma is a heterogeneous condition. Increased Th1- and/or Th17- responses (Liu et al., 2004b; Kumar et al., 2006; Truyen et al., 2006; Bullens, 2007), and non-eosinophilic, predominantly monocytic or neutrophilic, rather than eosinophilic, airway inflammation prevail in moderate to severe asthma (Ordonez et al., 2000; Gibson et al., 2001).

Corticosteroids suppress the immunopathological processes that drive inflammation and AHR and are the mainstay of asthma therapy (Currie et al., 2005; Fanta, 2009). However, 5-10% of asthmatics do not respond to steroid treatment. These steroid-insensitive patients typically have more severe disease and account for
~50% of asthma associated health-care-costs (Barnes and Woolcock, 1998; Chung et al., 1999; Ito et al., 2006c; Barnes and Adcock, 2009). Importantly, steroid-insensitive severe asthma is associated with non-eosinophilic endotypes of the disease, including neutrophilic asthma (Green et al., 2002; Hansbro et al., 2011). These patients (and those with other steroid-insensitive conditions) are sub-optimally treated and the development of efficacious therapeutics is dependent on improving our understanding of the mechanisms involved in driving disease.

The anti-inflammatory activity of corticosteroids is largely mediated through the activation of the cytosolic GR-α (Ito et al., 2006b). Upon activation by corticosteroid binding, GR-α translocates to the nucleus and inhibits the expression of pro-inflammatory genes. This process is mediated, at least in part, through the recruitment of the enzyme HDAC2 that deacetylates core histone proteins and suppresses gene transcription (Ito et al., 2001). Reduced HDAC2 expression and activity has been associated with increased disease severity and steroid insensitivity in both severe asthma and COPD (Ito et al., 2005; Barnes, 2008b; Marwick et al., 2009).

Steroid insensitivity and reduced HDAC2 activity have both been linked to aberrant PI3K-dependent signalling. Pharmacological and genetic interruption of PI3K reinstated steroid sensitivity and HDAC2 activity in a cigarette smoke-induced model of COPD (Marwick et al., 2009; To et al., 2010). Thus, amplified PI3K activity may promote steroid insensitivity by reducing the expression and activity of HDAC2.

Clinical evidence links respiratory infections, such as those with C. pneumoniae and H. influenzae, with severe, neutrophilic, steroid-insensitive asthma. We, and others, have shown that Chlamydia and Haemophilus induce neutrophilic, T_{H1} and/or T_{H17} responses in experimental models of asthma replicating the effects in patients with this form of asthma (Zhou et al., 2009; Horvat et al., 2010a; Essilfie et
al., 2011; Essilfie et al., 2012). T\(_{H17}\)-inducing cytokines such as IL-6 and IL-1\(\beta\) activate PI3K signalling (Hideshima et al., 2001; Neumann et al., 2002). Furthermore, PI3K activity can promote T\(_{H17}\) immune responses (Haylock-Jacobs et al., 2011) and plays a key role in facilitating Chlamydia and Haemophilus entry into cells (Coombes and Mahony, 2002; Marti-Lliteras et al., 2009).

MiRNAs are highly conserved, single-stranded RNA molecules that silence genes at the post-transcriptional level (Bartel, 2009; Winter et al., 2009). Several miRNAs have been implicated in asthma pathogenesis, and miR-21 in particular has consistently been shown to be important in multiple murine models of AAD (Lu et al., 2009; Mattes et al., 2009). MiR-21 is highly up-regulated during pulmonary inflammation (Lu et al., 2009) and promotes T\(_{H2}\) immune polarisation through the suppression of IL-12p35 (Lu et al., 2009; Lu et al., 2011). Indeed, miR-21-deficient mice exhibit reduced eosinophilic inflammation and IL-4 levels with a concomitant increase in IFN-\(\gamma\) during Ova-induced AAD (Lu et al., 2011). However, the inhibition of miR-21 during the challenge phase of HDM-induced AAD in WT mice had no effect on the production of T\(_{H2}\) cytokines or eosinophilic inflammation (Collison et al., 2011). These studies suggest that miR-21 exerts potent immune polarising effects during allergic sensitisation rather than during the exacerbation of disease (Lu and Rothenberg, 2013). MiR-21 also down-regulates the expression of PTEN (Meng et al., 2007; Yamanaka et al., 2009), which antagonises PI3K-dependent signalling (Meng et al., 2007; Carracedo and Pandolfi, 2008; Yamanaka et al., 2009; Miletic et al., 2010). We hypothesised that overexpression of miR-21 and its attenuation of PTEN could promote steroid insensitivity by amplifying PI3K-mediated, suppression of HDAC2.
Here we developed a novel mouse model of infection-induced, severe, steroid-insensitive AAD that recapitulates the hallmark features of this form of asthma. This was achieved by overlaying respiratory infection with *Chlamydia* in mice with established Ova-induced AAD. *Chlamydia* infection induced neutrophilic inflammatory responses, whilst suppressing eosinophil infiltration, in the airways in AAD. Infection-induced neutrophilic inflammation and AHR was insensitive to steroid treatment, unlike in AAD without infection. We utilised this model to demonstrate an important role for infection-induced miR-21 in promoting severe, neutrophilic, steroid-insensitive AAD through the amplification of PI3K-mediated phosphorylation of Akt at serine 473 (pAkt) and suppression of HDAC2 levels. These effects could be inhibited, and steroid sensitivity restored, by the suppression of miR-21 and/or PI3K. Additionally, we show that inhibition of miR-21 also suppresses the key features of *Haemophilus*-induced, severe, steroid-insensitive AAD. This study defines a novel miR-21/PI3K/pAkt/HDAC2 axis in a previously unrecognised pathogenic role and highlights miR-21 as a novel therapeutic target for the treatment of severe, steroid-insensitive asthma, and possibly other steroid-insensitive diseases.
4.3 Methods

4.3.1 Ethics statement

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

4.3.2 Murine model of established AAD

Female WT BALB/c mice, 6-8 weeks old, were sensitised to Ova (50µg, i.p. injection, Sigma-Aldrich, Sydney, Australia), in Rehydragel® (1mg, Reheis, Berkeley Heights, New Jersey, USA) in sterile saline (200µL). They were subsequently challenged i.n. with the same antigen (10µg/50µL sterile saline) under isoflurane anaesthesia on days 12-13 to induce AAD and again on days 33-34 to recapitulate AAD. Sham-sensitised controls received saline sensitisation with Rehydragel® by i.p. injection and subsequent Ova challenges on days 12-13 and 33-34 (Figure 4.1). Features of AAD were characterised on days 32 (tissues collected for RNA and protein analyses) or 35 (lung function assessment and tissues collected for RNA and protein analyses).

4.3.3 C. muridarum and H. influenzae respiratory infections

Mice with established AAD were inoculated under isoflurane anaesthesia on day 14 i.n. with the natural mouse pathogen C. muridarum (100 IFU, ATCC VR-123, in 30µL SPG) or intratracheally (i.t.) with non-typeable H. influenzae (2x10⁶ colony
forming units, NTHi-289, in 30µL phosphate-buffered saline (PBS)). Sham-infected controls received the equivalent volume of SPG or PBS, respectively.

4.3.4 Treatment with dexamethasone during AAD

Some mice were treated with DEX (Sigma-Aldrich, Sydney, Australia; 2 mg/kg) administered i.n. on days 32-34. This dosing regime was optimised prior to conducting the work outlined in this Thesis.

4.3.5 In vivo administration of miRNA inhibitor (antagomir)

We inhibited miRNAs in vivo in Chlamydia- and Haemophilus-induced AAD using miRNA-specific antagomirs. The miR-21 sequence was downloaded from miRBase University of Manchester, UK (http://www.mirbase.org/) and complementary miRNA antagomirs were designed. Ant-21 and scrambled antagomir control (Ant-Scram; nonspecific RNA VIII, BLAST searched against the mouse genome) were synthesised by Sigma-Aldrich (refer to Table 3.1 for details). Lyophilised antagomirs were reconstituted in sterile nuclease-free water and the concentrations quantified by spectrophotometry prior to final stock dilution. Groups of Chlamydia- or Haemophilus-infected, allergic WT BALB/c mice were treated i.n. with Ant-21 (50µg delivered in 50µL sterile saline) or an equivalent amount of Ant-Scram on day 32 in the absence, or presence, of DEX (Figure 4.1), as described previously (Krutzfledt et al., 2005; Mattes et al., 2009; Collison et al., 2011). The effects of treatment with Ant-21 were assessed on day 35.

4.3.6 In vivo administration of LY294002; PI3K inhibition
Groups of *Chlamydia*-infected, allergic WT BALB/c mice were treated i.n. with the class I pan-PI3K inhibitor LY294002 (Selleck, Houston, USA; 2 mg/kg in 3% DMSO vehicle) on days 32-34 in the absence, or presence, of DEX. Control groups (sham- and *Chlamydia*-infected, allergic mice) were treated with DMSO vehicle in the absence, or presence, of DEX (Figure 4.1). This dosing regime was optimised prior to conducting the work outlined in this Thesis.

### 4.3.7 Airway inflammation

Airway inflammation was assessed in cytospin preparations of cells from BALF collected on day 35 of the study protocol (Figure 4.1) (Horvat et al., 2007; Horvat et al., 2010a). BALF was collected by two 1mL washes with Hank’s Balanced Salt Solution (Life Technologies, Australia) via a cannula inserted into the trachea, centrifuged (300xg, 10 min, 4°C), treated with red blood cell lysis buffer (200µL, Tris-buffered NH₄Cl) and pelleted before total leukocyte numbers were determined using a haemocytometer. Cells were cytocentrifuged and stained with May-Grunwald-Giemsa. Differential leukocyte counts were determined using morphological criteria (≈175 cells by light microscopy [x40 magnification]) (Horvat et al., 2007; Horvat et al., 2010a). All samples were coded and counts were performed in a blinded fashion.

### 4.3.8 Lung function

Mice were anaesthetised with ketamine (100mg/kg) and xylazine (10mg/kg, Troy Laboratories, Smithfield, Australia) and their tracheas were cannulated (tracheostomy with ligation) (Horvat et al., 2007; Horvat et al., 2010a; Horvat et al., 2010b; Starkey et al., 2012; Starkey et al., 2013a; Starkey et al., 2013b). FlexiVent
apparatus (FX1 System; SCIREQ, Montreal, Canada) was used to assess airway-specific resistance (Rn; tidal volume of 8 mL/kg at a respiratory rate of 450 breaths/min) (Beckett et al., 2013) in response to increasing doses of nebulised methacholine (Sigma-Aldrich, Sydney, Australia). This combination of anaesthesia and ventilation is common and recommended by the manufacturer (Horvat et al., 2010a; Li et al., 2010; Beckett et al., 2013). Assessments were performed at least three times per dose of saline/methacholine and the average calculated.

4.3.9 Quantification of mRNA and miRNA expression by real-time qPCR

Total RNA was isolated from homogenised lungs with TRIzol® Reagent (Invitrogen, Life Technologies, Australia). Random-primed reverse transcriptions were performed for mRNA real-time qPCRs. Gene expression was normalised to the transcript of the housekeeping gene HPRT (Horvat et al., 2010b). Expression of miR-21 was assessed by real-time qPCR, as described previously (Beveridge et al., 2008; Beveridge et al., 2010; Santarelli et al., 2011). Briefly, multiplex reverse transcriptions were performed on DNase I-treated total RNA using a combination of reverse primers specific for mature mmu-miR-21 and the endogenous controls U6 snRNA and U49 snoRNA, to a final concentration of 40nM each. The relative abundance of miR-21 was calculated against the geometric mean of U6 and U49. For primer sequences refer to Table 4.1. All reactions were performed using BioScript™ reverse transcriptase in one times first-strand buffer according to manufacturer’s instructions (Bioline Pty. Ltd., NSW, Australia). Real-time qPCR assays were performed with SYBR Green Supermix (KAPA Biosystems, Inc., MA, USA) and a Mastercycler® ep realplex² system (Eppendorf South Pacific Pty. Ltd., NSW, Australia).
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<td>mmu-miR-21</td>
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1 LNA™-modified bases are preceded by a [+] symbol
4.3.10 Immunoblot analysis

Nuclear protein fractions were isolated from lung tissues using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo, Scientific) with added Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo, Scientific). Sample protein and Precision Plus Protein™ WesternC™ Standards (Bio-Rad, CA, USA) were resolved on 4-15% gradient Mini-PROTEAN® TGX Stain-Free™ polyacrylamide gels (Bio-Rad) and transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) and Tween 20 (TBS-T) and incubated with primary antibodies overnight before adding the relevant secondary antibody with Precision Protein™ StrepTactin-horseradish peroxidase (HRP) Conjugate (Bio-Rad). Primary antibodies employed were; anti-pAkt (Ser473) and anti-Akt antibodies (Cell Signaling Technology, MA, USA), and anti-HDAC2 and anti-TATA binding protein (TBP) [1TBP18] antibodies (Abcam, MA, USA), and were used according to manufacturer’s instructions. Secondary antibodies used were anti-Rabbit IgG HRP (R&D Systems, MN, USA) and anti-Mouse IgG (whole molecule)-Peroxidase antibodies (Sigma-Aldrich, Sydney, Australia). SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo, Scientific) was used to develop and visualise membranes by chemiluminescence (Bio-Rad, ChemiDoc MP System).

4.3.11 Statistics

Comparisons between two groups were made using unpaired t-Tests or a non-parametric equivalent where appropriate. Comparisons between multiple groups were made using a One-way ANOVA and an appropriate Post Test or a non-parametric equivalent where appropriate. Lung function data were assessed using a Two-way
ANOVA and an appropriate Post Test or a non-parametric equivalent. Analyses were performed using GraphPad Prism Software (San Diego, California). All data shown are representative of individual mice. No data has been pooled.
4.4 Results

4.4.1 *Chlamydia* respiratory infection induces severe, neutrophilic, steroid-insensitive AAD

To investigate the role of *Chlamydia* respiratory infection in the pathogenesis of severe, neutrophilic, steroid-insensitive asthma we generated a novel mouse model of disease (*Figure 4.1*). Acute Ova-induced AAD was established in WT BALB/c mice, which were then i.n. infected with the natural mouse respiratory pathogen *C. muridarum*. This is the most appropriate strain for studying host: pathogen relationships in mice (Horvat et al., 2007; Horvat et al., 2010a; Horvat et al., 2010b; Fung et al., 2013). The infection and inflammation peak at day 10 and 14-15, respectively (Horvat et al., 2007; Horvat et al., 2010b). The phenotype of AAD in the acute Ova model wains over time (data not shown - part of a separate manuscript not presented in this Thesis) and so we recapitulated the phenotype with two additional Ova challenges 19 days after infection. This is a similar scenario that occurs during allergen-induced increases in asthma severity in humans. This model enabled us to assess the impact of a resolved infection on established AAD. Hallmark disease features were assessed on day 35 in the absence, or presence, of treatment with the steroid DEX.

As expected, in the absence of infection, airway inflammation, which was predominantly eosinophilic, was increased in Ova-induced AAD (Ova/SPG) compared to non-allergic (Sal/SPG) controls (*Figure 4.2 A-C*). When *Chlamydia* infection was overlayed, it suppressed eosinophilic but increased neutrophilic airway inflammation in AAD (Ova/Cmu) compared to sham-infected, allergic (Ova/SPG) controls. Gene expression profiling showed increased mRNA expression of $T_{H1}$-associated (TLR2, STAT1, IFN-$\gamma$, chemokine (C-X-C motif) ligand(CXCL)-9 and -
10 and TNF-α; Figure 4.2 D) and T_{H17}-associated (IL-17, IL-6, transforming growth factor(TGF)-β, IL-1β; Figure 4.2 D), but reduced expression of T_{H2}-associated (IL-5, IL-13; data not shown - part of a separate manuscript not presented in this Thesis) cytokines and factors in the lungs of mice with infection and AAD (Ova/Cmu) compared to sham-infected, allergic (Ova/SPG) controls. Thus, Chlamydia infection resulted in a switch from T_{H2}-dominated, eosinophilic inflammatory responses to T_{H1}/T_{H17}-dominated, neutrophilic inflammatory responses in Ova-induced AAD. In the absence of infection AHR increased in Ova-induced AAD and this was not altered by infection (Figure 4.2 E and F). Chlamydia infection in sham-sensitised, non-allergic (Sal/Cmu) mice did not increase airway inflammation or AHR compared to sham-infected, non-allergic (Sal/SPG) controls (Figure 4.2 A-C, E and F), suggesting that Chlamydia-induced neutrophilic AAD is due to a change in the AAD phenotype rather than the additive effects of infection and AAD on inflammation and AHR.

We then assessed the efficacy of inhaled steroids in both Chlamydia-infected and sham-infected groups with AAD. Treatment of sham-infected, allergic mice with DEX (Ova/SPG/DEX, Figure 4.1) suppressed airway inflammation and AHR compared to sham-infected, sham-treated, allergic (Ova/SPG) controls (Figure 4.2 A-C, E and F). However, when Chlamydia-infected, allergic mice were treated with steroids (Ova/Cmu/DEX), neutrophilic inflammation and AHR were completely steroid-insensitive.

Collectively, these data demonstrate that Chlamydia respiratory infection induces T_{H1}/T_{H17}-dominated, neutrophilic AAD that is insensitive to steroid treatment, a phenotype that resembles severe, neutrophilic, steroid-insensitive asthma in humans.
Figure 4.1: Experimental protocol. Investigation and treatment of Chlamydia- and Haemophilus-induced, severe, neutrophilic, steroid-insensitive allergic airways disease (AAD). Wild-type (WT) BALB/c mice were intraperitoneally (i.p.) sensitised to ovalbumin (Ova) (d0) and AAD was induced by intranasal (i.n.) Ova challenge (d12 and 13) followed by re-challenge (d33 and 34). Non-allergic controls were sham-sensitised with saline (Sal). Some groups were inoculated i.n. with 100 inclusion-forming units (IFU) of C. muridarum (Cmu; d14), or intratracheally (i.t.) with 2x10⁶ colony-forming units (CFU) of H. influenzae (Hinf; d14). Controls were sham-infected with sucrose phosphate glutamate (SPG) or phosphate buffered saline (PBS), respectively. Steroid responses were assessed by i.n. treatment with dexamethasone (DEX; d32-34). MiR-21-depleting antagonirs (d32) and pan-phosphoinositide-3-kinase inhibitor LY294002 (d32-34) were administered i.n. to suppress miR-21 and PI3K, respectively. Controls received scrambled antagonir or DMSO vehicle.
Figure 4.2: *Chlamydia* infection induces severe, neutrophilic, steroid-insensitive allergic airways disease (AAD). Total leukocyte (A), eosinophil (B) and neutrophil numbers (C) were enumerated in BAL fluid, and lung expression of T\(_{H1}\)- and T\(_{H17}\)-associated factors (D) was determined on d35 of the study protocol (Figure 4.1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the presence, and/or absence,
of steroid (DEX) treatment compared to non-allergic controls (Sal). Airways hyper-
responsiveness in terms of airways resistance (Rn) in response to increasing doses of
methacholine (Mch) (E), and 10 mg/mL of Mch (F; shows statistics for maximal resistance
from AHR curves [Fig. 4.2 F]) was also determined. All data are presented as mean±s.e.m
(≥two experiments; n=4-10). *P< 0.05; ** P<0.01; ***P<0.001; ****P< 0.0001.
4.4.2 *Chlamydia* infection induces a persistent increase in miR-21 expression in severe, steroid-insensitive AAD

Since miR-21 has been implicated in the pathogenesis of asthma we assessed whether this miRNA is overexpressed in experimental *Chlamydia*-induced, severe, steroid-insensitive asthma. We observed a robust increase in the expression of miR-21 in the lungs of *Chlamydia*-infected mice with AAD (Ova/Cmu), compared to sham-infected, non-allergic (Sal/SPG) controls (Figure 4.3 A). This increase was not limited to *Chlamydia*-infected mice, with all allergic groups, regardless of infection status, exhibiting significantly increased expression of miR-21 compared to sham-infected, non-allergic (Sal/SPG) controls. Steroid treatment did not suppress miR-21 expression in either of the allergic (Ova/SPG/DEX, Ova/Cmu/DEX) groups, suggesting that miR-21 expression is not steroid-sensitive. Importantly, the *Chlamydia*-infected, non-allergic (Sal/Cmu) group had substantially increased miR-21 expression compared to sham-infected, non-allergic (Sal/SPG) controls. Indeed miR-21 expression was similar across all infected groups regardless of allergic status. Most significantly, miR-21 expression was substantially increased on day 32 of the study protocol (Figure 4.1) in *Chlamydia*-infected, allergic groups (Ova/Cmu), compared to sham-infected, allergic (Ova/SPG) controls (Figure 4.3 B).

These observations demonstrate that infection induces an environment within the lungs of allergic mice where miR-21 expression is chronically increased (6-fold) prior to subsequent Ova challenges and therapeutic steroid administration. This may promote steroid insensitivity of the hallmark features of infection-induced, severe, AAD.
Figure 4.3: *Chlamydia* respiratory infection induces microRNA (miR)-21 expression in severe, steroid-insensitive allergic airways disease (AAD). Expression of miR-21 in whole lung tissue was assessed by qPCR (A) on d35 of the study protocol (Figure 4.1) in *Chlamydia* (Cmu) and sham (SPG)-infected mice with ovalbumin (Ova)-induced AAD in the absence, or presence, of steroid (DEX) treatment compared to non-allergic controls (Sal; ≥two experiments; n=4-6). MiR-21 lung expression was also assessed in allergic groups on d32 (B) prior to recapitulation of AAD (Figure 4.1) (one experiment; n=8). All data are presented as mean±s.e.m. **P<0.01; ***P<0.001.
4.4.3 *Chlamydia* infection primes steroid-insensitive responses in AAD

The data described above suggests that *Chlamydia* infection may induce an environment that primes for steroid-insensitive responses. To investigate this possibility further, we examined whether the increased miR-21 expression in *Chlamydia*-induced, severe, steroid-insensitive AAD was associated with the suppression of key factors involved in responses to steroids. *Chlamydia* infection-induced increases in miR-21 levels were associated with reduced expression of GR-α, PTEN and HDAC2 in Ova-induced AAD (Ova/Cmu) on day 32 of the study protocol (Figure 4.1) compared to sham-infected, allergic (Ova/SPG) controls (Figure 4.4 A-C).

Using pAkt as a surrogate marker of PI3K activity, we next evaluated the potential mechanistic link between *Chlamydia*-induced miR-21 and amplified PI3K-dependent signalling. Immunoblot analyses of nuclear protein fractions showed that *Chlamydia* infection in AAD (Ova/Cmu) significantly increased pAkt (Figure 4.4 D) and reduced active HDAC2 (60kDa) levels (Figure 4.4 E) levels on day 32 compared to sham-infected, allergic (Ova/SPG) controls.

Thus, *Chlamydia*-induced increases in miR-21 expression at the time point of therapeutic administration of steroids is associated with reduced GR-α expression, increased PI3K signalling and reduced HDAC2 production in severe, steroid-insensitive AAD.
Figure 4.4: *Chlamydia* respiratory infection promotes steroid-insensitive responses in severe, allergic airways disease (AAD). Lung expression of Gr-α (A), Pten (B) and Hdac2 (C) was assessed by qPCR (one experiment; n=8) and nuclear protein levels of Akt and pAkt (D; pAkt, Akt and pAkt:Akt ratio) and Hdac2 (E; active Hdac2 [60kDa], degraded Hdac2 [20kDa], active to degraded Hdac2 ratio [60kDa/20kDa]) normalised to TATA binding protein (TBP) were determined by immunoblot (top panels; representative immunoblots) and quantification by densitometry (bottom panels) (≥two experiments; n=4) on d32 prior to recapitulation of AAD (Figure 4.1) in *Chlamydia* (Cmu) and sham (SPG)-infected mice during ovalbumin (Ova)-induced AAD. All data are presented as mean±s.e.m. **P<0.01; ***P<0.001; ****P<0.0001.
4.4.4 MiR-21 increases pAkt and reduces HDAC2 levels in *Chlamydia*-induced, severe, steroid-insensitive AAD

Given that *Chlamydia*-induced miR-21 expression is associated with increased PI3K signalling and decreased active HDAC2 (60kDa) levels, we assessed whether there are direct interactions between these factors. To do this we examined the effects of inhibiting miR-21 during *Chlamydia*-induced, severe, steroid-insensitive AAD. A single i.n. administration of miR-21-specific inhibitor (antagomir, Ant-21) on day 32 of the study protocol (Figure 4.1) completely ablated lung miR-21 expression on day 35 in infected, allergic groups in the absence or presence of steroids (Ova/Cmu/Ant-21 and Ova/Cmu/Ant-21/DEX) compared to scrambled antagomir-treated (Ova/Cmu/Scram and Ova/Cmu/Scram/DEX) controls (Figure 4.5 A). Significantly, Ant-21 treatment reversed *Chlamydia*-induced suppression of PTEN (Figure 4.5 B) and HDAC2 (Figure 4.5 C) expression in infected mice with AAD (Ova/Cmu/Ant-21) compared to scrambled antagomir-treated (Ova/Cmu/Scram) controls.

We next determined if miR-21 inhibition reversed infection-induced changes in the levels of nuclear pAkt and HDAC2. Infection, as expected, increased the relative levels of nuclear pAkt in the lungs of scrambled antagomir-treated, *Chlamydia*-infected, allergic (Ova/Cmu/Scram) groups compared to sham-infected, allergic (Ova/SPG/Scram) controls (Figure 4.5 D). Treatment with Ant-21 suppressed *Chlamydia*-induced pAkt in AAD in the absence, or presence, of steroid treatment (Ova/Cmu/Ant-21 and Ova/Cmu/Ant-21/DEX) compared to scrambled antagomir-treated (Ova/Cmu/Scram and Ova/Cmu/Scram/DEX) controls. In the absence of Ant-21 treatment, steroid treatment had no effect on nuclear pAkt levels.

We then assessed whether *Chlamydia*-induced miR-21 is required for the suppression of HDAC2. Infection, as expected, reduced the levels of nuclear HDAC2
protein in the lungs of scrambled antagomir-treated, *Chlamydia*-infected, allergic (Ova/Cmu/Scram) groups compared to sham-infected, allergic (Ova/SPG/Scram) controls (Figure 4.5 E). Importantly, treatment with Ant-21 restored the levels of HDAC2 during infection-induced AAD in the absence (Ova/Cmu/Ant-21), or presence (Ova/Cmu/Ant-21/DEX), of steroids compared to scrambled antagomir-treated (Ova/Cmu/Scram and Ova/Cmu/Scram/DEX) controls (Figure 4.5 E). In the absence of Ant-21, steroid treatment had no effect on HDAC2 levels.

Thus, the inhibition of *Chlamydia*-induced miR-21 in severe, steroid-insensitive AAD reverses infection-induced increases in PI3K signalling and decreases in HDAC2 levels.
Figure 4.5: *Chlamydia*-induced microRNA (miR)-21 increases PI3K signalling and decreases HDAC2 levels in severe, steroid-insensitive allergic airways disease (AAD).

Lung expression of miR-21 (A) was determined by qPCR on d35 of the study protocol (Figure 4.1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or anti-miR-21 (Ant-21) or scrambled (Scram) antagonir treatment (≥two experiments; n=4-5). The effect of Ant-21 treatment on the expression of Pten (B) and Hdac2 (C) was assessed by qPCR after the second Ova challenges in allergic groups not treated with DEX (≥two experiments; n=4-5).

Nuclear protein levels of Akt and pAkt (D; pAkt, Akt and pAkt:Akt ratio) and Hdac2 (E; active Hdac2 [60kDa], degraded Hdac2 [20kDa], active to degraded Hdac2 ratio [60kDa/20kDa]) normalised to TATA binding protein (TBP) were also determined by immunoblot (top panels; representative immunoblots) and quantification by densitometry (bottom panels) (≥two experiments; n=5). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
4.4.5 Inhibition of miR-21 restores steroid sensitivity in *Chlamydia*-induced, severe, steroid-insensitive AAD

To define the functional role of miR-21 in steroid insensitivity we next assessed the effects of inhibiting this miRNA on inflammation and AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD. In support of our initial observations (Figure 4.2), the administration of steroids reduced airway inflammation and AHR in AAD in scrambled antagonir-treated, sham-infected, allergic (Ova/SPG/Scram/DEX) groups compared to non-steroid treated (Ova/SPG/Scram) controls (Figure 4.6 A-G). Again, by contrast, steroids did not suppress inflammation and AHR in AAD in scrambled antagonir-treated, *Chlamydia*-infected, allergic (Ova/Cmu/Scram/DEX) groups compared to non-steroid treated (Ova/Cmu/Scram) controls. Importantly, treatment with Ant-21, in the presence of steroids, suppressed inflammation in *Chlamydia*-induced, severe, steroid-insensitive AAD (Ova/Cmu/Ant-21/DEX) compared to sham-treated (Ova/Cmu/Scram and Ova/Cmu/Scram/DEX) controls. This suppression did not occur in the absence of steroids (Ova/Cmu/Ant-21 vs Ova/Cmu/Ant-21/DEX). Ant-21 also suppressed AHR irrespective of whether steroids were present or not. Indeed, Ant-21-mediated suppression of AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD was potent and similar to that achieved in steroid-treated, sham-infected, allergic (Ova/SPG/Scram/DEX) groups.

Collectively, these findings demonstrate that the key features of *Chlamydia*-induced, severe, steroid-insensitive AAD are miR-21-dependent.
Figure 4.6: Inhibition of microRNA (miR)-21 reinstates steroid sensitivity in *Chlamydia*-induced, severe, steroid-insensitive allergic airways disease (AAD). Total leukocyte (A), eosinophil (B), neutrophil (C), macrophage (D), and lymphocyte numbers (E) were determined in BAL fluid on d35 of the study protocol (Figure 4.1) in *Chlamydia* (Cmu) and
sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or anti-miR-21 (Ant-21) or scrambled (Scram) antagonim treatment (≥two experiments; n=4-6). Airways hyper-responsiveness in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (F), and 10 mg/mL of Mch (G; shows statistics for maximal resistance from AHR curves [Figure 4.6 F]) was also determined in all allergic groups on d35 (≥three experiments; n=5-10). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
4.4.6 PI3K-dependent signalling regulates pAkt and HDAC2 levels in Chlamydia-induced, severe, steroid-insensitive AAD

Given that Chlamydia infection induced the nuclear accumulation of pAkt and reduced nuclear HDAC2 levels (Figure 4.4 D and E, 4.5 D and E), we next examined the effects of inhibiting PI3K-dependent signalling with the pan-PI3K inhibitor, LY294002 (LY29), during severe, steroid-insensitive AAD. Significantly, treatment with LY29 reversed the suppression of HDAC2 expression in infected mice with AAD (Ova/Cmu/LY29) compared to sham-treated (Ova/Cmu/DMSO) controls (Figure 4.7 A).

We then determined if the inhibition of PI3K signalling reversed infection-induced changes in the levels of nuclear pAkt and HDAC2. Infection, as expected, increased the levels of nuclear pAkt in the lungs of sham-treated, infected, allergic (Ova/Cmu/DMSO) mice compared to sham-infected, allergic (Ova/SPG/DMSO) controls (Figure 4.7 B). Importantly, treatment with LY29 suppressed Chlamydia-induced nuclear pAkt in the absence (Ova/Cmu/LY29), or presence (Ova/Cmu/LY29/DEX), of steroids compared to sham-treated (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls. In the absence of LY29, steroid treatment had no effect on pAkt levels.

We then assessed whether Chlamydia-induced PI3K signalling was required for the suppression of active HDAC2 (60kDa) levels in infection-induced, severe, steroid-insensitive AAD. Infection, as expected, decreased the levels of nuclear HDAC2 in the lungs of sham-treated, infected, allergic (Ova/Cmu/DMSO) groups compared to sham-infected (Ova/SPG/DMSO) controls (Figure 4.7 C). Treatment with LY29 restored HDAC2 levels in Chlamydia-induced, severe, steroid-insensitive AAD in the absence (Ova/Cmu/LY29), or presence (Ova/Cmu/LY29/DEX) of
steroids compared to sham-treated (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls. In the absence of LY29, steroid treatment had no effect on HDAC2 levels.

Thus, the inhibition of *Chlamydia*-induced PI3K signalling in severe, steroid-insensitive AAD reverses infection-induced increases in pAkt and decreases in HDAC2 levels.
Figure 4.7: *Chlamydia*-induced PI3K signalling suppresses HDAC2 levels in severe, steroid-insensitive allergic airways disease (AAD). Lung expression of Hdac2 (A) was determined by qPCR on d35 of the study protocol (Figure 4.1) in *Chlamydia* (Cmu) and sham (SPG)-infected mice with ovalbumin (Ova)-induced AAD that were treated with LY294002 (LY29) or vehicle (DMSO) (≥two experiments; n=4-5). Nuclear protein levels of Akt and pAkt (B; pAkt, Akt and pAkt:Akt ratio) and Hdac2 (C; active Hdac2 [60kDa], degraded Hdac2 [20kDa], active to degraded Hdac2 ratio [60kDa/20kDa]) normalised to TATA binding protein (TBP) were also determined on d35 in *Chlamydia* (Cmu) and sham (SPG)-infected groups following Ova-induced AAD, in the absence, and presence of steroid (DEX) and LY29 treatment by immunoblot (top panels; representative immunoblots) and quantification by densitometry (bottom panels) (≥two experiments; n=5). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
4.4.7 Inhibition of PI3K-dependent signalling reinstates steroid sensitivity in 
*Chlamydia*-induced, severe, steroid-insensitive AAD

To define the functional role of PI3K in steroid insensitivity, we next assessed the effects of LY29-mediated inhibition of this signalling pathway on inflammation and AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD. Again, the administration of steroids decreased airway inflammation and AHR during AAD in sham-treated, sham-infected, allergic (Ova/SPG/DMSO/DEX) groups compared to non-steroid-treated (Ova/SPG/DMSO) controls (Figure 4.8 A-G). Also again, steroids did not suppress inflammation or AHR during AAD in sham-treated, *Chlamydia*-infected, allergic (Ova/Cmu/DMSO/DEX) groups compared to non-steroid-treated (Ova/Cmu/DMSO) controls. Importantly, treatment with LY29, in the presence of steroids, suppressed inflammation in *Chlamydia*-infected, allergic (Ova/Cmu/LY29/DEX) groups compared to sham-treated (Ova/Cmu/DMSO/DEX) controls. This suppression did not occur in the absence of steroids where in fact inflammation was increased (Ova/Cmu/LY29 vs Ova/Cmu/DMSO). LY29, like miR-21 inhibition, also suppressed AHR irrespective of whether steroids were present or not, although the effects were greater in the presence of steroids. Indeed, the LY29-induced suppression of AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD was potent and similar to that achieved in steroid-treated, sham-infected, allergic (Ova/SPG/Scram/DEX) groups.

Collectively, these findings demonstrate that the key features of *Chlamydia*-induced, severe, steroid-insensitive AAD are also PI3K-dependent and that steroid-insensitive airway inflammation and AHR are potentially induced by a miR-21-dependent, PI3K-mediated signalling axis that increases pAkt and reduces HDAC2 levels.
Figure 4.8: Inhibition of *Chlamydia*-induced PI3K signalling reinstates steroid sensitivity in severe, steroid-insensitive allergic airways disease (AAD). Total leukocyte (A), eosinophil (B), neutrophil (C), macrophage (D), and lymphocyte numbers (E) were determined in BAL fluid on d35 of the study protocol (Figure 4.1) in *Chlamydia* (Cmu) and
sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or LY294002 (LY29) or vehicle (DMSO) treatment (≥two experiments; n=4-8). Airways hyper-responsiveness in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (F), and 10 mg/mL of Mch (G; shows statistics for maximal resistance from AHR curves [Figure 4.8 F]) was also determined in all allergic groups on d35 (≥two experiments; n=5-8). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
4.4.8 Inhibition of miR-21 suppresses hallmark features of Haemophilus-induced, severe, neutrophilic, steroid-insensitive AAD

We then assessed the applicability of our observations to another model of respiratory infection-induced, severe, steroid-insensitive asthma. Respiratory infection with *H. influenzae* is associated with steroid insensitivity and more severe disease in both experimental and clinical asthma (Wood et al., 2010; Essilfie et al., 2012). Thus, we developed a novel model of Haemophilus-induced, severe, neutrophilic, steroid-insensitive AAD. WT BALB/c mice were infected with NTHi (NTHi-289; Hinf; i.t.) after the establishment of Ova-induced AAD, but before the final Ova challenges (Figure 4.1). Hallmark features of disease were assessed on day 35 in the absence, or presence, of treatment with DEX (Figure 4.9 A-G). Like Chlamydia, Haemophilus infection suppressed eosinophilic, and increased neutrophilic, airway inflammation in AAD (Ova/Hinf/Scram) compared to sham-infected, allergic (Ova/PBS/Scram) controls (Figure 4.9 B and C). Furthermore, the hallmark features of Haemophilus-induced, severe AAD (inflammation and AHR) were steroid-insensitive (Figure 4.9 A-G). Similarly, treatment with Ant-21 suppressed inflammation in Haemophilus-induced, severe, steroid-insensitive AAD in combination with steroids (Ova/Hinf/Ant-21/DEX) compared to scrambled antagomir-treated (Ova/Hinf Scram/DEX and Ova/Hinf/Scram) controls. Interestingly, unlike with Chlamydia, treatment with Ant-21 in Haemophilus-induced, severe, steroid-insensitive AAD also suppressed inflammation in the absence of steroids (Ova/Hinf/Ant-21) compared to scrambled antagomir-treated (Ova/Hinf/Scram and Ova/Hinf/Scram/DEX) controls. Importantly, like with Chlamydia, treatment with Ant-21 in Haemophilus-induced, severe, steroid-insensitive AAD also suppressed AHR irrespective of whether steroids were present or not (Ova/Hinf/Ant-21 and Ova/Hinf/Ant-21/DEX) and was similar to
that achieved in steroid-treated, sham-infected, allergic (Ova/PBS/Scram/DEX) groups.

Collectively, these findings demonstrate that the key features of both Chlamydia- and Haemophilus-induced, severe, steroid-insensitive AAD are induced by a common miR-21-dependent mechanism.
Figure 4.9: Inhibition of microRNA (miR)-21 reinstates steroid sensitivity in *Haemophilus*-induced, severe, steroid-insensitive allergic airways disease (AAD). Total leukocyte (A), eosinophil (B), neutrophil (C), macrophage (D) and lymphocyte numbers (E)
were determined in BAL fluid on d35 of the study protocol (Figure 4.1) in *Haemophilus* (Hinf) and sham (PBS)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or anti-miR-21 (Ant-21) or scrambled (Scram) antagonir treatment (one experiment; n=5-6). Airways hyper-responsiveness in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (F), and 10 mg/mL of Mch (G; shows statistics for maximal resistance from AHR curves [Figure 4.9 F]) was also determined in all allergic groups on d35 (one experiment; n=5-6). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
4.5 Discussion

In this study, we established novel experimental models of severe, neutrophilic, steroid-insensitive asthma that are driven by *Chlamydia* and *Haemophilus* respiratory infections. Our models recapitulate the hallmark features of this form of human asthma including exaggerated T\textsubscript{H}1/T\textsubscript{H}17-associated responses and steroid-insensitive airway inflammation and AHR. We showed that increased miR-21 and PI3K signalling were associated with pathogenesis. We then used a combination of novel miR-21 antagonirs that specifically target and deplete this miRNA *in vivo* and the pan-PI3K inhibitor, LY29, to define a previously unrecognised effector pathway that induces infection-induced, severe, steroid-insensitive AAD. Collectively our data show that infection-induced miR-21 expression promotes PI3K-mediated phosphorylation and nuclear translocation of pAkt that suppresses HDAC2 and leads to steroid insensitivity (Figure 4.10).

We previously showed that *Chlamydia* respiratory infection during systemic sensitisation to Ova leads to exaggerated T\textsubscript{H}1 (IFN-γ)/T\textsubscript{H}17 (IL-17) responses and neutrophilic inflammation in Ova-induced AAD (Horvat et al., 2010a). In that model we inoculated with *Chlamydia* during Ova sensitisation. In severe, neutrophilic, steroid-insensitive asthma we propose that it is infection in patients with established asthma that drives the development of this form of the disease. Thus, in this study, we advance our earlier findings by developing a model that more accurately reflects the human scenario. We established AAD in mice and then induced a *Chlamydia* infection. We know that AAD wains over 20 days (unpublished observations) and so to test the impact of infection on established AAD it was necessary to recapitulate AAD with a second set of Ova challenges. This is representative of patients with established asthma that are exposed to respiratory infection and allergens and is
reflective of what occurs in the community. Expanding on our previous findings we now show that *Chlamydia* infection-induced, severe, neutrophilic AAD is characterised by exaggerated expression of a broad range of T\(_H\)1-associated (TLR2, STAT1, IFN-\(\gamma\), CXCL-9 and -10, TNF-\(\alpha\)) and T\(_H\)17-associated (IL-17, IL-6, TGF-\(\beta\), IL-1\(\beta\)) factors in the lung. Importantly, we now also show that both inflammation and AHR are steroid-insensitive in this model (*Figures 4.2, 4.6 and 4.8*). This indicates that infection may drive an abnormal array of pathogenic processes that are not adequately attenuated by treatment with anti-inflammatory steroids.

Our findings expand our understanding of clinical studies that associate *Chlamydia*, and other, respiratory infections with non-eosinophilic forms of asthma that are refractory to steroid treatment. Neutrophil numbers are increased in the lungs of exacerbating asthmatics with evidence of *Chlamydia* respiratory infection (Wark et al., 2002) and asthmatic patients with evidence of this infection are resistant to steroid treatment (Cho et al., 2005; Patel et al., 2010). In a cohort of asthmatics with severe refractory asthma, airway neutrophil numbers predict the presence of *Chlamydia* lung infection (Patel et al., 2010).

Multiple mechanisms are implicated in the pathogenesis of steroid-insensitive asthma, including altered immune responses and increased activity of transcription factors, as well as defective GR activation, nuclear translocation and association with pro/anti-inflammatory responsive elements (Adcock and Ito, 2004; Ito et al., 2006c; Wang et al., 2010b). Targeting pivotal molecules that control multi-functional pathways may be needed to overcome the multiple factors involved in steroid insensitivity and may be the most effective therapeutic strategy for treating this form of asthma.
**Figure 4.10: Mechanisms and potential treatment of severe, steroid-insensitive asthma.**  

Infection in allergic airways disease/asthma induces miR-21 that inhibits Pten and promotes PI3K-induced inhibition of Hdac2 and steroid insensitivity. This pathway may be targeted therapeutically by inhibition of miR-21 and/or PI3K with Ant-21 and LY294002, respectively.
miRNAs have emerged as master regulators of gene expression that have potent influences on immunity. However, only recently have their functional contributions to inflammatory diseases of the lung, such as asthma been defined (Mattes et al., 2009; Xiao and Rajewsky, 2009; Collison et al., 2011; Foster et al., 2013; Plank et al., 2013). MiR-21 has recently been shown to play an important role in the pathogenesis of AAD. This miRNA is highly induced in inflamed lungs (Lu et al., 2009) and several studies have shown its role in promoting eosinophilic inflammation and Th2 responses, whilst suppressing Th1 immunity through the disruption of IL-12p35 (Lu et al., 2009; Lu et al., 2011; Sawant et al., 2013). Consequently, the focus of miR-21 studies in asthma have been in investigating its role in immune polarisation during the inception of allergic sensitisation (Lu and Rothenberg, 2013). Here we uncover a novel role for miR-21 in steroid insensitivity using our novel models of infection-induced, severe, steroid-insensitive AAD.

We show that miR-21 expression was increased in both infected and sham-infected mice with AAD compared to sham-infected, non-allergic controls, however, sham-infected mice only experienced an increase in miR-21 expression following the repeat administration of Ova (Figures 4.3 and 4.5 A). This suggests that the up-regulation of miR-21 expression in sham-infected, allergic mice is a transient phenomenon acutely induced by the allergic inflammatory response. Importantly, we show that infection resulted in an increase in miR-21 expression on day 32 of our model of AAD (Figure 4.3 B) which was 18 days after infection when Chlamydia-induced inflammation had subsided to baseline levels prior to Ova re-challenge. Day 32 also represents the time point prior to recapitulation of disease in our model when steroid administration commenced. Thus, the infection-induced miR-21-influenced
environment within the lung at this time point is likely crucial in driving the severe phenotype in response to airway allergen exposure and steroid sensitivity.

Significantly, the increase in miR-21 expression in infected mice at day 32 was accompanied by concomitant reductions in GR-α, PTEN and HDAC2 expression (Figure 4.4 A-C). MiR-21 directly inhibits PTEN, which in turn antagonises PI3K activity (Meng et al., 2007; Yamanaka et al., 2009; Miletic et al., 2010). Thus, this miRNA may potentiate PI3K-dependent signalling (Figure 4.10). Several studies strongly associate PI3K-dependent signalling with inflammation and AHR in AAD and one showed that exposure to rhinovirus induced PI3K-dependent neutrophilic airway inflammation (Newcomb et al., 2008). Here we show that Chlamydia-infected mice have increased levels of nuclear pAkt (Figure 4.4 D) a well-established indicator of PI3K-dependent signalling (Lee et al., 2006; Newcomb et al., 2008).

These Chlamydia-induced effects on miR-21, PTEN and pAkt were associated with reductions in the nuclear levels of active HDAC2 (60kDa) (Figure 4.4 E). This is supported by other studies that show a PI3K-mediated reduction in HDAC2 activity as a mechanism of steroid insensitivity in COPD (Barnes et al., 2004; Ito et al., 2006a; Barnes and Adcock, 2009; Marwick et al., 2009; To et al., 2010). These studies showed that the non-selective inhibition of PI3K activity with LY29 restores HDAC2 activity and steroid sensitivity in a cigarette smoke-induced model of COPD, and that smoke-exposed PI3Kδ kinase dead knock-in transgenic mice had no deficit in steroid sensitivity, which correlated with reduced tyrosine nitrination of HDAC2 (Marwick et al., 2009; To et al., 2010). Nitration of HDAC2 reduces its catalytic activity and leads to its ubiquitination and proteasomal degradation (Barnes, 2009; Osoata et al., 2009). Together, these findings suggest that Chlamydia-induced miR-21 expression may disrupt PTEN activity and amplify PI3K-dependent signalling. This leads to
inappropriate increases in PI3K-mediated phosphorylation and nuclear translocation of pAkt, resulting in reduced HDAC2 levels and steroid insensitivity. To our knowledge our study is the first to identify the axis encompassing the miR-21-dependent, PI3K-mediated suppression of HDAC2 in the pathogenesis of steroid insensitivity. This identifies miR-21 as a modifiable therapeutic target that can be effectively attenuated in vivo with specific agents (i.e. antagomirs) to reverse steroid insensitivity.

We used two approaches to verify the functional contribution of the *Chlamydia*-induced, miR-21-dependent, PI3K-mediated signalling axis in severe, steroid-insensitive AAD and identify their potential for therapeutic targeting. First, we directly inhibited miR-21 expression in vivo with antagomir treatment (Figure 4.1), as previously described (Krutzfeldt et al., 2005; Mattes et al., 2009; Collison et al., 2011). Treatment suppressed *Chlamydia*-induced miR-21 expression (Figure 4.5 A) back to levels in sham-infected groups, confirming effective inhibition in the lungs. Inhibition restored the expression of PTEN and HDAC2 in the lungs (Figure 4.5 B and C), and also reduced nuclear pAkt (Figure 4.5 D) and increased active HDAC2 (60kDa) (Figure 4.5 E) levels in severe, steroid-insensitive AAD. Most importantly, treatment with Ant-21 reversed steroid insensitivity (Figure 4.6 A-G). Interestingly, treatment with Ant-21 only suppressed steroid-insensitive airway inflammation when co-administered with steroids, whereas AHR was suppressed by antagomir treatment alone. This suggests that miR-21 may have additional functions that are independent of its regulation of PI3K-mediated suppression of HDAC2. Steroid-insensitive airway inflammation and AHR may have different aetiologies but require the overexpression of miR-21 to maintain steroid insensitivity. It is widely accepted that pulmonary
inflammation and AHR may be linked but may also be independent (Crimi et al., 1998).

To verify a functional contribution of PI3K signalling to the pathogenesis of severe, steroid-insensitive AAD and to substantiate the existence of a pathogenic, infection-driven miR-21/PI3K signalling axis, we next inhibited its activity and assessed the impact on the phenotype. PI3K signalling is strongly implicated in steroid insensitivity and can be activated by factors associated with severe, steroid-insensitive asthma, such as IL-6 and IL-1β (Hideshima et al., 2001; Neumann et al., 2002). PI3K-dependent signalling also promotes increased activity of the pro-inflammatory transcription factors NF-κB and AP-1 (Reddy et al., 1997). We inhibited PI3K signalling in vivo with the pan-PI3K inhibitor LY29 (Figure 4.1), as used previously (Newcomb et al., 2008; To et al., 2010). The therapeutic administration of LY29 had similar effects to those observed with Ant-21 treatment. Treatment with LY29 reduced nuclear pAkt back to levels observed in sham-infected groups (Figure 4.7 B) in a similar manner to treatment with Ant-21 (Figure 4.5 D). Interestingly, treatment with steroids alone did not affect nuclear pAkt levels, indicating that PI3K-dependent signalling is steroid-insensitive. LY29 treatment also restored lung HDAC2 expression (Figure 4.7 A) and HDAC2 protein levels (Figure 4.7 C). Most importantly, treatment restored steroid sensitivity to airway inflammation (Figure 4.8 A-E) and AHR (Figure 4.8 F and G) in a similar manner to Ant-21 treatment. The similar effects of treatment with Ant-21 and LY29 strongly suggest that Chlamydia initiates, and persistently maintains the activation of a pathogenic signalling axis comprised of both miR-21 and PI3K and that this axis suppresses HDAC2 that leads to the induction of severe, steroid-insensitive AAD.
To demonstrate a widespread role for miR-21 in the induction of severe, steroid-insensitive asthma, and that it is not specific to *Chlamydia*-associated disease, we investigated the effects of inhibition of miR-21 in *Haemophilus*-induced, severe, neutrophilic, steroid-insensitive AAD. *H. influenzae* is one of the most commonly isolated bacteria from the lungs of non-eosinophilic asthmatics. Indeed 60% of stable asthmatics with high levels of bacteria in their airways were culture-positive for *H. influenzae* and these individuals were more likely to have non-eosinophilic asthma and be taking high doses of inhaled steroids (Wood et al., 2010). Significantly, both *Chlamydia* and *Haemophilus* respiratory infections induce $T_{H1}$ and/or $T_{H17}$ responses (Zhou et al., 2009; Horvat et al., 2010a; Essilfie et al., 2011; Essilfie et al., 2012) and require PI3K for entry into host cells (Coombes and Mahony, 2002; Marti-Lliteras et al., 2009). Thus, we hypothesised that *Haemophilus*, like *Chlamydia*, may induce severe, steroid-insensitive AAD through a miR-21-dependent mechanism. We previously showed that *H. influenzae* infection induces $T_{H17}$-dominant immunity that drives neutrophilic, as opposed to eosinophilic, inflammatory responses during Ova-induced AAD (Essilfie et al., 2011) and that inflammation and AHR in this model are steroid-insensitive (Essilfie et al., 2012). We first developed a novel and refined model of *Haemophilus*-induced, severe, neutrophilic, steroid-insensitive AAD as we did with *Chlamydia* (Figure 4.1). We then showed that treatment with Ant-21 restored steroid sensitivity in *Haemophilus*-induced disease (Figure 4.9). Thus, targeting miR-21 in the lung may have broad therapeutic relevance to respiratory infection-induced, severe, steroid-insensitive asthma and may be a new and effective treatment for this disease. Furthermore, elevated miR-21 occurs in other steroid-insensitive diseases including IBD and arthritis (Takagi et al., 2010; Iliopoulos et al.,
In summary, our study demonstrates for the first time that miR-21 is pivotal in promoting steroid-insensitive inflammation and AHR in respiratory infection-induced, severe, steroid-insensitive AAD. Significantly, we have defined the functional relevance of infection-induced activation, and maintenance, of a novel miR-21/PI3K/pAkt/HDAC2 signalling axis in steroid insensitivity. Our data suggest that the therapeutic inhibition of miR-21 expression may reinstate steroid sensitivity in severe, steroid-insensitive asthma. This is more attractive than targeting PI3K pathways as inhibition of miR-21 is more specific and may broadly affect steroid-insensitive networks. Targeting miR-21 may also be applicable for the treatment of other steroid-insensitive inflammatory conditions.
Chapter 5:

NLRP3 inflammasome activation by bacterial respiratory infections promotes steroid insensitivity in experimental asthma

In this chapter, I show that *Chlamydia* respiratory infection in established AAD increases the levels of IL-1β through an NLRP3 inflammasome-dependent, Caspase-1-mediated mechanism to promote severe, steroid-insensitive AAD. I show that these responses are also important in *Haemophilus*-induced, severe, steroid-insensitive AAD. This suggests that NLRP3 inflammasome activation is pivotal in inducing steroid-insensitive inflammation and AHR in severe AAD and the therapeutic targeting of the NLRP3 inflammasome/Caspase-1/IL-1β axis may be an effective therapeutic strategy for steroid-insensitive asthmatics with evidence of increased inflammasome/IL-1β responses.

This chapter is currently in preparation for submission as an original research article.
5.1 Abstract

Severe, corticosteroid-insensitive asthma is often uncontrolled and urgently requires effective therapies, however, their development depends upon improving our understanding of the mechanisms that underpin the pathogenesis of steroid-insensitive disease. Clinical evidence implicates NLRP3 inflammasome activation and increased levels of IL-1β in the pathogenesis of severe, steroid-insensitive asthma. Importantly, Chlamydia respiratory infection can induce these factors and is linked with severe, steroid-insensitive asthma. In this study, we show that Chlamydia respiratory infection induces an NLRP3 inflammasome/Caspase-1/IL-1β signalling axis to promote steroid-insensitive airway inflammation and AHR and the therapeutic inhibition of each axis component restores steroid sensitivity in experimental asthma. Furthermore, we show that therapeutic inhibition of each axis component also restores steroid sensitivity in Haemophilus-induced, severe, steroid-insensitive AAD. Thus, our results implicate NLRP3 inflammasome activation as a key determinant in the pathogenesis of infection-induced, severe, steroid-insensitive asthma.
5.2 Introduction

Asthma is a chronic inflammatory disease of the airways typically mediated by aberrant \( T_{H2} \) lymphocyte responses that drive eosinophilic airway inflammation, mucous hypersecretion and AHR (Bochner et al., 1994; Wills-Karp, 1999; Umetsu et al., 2002; Asher et al., 2006; Eder et al., 2006; Kim et al., 2010; Thorburn and Hansbro, 2010). The majority of asthmatics respond well to anti-inflammatory corticosteroid-based therapies that control their symptoms. However, 5-10% of asthmatics, typically those with more severe disease, have poor sensitivity to steroid treatment even at high doses (Barnes and Woolcock, 1998; Chung et al., 1999; Rhen and Cidlowski, 2005; Ito et al., 2006c; Barnes and Adcock, 2009; Barnes, 2010). Recent studies have shown that asthma is a heterogeneous disease with moderate to severe forms of asthma associated with increased \( T_{H1} \)- and/or \( T_{H17} \)-responses and monocytic or neutrophilic, rather than eosinophilic, airway inflammation (Ordonez et al., 2000; Gibson et al., 2001; Liu et al., 2004b; Kumar et al., 2006; Truyen et al., 2006; Bullens, 2007). There are currently no effective therapies for severe, steroid-insensitive asthma and the development of effective therapies is hampered by the lack of understanding of the mechanisms and factors involved.

Increasing clinical and experimental evidence strongly implicates inflammasome activation and the excess production of IL-1\( \beta \) in the pathogenesis of severe, steroid-insensitive asthma (Konno et al., 1996; Wanderer, 2009; Hastie et al., 2010; Baines et al., 2011; Besnard et al., 2012; Kim et al., 2014; Simpson et al., 2014). Inflammasomes are multi-protein signalling complexes that induce the maturation of the inflammatory cytokines IL-1\( \beta \) and IL-18 (Martinon et al., 2002; Schroder and Tschopp, 2010). At present, five different inflammasomes have been identified, including NLRP1, NLRP3, NLRC4, AIM2 and retinoic acid-inducible
gene 1 (RIG-I). The NLRP3 inflammasome is the best characterised and widely implicated in inflammatory diseases and is comprised of NLRP3, ASC and pro-Caspase-1 domains (Schroder and Tschopp, 2010). Importantly, inflammasomes require two separate events to exert their effector functions, the first of which involves the expression and assembly of inflammasome components. This process is induced by PAMPs, such as LPS and dsDNA, that trigger TLR and NLR signalling (Schroder and Tschopp, 2010). DAMPs such as ATP and, reportedly, monosodium urate crystals, provide the second signal and activate the assembled inflammasome by stimulating the P2X7R (Stutz et al., 2009; Schroder and Tschopp, 2010). The NLRP3 inflammasome potently induces inflammation through the cleavage, and activation, of pro-Caspase-1 into Caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 causing the release of active IL-1β and IL-18 (Schroder and Tschopp, 2010).

Recent studies have shown that IL-1β-related genes (IL-1β, IL-1Rs, IRAKs) are overexpressed in severe, steroid-insensitive asthma and increased IL-1β signalling predicted more severe disease (Baines et al., 2011). Significantly, ATP and P2X7R levels are increased in asthma and inhibition of ATP:P2X7R-signalling suppresses disease in experimental asthma (Idzko et al., 2007; Muller et al., 2011). Most significantly, NLRP3 and Caspase-1 are also elevated in these patients (Simpson et al., 2014). IL-1β promotes T\textsubscript{H}17 differentiation and the production of IL-17 that induces steroid-insensitive neutrophilic inflammation and AHR (McKinley et al., 2008; Chung et al., 2009; Korn et al., 2009; Hastie et al., 2010; Besnard et al., 2012; Kim et al., 2014) and treatment with IL-1R antagonist during experimental asthma reduced inflammation and AHR (Wang et al., 2006).
Collectively, these studies strongly implicate the activation of the NLRP3 inflammasome and elevated IL-1β levels in the pathogenesis of severe, steroid-insensitive asthma.

Substantial clinical evidence links *Chlamydia* respiratory infections with severe, steroid-insensitive asthma. *Chlamydia*-associated asthma is less responsive to steroids, asthmatics with acute antibody responses to *Chlamydia* have increased sputum neutrophil numbers (Wark et al., 2002; Cho et al., 2005; Patel et al., 2010) and airway neutrophilia predicted the presence of *Chlamydia* in severe, steroid-insensitive asthma (Patel et al., 2010). Furthermore, *Haemophilus* is commonly isolated from the airways of severe, steroid-insensitive asthmatics compared to patients with less severe disease (Simpson et al., 2007; Wood et al., 2010) and we, and others, have shown that both *Chlamydia* and *Haemophilus* induce neutrophilic, T\(_{H}1\) and/or T\(_{H}17\) responses in experimental asthma (Zhou et al., 2009; Horvat et al., 2010a; Essilfie et al., 2011; Essilfie et al., 2012). Significantly, both *Chlamydia* and *Haemophilus* infections induce the release of active IL-1β through an NLRP3 inflammasome-dependent, Caspase-1-mediated mechanism (He et al., 2010; Rotta Detto Loria et al., 2013). Thus, we propose that infection-induced, NLRP3 inflammasome-mediated IL-1β responses may be important in the pathogenesis of severe, steroid-insensitive asthma.

In this study, we used our murine models of *Chlamydia* and *Haemophilus* infection-induced AAD (described in Chapter 4) to demonstrate an important role for NLRP3 inflammasome-mediated IL-1β responses in promoting severe, steroid-insensitive asthma. We show that the inhibition of increased NLRP3 inflammasome, Caspase-1 and IL-1β responses in infection-induced, severe, steroid-insensitive AAD were able to suppress inflammation and AHR where steroids were ineffective. This
study defines the NLRP3 inflammasome/Caspase-1/IL-1β axis in a previously unrecognised pathogenic role and highlights the NLRP3 inflammasome as a novel therapeutic target for severe, steroid-insensitive asthma.
5.3 Methods

5.3.1 Ethics statement

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

5.3.2 Murine model of established AAD, *C. muridarum* and *H. influenzae* respiratory infections, and treatment with dexamethasone

Female WT BALB/c mice, 6-8 weeks old, were sensitised to Ova (50µg, i.p. injection, Sigma-Aldrich, Sydney, Australia), in Rehydragel® (1mg, Reheis, Berkeley Heights, New Jersey, USA) in sterile saline (200µL). They were subsequently challenged i.n. with Ova (10µg/50µL sterile saline) under isoflurane anaesthesia on days 12-13 to induce AAD and again on days 33-34 to recapitulate AAD. Sham-sensitised controls received saline sensitisation with Rehydragel® by i.p. injection and subsequent Ova challenges on days 12-13 and 33-34 (model previously described in Chapter 4). Features of AAD were characterised on days 30 (tissues collected for RNA and protein analyses) or 35 (lung function assessment and tissues collected for RNA and protein analyses) of the study protocol (Figure 5.1).

Mice with established AAD were inoculated under isoflurane anaesthesia on day 14 i.n. with the natural mouse pathogen *C. muridarum* (100 IFU, ATCC VR-123, in 30µL SPG) or i.t. with NTHi (2x10^6 colony forming units, NTHi-289, in 30µL PBS). Controls were sham-infected with the equivalent volume of SPG or PBS, respectively (Figure 5.1).
Some mice were treated with DEX (Sigma-Aldrich, Sydney, Australia; 2 mg/kg) administered i.n. on days 32-34 (Figure 5.1). This dosing regime was optimised prior to conducting the work outlined in this Thesis.

5.3.3 In vivo neutralisation of IL-1β and administration of caspase and NLRP3 inhibitors during infection-induced, severe, steroid-insensitive AAD

Groups were treated i.n. with anti(α)-IL-1β neutralising antibody (R&D Systems, Minneapolis, USA; 10µg in 50µL PBS) on days 30, 32 and 34 in the absence, or presence, of DEX. Controls were treated with isotype control antibodies (BioXCell, West Lebanon, USA; 50µg in 50µL PBS) (Figure 5.1 A). Other groups were treated i.n. with the pan-caspase inhibitor z-VAD-fmk (Invivogen, San Diego, USA; 1mg/kg in 3% DMSO vehicle in 50µL PBS) on days 32-34 in the absence, or presence, of DEX. Other groups were treated i.n. with the Caspase-1-specific inhibitor Ac-YVAD-cho (Enzo Life Sciences, NY, USA; 1mg/kg in 3% DMSO vehicle in 50µL PBS) on days 32-34 in the absence, or presence, of DEX. Additional groups were treated i.n. with a novel NLRP3 inhibitor MCC950 (compound synthesised by collaborator Prof. Matt Cooper’s research team, The University of Queensland, Australia; 1mg/kg or 10mg/kg in 3% DMSO vehicle in 50µL PBS) on days 32-34 in the absence, or presence, of DEX. Controls were treated with DMSO vehicle (Figure 5.1 B). The effects of treatment with α-IL-1β, z-VAD-fmk, Ac-YVAD-cho and MCC950 were assessed on day 35. These dosing regimes were optimised prior to conducting the work outlined in this Thesis.
Figure 5.1: Experimental protocol. Investigation and treatment of *Chlamydia* and *Haemophilus*-induced, severe, neutrophilic, steroid-insensitive allergic airways disease (AAD). Wild-type (WT) BALB/c mice were intraperitoneally (i.p.) sensitised to ovalbumin (Ova) (d0) and AAD was induced by intranasal (i.n.) Ova challenge (d12 and 13) followed by re-challenge (d33 and 34). Non-allergic controls were sham-sensitised with saline (Sal). Some groups were inoculated i.n. with 100 inclusion-forming units (IFU) of *C. muridarum* (Cmu; d14), or intratracheally (i.t.) with $2 \times 10^6$ colony-forming units (CFU) of *H. influenzae* (Hinf; d14). Controls were sham-infected with sucrose phosphate glutamate (SPG) or phosphate buffered saline (PBS), respectively. Steroid responses were assessed by i.n. treatment with dexamethasone (DEX; d32-34). (A) In some groups, anti($\alpha$)-IL-1$\beta$ neutralising antibody (d30, 32, 34) was administered i.n. to neutralise IL-1$\beta$. Controls received isotype control antibody.
In other groups, the pan-caspase inhibitor, z-VAD-fmk, the Caspase-1-specific inhibitor, Ac-YVAD-cho, or a novel NLRP3 inhibitor, MCC950, were administered i.n. to suppress caspases, Caspase-1 and the NLRP3 inflammasome, respectively. Controls received DMSO vehicle.

5.3.4 Airway inflammation

Airway inflammation was assessed in cytospin preparations of cells from BALF collected on day 35 of the study protocol (Figure 5.1) (Horvat et al., 2007; Horvat et al., 2010a). BALF was collected by two 1mL washes with Hank’s Balanced Salt Solution (Life Technologies, Australia) via a cannula inserted into the trachea, centrifuged (300xg, 10 min, 4°C), treated with red blood cell lysis buffer (200µL, Tris-buffered NH₄Cl) and pelleted before total leukocyte numbers were determined using a haemocytometer. Cells were cytocentrifuged and stained with May-Grunwald-Giemsa. Differential leukocyte counts were determined using morphological criteria (≈175 cells by light microscopy [x40 magnification]) (Horvat et al., 2007; Horvat et al., 2010a). All samples were coded and counts were performed in a blinded fashion.

5.3.5 Lung function

Mice were anaesthetised with ketamine (100mg/kg) and xylazine (10mg/kg, Troy Laboratories, Smithfield, Australia) and their tracheas were cannulated (tracheostomy with ligation) (Horvat et al., 2007; Horvat et al., 2010a; Horvat et al., 2010b; Starkey et al., 2012; Starkey et al., 2013a; Starkey et al., 2013b). FlexiVent apparatus (FX1 System; SCIREQ, Montreal, Canada) was used to assess airway-specific resistance (Rn; tidal volume of 8 mL/kg at a respiratory rate of 450
breaths/min) (Beckett et al., 2013) in response to increasing doses of nebulised methacholine (Sigma-Aldrich, Sydney, Australia). This combination of anaesthesia and ventilation is common and recommended by the manufacturer (Horvat et al., 2010a; Li et al., 2010; Beckett et al., 2013). Assessments were performed at least three times per dose of saline/methacholine and the average calculated.

5.3.6 Quantification of mRNA expression by real-time qPCR

Total RNA was isolated from homogenised lungs with TRIzol® Reagent (Invitrogen, Life Technologies, Australia). Random-primed reverse transcriptions were performed for mRNA real-time qPCRs. Gene expression was normalised to the transcript of the housekeeping gene HPRT (Horvat et al., 2010b). For primer sequences refer to Table 5.1. All reactions were performed using BioScript™ reverse transcriptase in one times first-strand buffer according to manufacturer’s instructions (Bioline Pty. Ltd., NSW, Australia). Real-time qPCR assays were performed with SYBR Green Supermix (KAPA Biosystems, Inc., MA, USA) and a Mastercycler® ep realplex2 system (Eppendorf South Pacific Pty. Ltd., NSW, Australia).
5.3.7 Immunoblot analysis

Cytoplasmic protein fractions were isolated from lung tissues using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo, Scientific) with added Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo, Scientific). Sample protein and Precision Plus Protein™ WesternC™ Standards (Bio-Rad, CA, USA) were resolved on 4-15% gradient Mini-PROTEAN® TGX Stain-Free™ polyacrylamide gels (Bio-Rad) and transferred onto PVDF membranes. The blots were then blocked with 5% BSA in TBS-T and incubated with primary antibodies overnight before adding the relevant secondary antibody with Precision Protein™ StrepTactin-HRP Conjugate (Bio-Rad). Primary antibodies employed were; anti-Caspase-1 (p10) (Santa Cruz Biotechnology, CA, USA) and anti-β-actin (Abcam, MA, USA) antibodies and were used according to manufacturer’s instructions. The secondary antibody used was anti-Rabbit IgG HRP (R&D Systems, MN, USA). SuperSignal® West Femto Maximum Sensitivity
Substrate (Thermo, Scientific) was used to develop and visualise membranes by chemiluminescence (Bio-Rad, ChemiDoc MP System).

5.3.8 Enzyme linked immunosorbent assay (ELISA) for IL-1β

Lung IL-1β protein levels were measured by ELISA. Briefly, 96-well flat-bottomed high binding ELISA plates (Corning Inc., Corning, USA) were coated with rat anti-mouse IL-1β (R&D Systems, MN, USA; 20µL, 2µg/mL at 4°C overnight). Coated plates were washed (3x 0.05% Tween 20 in PBS [PBS-T]), blocked (3% BSA [Sigma-Aldrich, Sydney, Australia] in PBS at room temperature [RT] for 90 minutes) and washed again (3x PBS-T). Samples (100µL of cleared lung homogenates) and standards (recombinant IL-1β [R&D Systems, MN, USA]; 100µL in 1% BSA in PBS; 100ng/mL - 0.006ng/mL range) were then added in duplicate and plates incubated (RT for 90 minutes). Plates were then washed (5x PBS-T), coated with biotinylated goat anti-mouse IL-1β (R&D Systems, MN, USA; 20µL, 1µg/mL at RT for 60 minutes), washed (5x PBS-T), and streptavidin-HRP conjugate added (Invitrogen, Carlsbad, USA; 20µL, 0.2µg/mL at RT for 60 minutes). Plates were then washed (3x PBS-T and 2x PBS). Colourimetric reactions were developed by incubation with tetramethylbenzidine (100µL, 1mg/mL at RT for approximately 5 minutes) and reactions were stopped with sulphuric acid (50µL; 1M). A microplate spectrophotometer (Fluostar Optima, BMG Labtech) was used to read optical absorbances and concentrations of IL-1β in samples calculated by interpolation with the standard curve.
5.3.9 Statistics

Comparisons between two groups were made using unpaired *t*-Tests or a non-parametric equivalent where appropriate. Comparisons between multiple groups were made using a One-way ANOVA and an appropriate Post Test or a non-parametric equivalent where appropriate. Lung function data were assessed using a Two-way ANOVA and an appropriate Post Test or a non-parametric equivalent. Analyses were performed using GraphPad Prism Software (San Diego, California). All data shown are representative of individual mice. No data has been pooled.
5.4 Results

5.4.1 *Chlamydia* respiratory infection induces severe, steroid-insensitive AAD that is associated with increased IL-1β expression and production of Caspase-1 in the lungs

Acute Ova-induced AAD was established in WT BALB/c mice that were then infected i.n. with the natural mouse respiratory pathogen *C. muridarum*. Since the phenotype of AAD in the acute Ova model wains over time (data not shown - part of a separate manuscript not presented in this Thesis) we recapitulated the phenotype with two additional Ova challenges 19 days after infection. This is representative of the human scenario of allergen-induced increases in asthma severity and allows us to assess the impact of a resolved respiratory infection on established AAD. Key disease features were assessed on day 35 in the absence, or presence, of treatment with the steroid, DEX (*Figure 5.1*).

In the absence of infection, airway inflammation, which was predominantly eosinophilic, was increased in Ova-induced AAD (Ova/SPG) compared to non-allergic (Sal/SPG) controls (*Figure 5.2 A-C*). *Chlamydia* infection suppressed eosinophilic, but increased neutrophilic, airway inflammation in AAD (Ova/Cmu) compared to sham-infected, allergic (Ova/SPG) controls, which replicates our previous observations (*Chapter 4, Figure 4.2*). AHR increased in Ova-induced AAD, the magnitude of which was not altered by *Chlamydia* infection (*Figure 5.2 D and E*). Infection of sham-sensitised, non-allergic mice (Sal/Cmu) did not increase airway inflammation or AHR on day 35 compared to sham-infected, non-allergic (Sal/SPG) controls (*Figure 5.2 A-E*). This suggests that *Chlamydia*-induced neutrophilic AAD is an altered phenotype of AAD rather than the additive effects of infection and AAD. Treatment of sham-infected, allergic mice with DEX (Ova/SPG/DEX) suppressed
airway inflammation and AHR compared to sham-infected, sham-treated, allergic (Ova/SPG) controls (Figure 5.2 A-E). In contrast, airway inflammation and AHR were completely steroid-insensitive in *Chlamydia*-infected, allergic mice that were treated with DEX (Ova/Cmu/DEX). These data demonstrate that *Chlamydia* respiratory infection induces neutrophilic AAD that is insensitive to steroid treatment.

To investigate the role of IL-1β in the pathogenesis of severe, steroid-insensitive asthma, we initially sought to assess IL-1β and Caspase-1 expression in whole lungs in our model of infection-induced, severe, steroid-insensitive AAD. We previously showed that *Chlamydia* infection of allergic mice induces a robust increase in the expression of TLR2 and IL-1β on day 35 of the study protocol (Figures 4.1, 4.2 D). In this study, we show that *Chlamydia* infection of allergic mice (Ova/Cmu) increases the expression of SAA3 on day 35, and of IL-1β and Caspase-1 on day 30, compared to sham-infected, allergic (Ova/SPG) controls (Figure 5.2 F-H). *Chlamydia* infection had no effect on Caspase-1 expression on day 35 (Figure 5.2 I). Thus, *Chlamydia* infection results in a switch from eosinophilic to neutrophilic inflammatory responses that are associated with increased TLR2, IL-1β, SAA3 and Caspase-1 expression in Ova-induced AAD.

Using Caspase-1 (10kDa) as a marker of the proteolytic cleavage and activation of pro-Caspase-1 (45kDa) precursor, we next examined whether *Chlamydia*-induced, severe, steroid-insensitive AAD was associated with increased levels of Caspase-1 in the lungs. Immunoblot analyses of cytoplasmic protein fractions showed that *Chlamydia* infection in AAD (Ova/Cmu) significantly decreased pro-Caspase-1 (45kDa) and increased Caspase-1 (10kDa) levels on day 35 (Figure 5.2 J) compared to sham-infected, allergic (Ova/SPG) controls. Taken together, we show that *Chlamydia* infection increases the expression of factors that
are associated with NLRP3 inflammasome-mediated responses in AAD. Importantly, all of these factors have been implicated in the pathogenesis of severe, steroid-insensitive asthma in humans.
Figure 5.2: *Chlamydia* infection induces severe, steroid-insensitive, neutrophilic allergic airways disease (AAD) that is associated with increased Caspase-1 and IL-1β responses. Total leukocyte (A), eosinophil (B) and neutrophil numbers (C) were enumerated in BAL fluid on d35 of the study protocol (Figure 5.1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) treatment compared to non-allergic controls (Sal). Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (D), and 10mg/mL of Mch (E; shows statistics for maximal resistance from AHR curves [Figure 5.2 D]) was also determined on d35 (≥two experiments; n=5-8). Lung mRNA expression of serum amyloid A (SAA)3, IL-1β and Caspase-1 (F-I) was determined on d30 and d35 of the study protocol (Figure 5.1) in Cmu and sham-infected, allergic groups. Cytoplasmic protein levels of Caspase-1 and pro-Caspase-1 (J; Caspase-1 (45kDa), pro-Caspase-1 (10kDa) and Caspase-1/Pro-Caspase-1 ratio) normalised to β-actin were also determined on d35 in Cmu and sham-infected groups following Ova-induced AAD by immunoblot (top panels) and quantification by densitometry (bottom panels) (one experiment; n=6). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
5.4.2 Inhibition of *Chlamydia*-induced IL-1β suppresses cardinal features of *Chlamydia*-induced, severe, steroid-insensitive AAD

We next sought to determine the role of IL-1β in *Chlamydia*-induced, severe, steroid-insensitive AAD. We observed a significant increase in the level of IL-1β in the lungs of *Chlamydia*-infected mice with AAD (Ova/Cmu/Iso), compared to sham-infected, allergic (Ova/SPG/Iso) controls (*Figure 5.3 A*). Steroid treatment did not suppress elevated IL-1β levels in infected, allergic mice (Ova/Cmu/Iso/DEX), suggesting that IL-1β expression is not steroid-sensitive.

In order to assess the potential of targeting IL-1β therapeutically, *Chlamydia*-infected, allergic mice were treated with neutralising anti-IL-1β antibody (α-IL-1β) on days 30, 32 and 34 of the study protocol (*Figure 5.1 A*), and the effect on infection-induced IL-1β, airway inflammation and AHR were assessed. The administration of α-IL-1β significantly reduced lung IL-1β levels on day 35 in infected, allergic groups in the absence, or presence, of steroids (Ova/Cmu/α-IL-1β and Ova/Cmu/α-IL-1β/DEX, respectively) compared to isotype-treated (Ova/Cmu/Iso and Ova/Cmu/Iso/DEX) controls (*Figure 5.3 A*). In support of our initial observations (*Figure 5.2 A-E*), the administration of steroids reduced airway inflammation and AHR in AAD in isotype-treated, sham-infected, allergic mice (Ova/SPG/Iso/DEX) compared to non-steroid treated (Ova/SPG/Iso) controls (*Figure 5.3 B-H*). In contrast, steroids did not suppress airway inflammation and AHR in AAD in isotype-treated, *Chlamydia*-infected, allergic mice (Ova/Cmu/Iso/DEX) compared to non-steroid treated (Ova/Cmu/Iso) controls (*Figure 5.3 B-H*). Significantly, neutralisation of IL-1β suppressed airway inflammation and AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD in the absence, or presence, of steroids (Ova/Cmu/α-IL-1β and Ova/Cmu/α-IL-1β/DEX, respectively) compared to isotype-treated (Ova/Cmu/Iso
and Ova/Cmu/Iso/DEX) controls (Figure 5.3 B-H). Indeed, α-IL-1β-mediated suppression of airway inflammation and AHR in Chlamydia-induced, severe, steroid-insensitive AAD was comparable to that achieved in steroid-treated, sham-infected, allergic (Ova/SPG/Iso/DEX) mice.

Collectively, these findings demonstrate that the key features of Chlamydia-induced, severe, steroid-insensitive AAD are IL-1β-dependent.
Figure 5.3: Inhibition of IL-1β suppresses cardinal features of Chlamydia-induced, severe, steroid-insensitive allergic airways disease (AAD). Lung IL-1β protein levels (A) were assessed by ELISA on d35 of the study protocol (Figure 5.1) in Chlamydia (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or anti(α)-IL-1β or isotype (Iso) antibody treatment (two experiments; n=4-5). Total leukocyte (B), eosinophil (C), neutrophil (D), macrophage (E), and lymphocyte numbers (F) were determined in BAL fluid on d35. Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (G), and 10 mg/mL of Mch (H; shows statistics for maximal resistance from AHR curves [Figure 5.3 G]) was also determined in all allergic groups on d35 (two experiments; n=5-12). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
5.4.3 Treatment with the pan-caspase inhibitor z-VAD-fmk (ZVAD) at 1mg/kg decreases IL-1β levels and suppresses steroid-insensitive AHR in Chlamydia-induced, severe, steroid-insensitive AAD

Active IL-1β is enzymatically cleaved from pro-IL-1β by caspase activity (Schroder and Tschopp, 2010). Since the key features of Chlamydia-induced, severe, steroid-insensitive AAD are dependent on IL-1β, we sought to determine the role of caspases in severe, steroid-insensitive AAD. To assess the potential of targeting caspases therapeutically, Chlamydia-infected, allergic mice were treated with the pan-caspase inhibitor ZVAD on days 32, 33 and 34 of the study protocol (Figure 5.1 B), and the effect on infection-induced IL-1β, airway inflammation and AHR were assessed.

As expected, we observed a significant increase in the levels of IL-1β in the lungs of Chlamydia-infected mice with AAD (Ova/Cmu/DMSO), compared to sham-infected, allergic (Ova/SPG/DMSO) controls (Figure 5.4 A). Steroid treatment did not suppress elevated IL-1β levels in infected, allergic mice (Ova/Cmu/DMSO/DEX), again suggesting that IL-1β expression is not steroid-sensitive. The administration of ZVAD trended towards reducing lung IL-1β levels on day 35 in infected, allergic mice (Ova/Cmu/ZVAD), however, a significant reduction was only achieved in the presence of steroids (Ova/Cmu/ZVAD/DEX) compared to DMSO-treated (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls (Figure 5.4 A).

In accordance with our earlier observations (Figure 5.2 A-E), treatment with steroids reduced airway inflammation and AHR in AAD in DMSO-treated, sham-infected, allergic mice (Ova/SPG/DMSO/DEX) compared to non-steroid treated (Ova/SPG/DMSO) controls (Figure 5.4 B-H). In contrast, steroids did not suppress airway inflammation and AHR in AAD in DMSO-treated, Chlamydia-infected,
allergic mice (Ova/Cmu/DMSO/DEX) compared to non-steroid treated (Ova/Cmu/DMSO) controls (Figure 5.4 B-H). Interestingly, treatment with ZVAD did not suppress airway inflammation, but suppressed AHR, in Chlamydia-induced, severe, steroid-insensitive AAD in the absence, or presence, of steroids (Ova/Cmu/ZVAD and Ova/Cmu/ZVAD/DEX, respectively) compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls. Indeed, ZVAD-mediated suppression of AHR in Chlamydia-induced, severe, steroid-insensitive AAD was comparable to that achieved in steroid-treated, sham-infected, allergic (Ova/SPG/DMSO/DEX) mice.

These findings demonstrate that pan-caspase inhibition can reverse some features of Chlamydia-induced, severe, steroid-insensitive AAD, including increased IL-1β responses and steroid-insensitive AHR.
Figure 5.4: Pan-caspase inhibition with z-VAD-fmk (ZVAD) decreases IL-1β levels and suppresses airways hyper-responsiveness (AHR) in Chlamydia-induced, severe, steroid-insensitive allergic airways disease (AAD). Lung IL-1β protein levels (A) were assessed by ELISA on d35 of the study protocol (Figure 5.1) in Chlamydia (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or ZVAD or DMSO (vehicle) treatment (two experiments; n=6). Total leukocyte (B), eosinophil (C), neutrophil (D), macrophage (E), and lymphocyte numbers (F) were determined in BAL fluid on d35. Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (G), and 10 mg/mL of Mch (H; shows statistics for maximal resistance from AHR curves [Figure 5.4 G]) was also determined in all allergic groups on d35. ELISA, BAL and AHR data for vehicle (Ova/SPG/DMSO±DEX and Ova/Cmu/DMSO±DEX) controls is repeated for each comparison of inhibitors that were administered in this vehicle (all experiments were performed concurrently) (≥ two experiments; n=5-15). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
5.4.4 Treatment with the Caspase-1 inhibitor Ac-YVAD-cho (YVAD) at 1mg/kg decreases IL-1β levels and suppresses steroid-insensitive airway inflammation and AHR in Chlamydia-induced, severe, steroid-insensitive AAD

Caspase-1, also referred to as IL-1 converting enzyme, plays a crucial role in the proteolytic cleavage, and biological activation, of pro-IL-1β (Martinon et al., 2002; Schroder and Tschopp, 2010). Importantly, recent evidence suggests that Caspase-1 is increased in severe asthma (Simpson et al., 2014) and we show that Chlamydia respiratory infection in AAD significantly increases the cytoplasmic levels of cleaved Caspase-1 (10kDa) on day 35 of the study protocol (Figure 5.2 J). Given that pan-caspase inhibition in Chlamydia-induced, severe, steroid-insensitive AAD was partially beneficial, we sought to examine and compare the therapeutic potential of specifically targeting Caspase-1. Chlamydia-infected, allergic mice were treated with the Caspase-1 inhibitor YVAD on days 32, 33 and 34 of the study protocol (Figure 5.1 B), and the effect on infection-induced IL-1β, airway inflammation and AHR were assessed.

As before, we observed a significant increase in the levels of IL-1β in the lungs of Chlamydia-infected mice with AAD (Ova/Cmu/DMSO), compared to sham-infected, allergic (Ova/SPG/DMSO) controls (Figure 5.5 A). Treatment with steroids did not suppress elevated IL-1β levels in infected, allergic mice (Ova/Cmu/DMSO/DEX), reinforcing the concept that the expression of IL-1β is not steroid-sensitive. Treatment with YVAD reduced lung IL-1β levels on day 35 in infected, allergic mice in the absence, or presence, of steroids (Ova/Cmu/YVAD and Ova/Cmu/YVAD/DEX, respectively), compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls (Figure 5.5 A).
As expected, treatment with steroids reduced airway inflammation and AHR in AAD in DMSO-treated, sham-infected, allergic mice (Ova/SPG/DMSO/DEX) compared to non-steroid treated (Ova/SPG/DMSO) controls (Figure 5.5 B-H). Steroids did not alter airway inflammation and AHR in AAD in DMSO-treated, *Chlamydia*-infected, allergic mice (Ova/Cmu/DMSO/DEX) compared to non-steroid treated (Ova/Cmu/DMSO) controls. Interestingly, and unlike with ZVAD, treatment with YVAD suppressed total airway inflammation in *Chlamydia*-induced, severe, steroid-insensitive AAD in the presence of steroids (Ova/Cmu/YVAD/DEX) and suppressed neutrophilic inflammation in the absence, or presence, of steroids (Ova/Cmu/YVAD and Ova/Cmu/YVAD/DEX, respectively), compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls (Figure 5.5 B-F). Importantly, treatment with YVAD suppressed AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD in the absence of steroids (Ova/Cmu/YVAD) but did not alter AHR when administered with steroids (Ova/Cmu/YVAD/DEX) when compared to DMSO-treated, infected allergic (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls (Figure 5.5 G and H). Indeed, YVAD-mediated suppression of AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD was comparable to that achieved in steroid-treated, sham-infected, allergic (Ova/SPG/DMSO/DEX) mice.

Collectively, our data suggest that Caspase-1-mediated activation of IL-1β in the lungs drives the key features of *Chlamydia*-induced, severe, steroid-insensitive AAD.
Figure 5.5: Inhibition of Caspase-1 with Ac-YVAD-cho (YVAD) decreases IL-1β levels and suppresses cardinal features of Chlamydia-induced, severe, steroid-insensitive allergic airways disease (AAD). Lung IL-1β protein levels (A) were assessed by ELISA on d35 of the study protocol (Figure 5.1) in Chlamydia (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or YVAD or DMSO (vehicle) treatment (two experiments; n=6). Total leukocyte (B), eosinophil (C), neutrophil (D), macrophage (E), and lymphocyte numbers (F) were determined in BAL fluid on d35. Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (G), and 10 mg/mL of Mch (H; shows statistics for maximal resistance from AHR curves [Figure 5.5 G]) was also determined in all allergic groups on d35. ELISA, BAL and AHR data for vehicle (Ova/SPG/DMSO±DEX and Ova/Cmu/DMSO±DEX) controls is repeated for each comparison of inhibitors that were administered in this vehicle (all experiments were performed concurrently) (≥ two experiments; n=5-15). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
5.4.5 Treatment with a novel NLRP3 inhibitor MCC950 at 1mg/kg suppresses steroid-insensitive airway inflammation in Chlamydia-induced, severe, steroid-insensitive AAD

There is a substantial body of evidence showing that the inflammasome-mediated recruitment and activation of pro-Caspase-1 plays a key role in the bio-activation of pro-IL-1β (Thornberry et al., 1992; Martinon et al., 2002; Stutz et al., 2009; Gabay et al., 2010; Schroder and Tschopp, 2010; Wittmann et al., 2011). The NLRP3 inflammasome is the most characterised Caspase-1-activating inflammasome and is widely implicated in inflammatory diseases (Schroder and Tschopp, 2010; Walsh et al., 2014). Importantly, clinical and experimental studies strongly implicate inflammasome-induced IL-1β in the pathogenesis of severe asthma (Idzko et al., 2007; Schroder and Tschopp, 2010; Muller et al., 2011; Simpson et al., 2014). Given that the key features of Chlamydia-induced, severe, steroid-insensitive AAD are dependent on both Caspase-1 and IL-1β (Figures 5.3, 5.4 and 5.5), we next examined the potential role of the NLRP3 inflammasome. To assess the possibility of targeting the NLRP3 inflammasome therapeutically, Chlamydia-infected, allergic mice were treated i.n. with a novel NLRP3 inflammasome inhibitor (MCC950), on days 32, 33 and 34 of the study protocol (Figure 5.1 B), and the effect on infection-induced IL-1β in the lungs, airway inflammation and AHR were assessed.

In accord with our earlier observations (Figures 5.4 A and 5.5 A), Chlamydia infection in AAD (Ova/Cmu/DMSO) significantly increased the levels of IL-1β in the lungs compared to sham-infected, allergic (Ova/SPG/DMSO) controls (Figure 5.6 A). Furthermore, steroid treatment of infected, allergic mice (Ova/Cmu/DMSO/DEX) did not affect IL-1β levels. Unexpectedly, treatment with MCC950 at 1mg/kg further increased lung IL-1β levels on day 35 in infected, allergic mice (Ova/Cmu/MCC950)
in the absence, or presence, of steroids (Ova/Cmu/MCC950 and Ova/Cmu/MCC950/DEX, respectively) compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls.

Treatment with steroids reduced airway inflammation and AHR in AAD in DMSO-treated, sham-infected, allergic mice (Ova/SPG/DMSO/DEX) compared to non-steroid treated (Ova/SPG/DMSO) controls (**Figure 5.6 B-H**). In contrast, steroids did not alter airway inflammation and AHR in AAD in DMSO-treated, *Chlamydia*-infected, allergic mice (Ova/Cmu/DMSO/DEX) compared to non-steroid treated, infected, allergic (Ova/Cmu/DMSO) controls. Treatment with MCC950 at 1mg/kg strongly trended towards suppressing airway inflammation and AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD in the absence of steroids (Ova/Cmu/MCC950) compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO) controls (**Figure 5.6 B-H**). Like with α-IL-1β and YVAD treatments, but unlike with ZVAD, treatment with MCC950 at 1mg/kg potently suppressed neutrophilic airway inflammation in *Chlamydia*-induced, severe, steroid-insensitive AAD in the absence of steroids (Ova/Cmu/MCC950) compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO) controls (**Figure 5.6 D**). Treatment with MCC950 at 1mg/kg in the presence of steroids (Ova/Cmu/MCC950/DEX) suppressed airway inflammation, but not AHR, in *Chlamydia*-induced, severe, steroid-insensitive AAD compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO/DEX) controls.
Figure 5.6: Treatment with a novel NLRP3 inhibitor MCC950 at 1mg/kg suppresses neutrophilic airway inflammation in *Chlamydia*-induced, severe, steroid-insensitive allergic airways disease (AAD). Lung IL-1β protein levels (A) were assessed by ELISA on d35 of the study protocol (Figure 5.1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or MCC950 at 1mg/kg or DMSO (vehicle) treatment (two experiments; n=5-6). Total leukocyte (B), eosinophil (C), neutrophil (D), macrophage (E), and lymphocyte numbers (F) were determined in BAL fluid on d35. Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (G), and 10 mg/mL of Mch (H; shows statistics for maximal resistance from AHR curves [Figure 5.6 G]) was also determined in all allergic groups on d35. ELISA, BAL and AHR data for vehicle (Ova/SPG/DMSO±DEX and Ova/Cmu/DMSO±DEX) controls is repeated for each comparison of inhibitors that were administered in this vehicle (all experiments were performed concurrently) (≥ two experiments; n=5-15). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
5.4.6 Treatment with MCC950 at 10mg/kg decreases IL-1β levels and suppresses steroid-insensitive airway inflammation and AHR in Chlamydia-induced, severe, steroid-insensitive AAD

Treatment with MCC950 at 1mg/kg partially suppressed the key features of Chlamydia-induced, severe, steroid-insensitive AAD (Figure 5.6). However, treatment with MCC950 at 1mg/kg did not reduce Chlamydia-induced lung IL-1β levels (Figure 5.6 A). Given that the key features of Chlamydia-induced, severe, steroid-insensitive AAD are dependent on both Caspase-1 and IL-1β (Figures 5.3, 5.4 and 5.5), we reassessed the therapeutic potential of targeting the NLRP3 inflammasome by administering a higher dose of MCC950. Chlamydia-infected, allergic mice were treated with MCC950 at 10mg/kg on days 32, 33 and 34 of the study protocol (Figure 5.1 B), and the effect on infection-induced IL-1β in the lungs, airway inflammation and AHR were assessed.

As before, we observed an increase in the levels of IL-1β in the lungs of Chlamydia-infected mice with AAD (Ova/Cmu/DMSO) compared to sham-infected, allergic (Ova/SPG/DMSO) controls (Figure 5.7 A). Furthermore, treatment with steroids did not affect Chlamydia-induced IL-1β in infected, allergic mice (Ova/Cmu/DMSO/DEX) compared to non-steroid treated, infected, allergic (Ova/Cmu/DMSO) controls. Importantly, unlike with MCC950 at 1mg/kg, and like with α-IL-1β, treatment with a higher dose of MCC950 at 10mg/kg reduced lung IL-1β levels on day 35 in infected, allergic groups in the absence of steroids (Ova/Cmu/MCC950[10mg/kg]) compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO and Ova/Cmu/DMSODEX) controls.

As expected, treatment with steroids reduced airway inflammation and AHR in AAD in DMSO-treated, sham-infected, allergic mice (Ova/SPG/DMSO/DEX)
compared to non-steroid treated (Ova/SPG/DMSO) controls (Figure 5.7 B-H). Steroids did not alter airway inflammation and AHR in AAD in DMSO-treated, Chlamydia-infected, allergic mice (Ova/Cmu/DMSO/DEX) compared to non-steroid treated, infected, allergic (Ova/Cmu/DMSO) controls. Importantly, and like with α-IL-1β, treatment with MCC950 at 10mg/kg suppressed airway inflammation and AHR in Chlamydia-induced, severe, steroid-insensitive AAD in the absence, or presence, of steroids (Ova/Cmu/MCC950[10mg/kg] and Ova/Cmu/MCC950[10mg/kg]/DEX, respectively) compared to DMSO-treated, infected, allergic (Ova/Cmu/DMSO and Ova/Cmu/DMSO/DEX) controls (Figure 5.7 B-H). Indeed, MCC950 at 10mg/kg-mediated suppression of airway inflammation and AHR in Chlamydia-induced, severe, steroid-insensitive AAD was comparable to that achieved in steroid-treated, sham-infected, allergic (Ova/SPG/DMSO/DEX) mice.

Collectively, these findings demonstrate that the key features of Chlamydia-induced, severe, steroid-insensitive AAD are NLRP3 inflammasome-dependent and that steroid-insensitive airway inflammation and AHR are potentially induced by a NLRP3 inflammasome-dependent, Caspase-1-mediated signalling axis that increases the levels of IL-1β in the lungs.
Figure 5.7: Treatment with a novel NLRP3 inhibitor MCC950 at 10mg/kg suppresses cardinal features of Chlamydia-induced, severe, steroid-insensitive allergic airways disease (AAD). Lung IL-1β protein levels (A) were assessed by ELISA on d35 of the study protocol (Figure 5.1) in Chlamydia (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or MCC950 at 10mg/kg or DMSO (vehicle) treatment (two experiments; n=5-6). Total leukocyte (B), eosinophil (C), neutrophil (D), macrophage (E), and lymphocyte numbers (F) were determined in BAL fluid on d35. Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (G), and 10 mg/mL of Mch (H; shows statistics for maximal resistance from AHR curves [Figure 5.7 G]) was also determined in all allergic groups on d35. ELISA, BAL and AHR data for vehicle (Ova/SPG/DMSO±DEX and Ova/Cmu/DMSO±DEX) controls is repeated for each comparison of inhibitors that were administered in this vehicle (all experiments were performed concurrently) (≥ two experiments; n=5-15). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
5.4.7 Inhibition of *Haemophilus*-induced IL-1β suppresses cardinal features of *Haemophilus*-induced, severe, steroid-insensitive AAD

To assess the applicability of our observations to other models of steroid-insensitive asthma, we repeated the most informative experiments in our model of *Haemophilus* infection-induced, severe, steroid-insensitive AAD. We examined and compared the effects of the most effective treatments on *Haemophilus*-induced IL-1β in the lungs, airway inflammation and AHR on day 35 of the study protocol (Figure 5.1) in the absence, or presence, of treatment with DEX. Like with *Chlamydia*, we observed a significant increase in the levels of IL-1β in the lungs of *Haemophilus*-infected mice with AAD (Ova/Hinf/Iso), compared to sham-infected, allergic (Ova/PBS/Iso) controls (Figure 5.8 A). Steroid treatment did not suppress elevated IL-1β levels in infected, allergic mice (Ova/Hinf/Iso/DEX), suggesting that *Haemophilus*-induced IL-1β expression, like with *Chlamydia* infection, is steroid-insensitive. To assess the potential of targeting IL-1β therapeutically, and to compare the effects with equivalent treatment in *Chlamydia*-induced, severe, steroid-insensitive AAD, *Haemophilus*-infected, allergic mice were treated with α-IL-1β on days 30, 32 and 34 of the study protocol (Figure 5.1 A). The administration of α-IL-1β significantly reduced lung IL-1β levels on day 35 in infected, allergic groups in the absence, or presence, of steroids (Ova/Hinf/α-IL-1β and Ova/Hinf/α-IL-1β/DEX, respectively) compared to isotype-treated (Ova/Hinf/Iso and Ova/Hinf/Iso/DEX) controls (Figure 5.8 A).

Treatment with steroids reduced airway inflammation and AHR in AAD in isotype-treated, sham-infected, allergic mice (Ova/PBS/Iso/DEX) compared to non-steroid treated (Ova/PBS/Iso) controls (Figure 5.8 B-H). In contrast, steroids did not affect neutrophilic airway inflammation and AHR in isotype-treated, *Haemophilus*-
infected, allergic mice (Ova/Hinf/Iso/DEX) compared to non-steroid treated, infected, allergic (Ova/Hinf/Iso) controls. Importantly, neutralisation of IL-1β suppressed neutrophilic airway inflammation and AHR in Haemophilus-induced, severe, steroid-insensitive AAD in the absence, or presence, of steroids (Ova/Hinf/α-IL-1β and Ova/Hinf/α-IL-1β/DEX, respectively) compared to isotype-treated, infected, allergic (Ova/Hinf/Iso and Ova/Hinf/Iso/DEX) controls. Indeed, α-IL-1β-mediated suppression of airway inflammation and AHR in Haemophilus-induced, severe, steroid-insensitive AAD was comparable to that achieved in steroid-treated, sham-infected, allergic (Ova/PBS/Iso/DEX) mice.

Collectively, these findings demonstrate that the key features of both Chlamydia- and Haemophilus-induced, severe, steroid-insensitive AAD are induced by a common IL-1β-dependent mechanism.
Figure 5.8: Inhibition of IL-1β suppresses cardinal features of Haemophilus-induced, severe, steroid-insensitive allergic airways disease (AAD). Lung IL-1β protein levels (A) were assessed by ELISA on d35 of the study protocol (Figure 5.1) in Haemophilus (Hinf) and sham (PBS)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or anti(α)-IL-1β or isotype (Iso) antibody treatment (one experiment; n=5-6). Total leukocyte (B), eosinophil (C), neutrophil (D), macrophage (E), and lymphocyte numbers (F) were determined in BAL fluid on d35. Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (G), and 10 mg/mL of Mch (H; shows statistics for maximal resistance from AHR curves [Figure 5.8 G]) was also determined in all allergic groups on d35 (two experiments; n=6-11). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
5.4.8 Treatment with the Caspase-1 inhibitor YVAD at 1mg/kg decreases IL-1β levels and suppresses steroid-insensitive airway inflammation and AHR in Haemophilus-induced, severe, steroid-insensitive AAD

Since the key features of Haemophilus-induced, severe, steroid-insensitive AAD are IL-1β-dependent, we sought to examine and compare the therapeutic potential of specifically targeting Caspase-1. Haemophilus-infected, allergic mice were treated with YVAD, on days 32, 33 and 34 of the study protocol (Figure 5.1 B), and the effects on infection-induced IL-1β in the lungs, airway inflammation and AHR were assessed. Given that YVAD suppressed Chlamydia-induced IL-1β levels, neutrophilic airway inflammation and AHR when administered alone (Figure 5.5 A), we assessed the effects of YVAD treatment in Haemophilus-induced, severe, steroid-insensitive AAD in the absence of steroids.

Like with α-IL-1β, treatment with YVAD suppressed lung IL-1β levels, neutrophilic airway inflammation and AHR on day 35 in Haemophilus-infected, allergic mice (Ova/Hinf/YVAD) compared to DMSO-treated, infected, allergic (Ova/Hinf/DMSO) controls (Figure 5.9 A, D, G and H). Treatment with YVAD also suppressed total airway inflammation in Haemophilus-induced, severe, steroid-insensitive AAD (Figure 5.9 B). Indeed, YVAD-mediated suppression of lung IL-1β levels, neutrophilic airway inflammation and AHR in Haemophilus-induced, severe, steroid-insensitive AAD was potent and similar to that achieved in steroid-treated, sham-infected, allergic (Ova/PBS/DMSO/DEX) mice and comparable to the effects of the equivalent treatment in Chlamydia-induced, severe, steroid-insensitive AAD.

Collectively, these findings indicate that Caspase-1-mediated activation of IL-1β in the lungs drives the key features of both Chlamydia- and Haemophilus-induced, severe, steroid-insensitive AAD.
Figure 5.9: Inhibition of Caspase-1 with Ac-YVAD-cho (YVAD) decreases IL-1β levels and suppresses cardinal features of *Haemophilus*-induced, severe, steroid-insensitive allergic airways disease (AAD). Lung IL-1β protein levels (A) were assessed by ELISA on d35 of the study protocol (Figure 5.1) in *Haemophilus* (Hinf) and sham (PBS)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or YVAD or DMSO (vehicle) treatment (two experiments; n=5-6). Total leukocyte (B), eosinophil (C), neutrophil (D), macrophage (E), and lymphocyte numbers (F) were determined in BAL fluid on d35. Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (G), and 10 mg/mL of Mch (H; shows statistics for maximal resistance from AHR curves [Figure 5.9 G]) was also determined in all allergic groups on d35. ELISA, BAL and AHR data for vehicle (Ova/PBS/DMSO±DEX and Ova/Hinf/DMSO±DEX) controls is repeated for each comparison of inhibitors that were administered in this vehicle (all experiments were performed concurrently) (two experiments; n=6-10). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
5.4.9 Treatment with MCC950 at 10mg/kg suppresses steroid-insensitive airway inflammation and AHR in Haemophilus-induced, severe, steroid-insensitive AAD

Since the key features of Haemophilus-induced, severe, steroid-insensitive AAD are IL-1β- and Caspase-1-dependent, we sought to examine and compare the therapeutic potential of specifically targeting the NLRP3 inflammasome. Haemophilus-infected, allergic mice were treated with MCC950 at 10mg/kg on days 32, 33 and 34 of the study protocol (Figure 5.1 B) and the effects on infection-induced airway inflammation and AHR were assessed.

The administration of steroids reduced airway inflammation and AHR in AAD in DMSO-treated, sham-infected, allergic mice (Ova/PBS/DMSO/DEX) compared to non-steroid treated (Ova/PBS/DMSO) controls (Figure 5.10 A-G). In contrast, steroids did not suppress airway inflammation and AHR in AAD in DMSO-treated, Haemophilus-infected, allergic mice (Ova/Hinf/DMSO/DEX) compared to non-steroid treated, infected, allergic (Ova/Hinf/DMSO) controls. Importantly, and like with α-IL-1β and YVAD, treatment with MCC950 at 10mg/kg suppressed airway inflammation and AHR in Haemophilus-induced, severe, steroid-insensitive AAD in the absence, or presence, of steroids (Ova/Hinf/MCC950[10mg/kg] and Ova/Hinf/MCC950[10mg/kg]/DEX, respectively) compared to DMSO-treated, infected, allergic (Ova/Hinf/DMSO and Ova/Hinf/DMSO/DEX) controls. Indeed, MCC950 at 10mg/kg-mediated suppression of airway inflammation and AHR in Haemophilus-induced, severe, steroid-insensitive AAD was comparable to that achieved in steroid-treated, sham-infected, allergic (Ova/PBS/DMSO/DEX) controls.

Collectively, these data show that the key features of both Chlamydia- and Haemophilus-induced, severe, steroid-insensitive AAD are NLRP3 inflammasome-
dependent and that steroid-insensitive airway inflammation and AHR are potentially induced by an NLRP3 inflammasome-dependent, Caspase-1-mediated, IL-1β signalling axis.
Figure 5.10: Treatment with a novel NLRP3 inhibitor MCC950 at 10mg/kg suppresses cardinal features of *Haemophilus*-induced, severe, steroid-insensitive allergic airways disease (AAD). Total leukocyte (A), eosinophil (B), neutrophil (C), macrophage (D), and lymphocyte numbers (E) were determined in BAL fluid on d35 in *Haemophilus* (Hinf) and sham (PBS)-infected groups with ovalbumin (Ova)-induced AAD in the absence, and presence, of steroid (DEX) and/or MCC950 at 10mg/kg or DMSO (vehicle) treatment. Airways hyper-responsiveness (AHR) in terms of airways resistance (Rn) in response to increasing doses of methacholine (Mch) (F), and 10 mg/mL of Mch (G; shows statistics for maximal resistance from AHR curves [Figure 5.10 F]) was also determined in all allergic groups on d35. BAL and AHR data for vehicle (Ova/PBS/DMSO±DEX and Ova/Hinf/DMSO±DEX) controls is repeated for each comparison of inhibitors that were administered in this vehicle (all experiments were performed concurrently) (two experiments; n=6-10). All data are presented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; n.s. (not significant).
5.5 Discussion

In this study, we show that Chlamydia and Haemophilus respiratory infections induce an NLRP3 inflammasome/Caspase-1/IL-1β signalling axis in the lungs to promote severe, steroid-insensitive AAD. We initially showed that increased expression of Caspase-1 and IL-1β were associated with Chlamydia-induced, severe, steroid-insensitive AAD. We then used a combination of neutralising anti-IL-1β antibodies that specifically target and inhibit this cytokine in vivo, the pan-caspase inhibitor, z-VAD-fmk, the Caspase-1 inhibitor, Ac-YVAD-cho, and a novel highly specific and potent NLRP3 inflammasome inhibitor, MCC950, to define a previously unrecognised role for this effector pathway in infection-induced, severe, steroid-insensitive AAD (Figure 5.1).

We have shown that Chlamydia respiratory infection results in a switch from T_{H}2-dominated, eosinophilic inflammatory responses to T_{H}1/T_{H}17-dominated, neutrophilic inflammatory responses in Ova-induced AAD and that both airway inflammation and AHR are steroid-insensitive in this model (Chapter 4). In this study, we expand on these observations by showing that Chlamydia infection-induced, severe, steroid-insensitive AAD is also characterised by increased gene expression (day 30 and 35) and lung protein levels (day 35) of IL-1β (Figures 5.2, 5.3, 5.4, 5.5, 5.6, 5.7). Significantly, this agrees with clinical evidence that links neutrophilic lung inflammation and increased IL-1β with severe asthma (Baines et al., 2011; Simpson et al., 2014). IL-1β promotes T_{H}17 differentiation and the production of IL-17 that induces neutrophilia (McKinley et al., 2008; Chung et al., 2009; Korn et al., 2009; Hastie et al., 2010; Besnard et al., 2012; Kim et al., 2014). Importantly, its expression is increased in the airways and serum in severe, steroid-insensitive, compared to mild, asthma and correlates with neutrophilic inflammation and
declining lung function (Konno et al., 1996; Wanderer, 2009; Hastie et al., 2010; Besnard et al., 2012; Kim et al., 2014). In this study, *Chlamydia* infection increased IL-1β expression on day 30 of our model of AAD (*Figure 5.2 G*), which was 16 days after infection and when infection-induced inflammation had subsided to baseline levels prior to Ova re-challenge (data not shown - part of a separate manuscript not presented in this Thesis). Day 30 also represents the time point prior to recapitulation of disease in our model and before steroid administration. Thus, the *Chlamydia*-induced, IL-1β-influenced environment within the lung at this time point may be important in driving the severe, steroid-insensitive phenotype in response to airway allergen exposure.

To verify the functional contribution of IL-1β in the pathogenesis of severe, steroid-insensitive AAD and to identify the potential for therapeutic targeting, we inhibited IL-1β *in vivo* with neutralising α-IL-1β antibody treatment (*Figure 5.3*). Treatment with α-IL-1β, unlike with steroids, suppressed *Chlamydia*-induced IL-1β back to levels in sham-infected groups, confirming effective inhibition in the lungs (*Figure 5.3 A*). Importantly, inhibition of IL-1β also suppressed *Chlamydia*-induced, steroid-insensitive airway inflammation and AHR in the absence, and presence, of steroids (*Figure 5.3 B-H*). Our data also shows that *Chlamydia*-induced IL-1β production in the lungs is not effectively reduced with steroid treatment, the key features of *Chlamydia*-induced, severe, steroid-insensitive AAD are IL-1β-dependent, and that targeting elevated IL-1β in the lungs *in lieu* of steroids may be relevant. Furthermore, our observations are consistent with other studies that show that treatment with IL-1R antagonist reduced inflammation and AHR in a murine asthma model (Wang et al., 2006) and that treatment with IL-1 receptor antagonist suppressed acute ozone exposure-induced BALF neutrophilia and AHR (Park et al., 2004).
Figure 5.11: Mechanisms and potential treatment of severe steroid-insensitive asthma.

Infection in allergic airways disease/asthma induces NLRP3 inflammasome activity that recruits and cleaves pro-Caspase-1 into Caspase-1, which then cleaves pro-IL-1β into active IL-1β, to promote steroid insensitivity. This pathway may be targeted therapeutically by inhibition of the NLRP3 inflammasome and/or Caspase-1 and/or IL-1β.

Interestingly, *Chlamydia* infection in AAD increased Caspase-1 mRNA expression on day 30 (Figure 5.2 H) and the cleavage of pro-Caspase-1 on day 35 (Figure 5.2 J). Several studies demonstrate that active IL-1β is proteolytically cleaved from its precursor, pro-IL-1β, by Caspase-1, otherwise referred to as IL-1 converting enzyme (Thornberry et al., 1992). Importantly, there is a substantial body of evidence showing that the NLRP3 inflammasome recruits, and activates, pro-Caspase-1 to potently induce pro-inflammatory IL-1β responses. Clinical evidence
shows that NLRP3 and Caspase-1, along with IL-1β, are increased in the airways of severe asthmatics (Baines et al., 2011; Simpson et al., 2014). Collectively, these studies strongly implicate a role for NLRP3 inflammasome-mediated IL-1β responses in the pathogenesis of severe, steroid-insensitive asthma, however, they do not demonstrate a direct contribution of an NLRP3 inflammasome/Caspase-1/IL-1β axis. In this study, and to extend our observations with α-IL-1β treatment, we used several approaches to verify the functional contribution of Chlamydia-induced, NLRP3 inflammasome-dependent, Caspase-1-mediated activation of IL-1β in severe, steroid-insensitive AAD and identify their potential for therapeutic targeting.

Treatment with the pan-caspase inhibitor, ZVAD, like with α-IL-1β, suppressed AHR in Chlamydia-induced, severe, steroid-insensitive AAD in the absence, and presence, of steroids (Figure 5.4 G and H), so it is possible that the activity of caspases, including Caspase-1, may contribute to steroid-insensitive AHR. However, unlike with α-IL-1β, treatment with ZVAD did not suppress steroid-insensitive airway inflammation and only trended towards reducing lung IL-1β levels in the absence of steroids (Figure 5.4 A-F). This was surprising given that ZVAD is a known inhibitor of Caspase-1 and Caspase-1-mediated activation of pro-IL-1β (Hara et al., 1997; Lipinska et al., 2014) but supports the concept that Chlamydia-induced IL-1β promotes steroid-insensitive airway inflammation. It is possible that treatment inhibited a number of caspases other than Caspase-1, including Caspase-4 (human homologue of murine Caspase-11; collectively referred to as Caspase-4/11), which is reported to act upstream of Caspase-1 and promote its activation (Sollberger et al., 2012), and Caspase-8, which promotes the apoptosis of epithelial and inflammatory cells during a Chlamydia infection (Kruidering and Evan, 2000; Vats et al., 2010; Wang et al., 2010c). Recent evidence also shows that Caspase-8 can activate IL-1β
(Maelfait et al., 2008; Dupaul-Chicoine and Saleh, 2012; Gringhuis et al., 2012), which obscures the interpretation of the effects of ZVAD treatment. Thus, treatment with ZVAD potentially lacks the specificity and potency that is necessary to examine the role of Caspase-1 in Chlamydia-induced, severe, steroid-insensitive AAD and may have inadvertently promoted the survival of inflammatory cells in the airways of infected mice in AAD. Dosing ZVAD at higher concentrations may have suppressed lung IL-1β levels, however, this would likely induce strong off-target effects. Importantly, ZVAD is formulated with a tripeptide structure that is most effective as a broad-range irreversible caspase inhibitor (Cain et al., 1996). By contrast, YVAD possesses a tetrapeptide structure that shows stronger but reversible selectivity to Caspase-1 (Garcia-Calvo et al., 1998). Thus, we reassessed the role of Caspase-1 in the pathogenesis of Chlamydia-induced, severe, steroid-insensitive AAD by treating infected mice with YVAD. By contrast to the effects of ZVAD, treatment with YVAD suppressed lung IL-1β levels in Chlamydia-induced, severe, steroid-insensitive AAD in the absence, and presence of steroids (Figure 5.5 A). This shows that YVAD treatment, like with α-IL-1β, inhibits Chlamydia-induced IL-1β in the lungs independently of the effects of steroids and implicates the Caspase-1-mediated activation of IL-1β in the pathogenesis of Chlamydia-induced, severe, steroid-insensitive AAD. Treatment with YVAD also reduced total airway inflammation in the presence of steroids (Figure 5.5 B), suppressed infection-induced neutrophilic airway inflammation in the absence, and presence, of steroids (Figure 5.5 D), and suppressed AHR in the absence of steroids (Figure 5.5 G and H). Importantly, these effects were similar to those observed following α-IL-1β treatment, which suggests that treatment with YVAD affects a common pathogenic pathway, that the Caspase-1-mediated activation of IL-1β promotes the key features of Chlamydia-induced, severe,
steroid-insensitive AAD, and that targeting infection-induced Caspase-1 activity in the lungs *in lieu* of steroids may be therapeutically relevant.

To verify a functional contribution of the NLRP3 inflammasome to the pathogenesis of severe, steroid-insensitive AAD and to substantiate the existence of a pathogenic, infection-driven NLRP3 inflammasome/Caspase-1 signalling axis, we next inhibited its activity and assessed the impact on the phenotype. NLRP3 inflammasome-induced IL-1β responses are important in the control of infections, however, excessive activation results in aberrant inflammation and has been associated with the pathogenesis of chronic diseases of the skin, joints, heart and importantly lungs, specifically severe, steroid-insensitive asthma and COPD (Schroder and Tschopp, 2010; Simpson et al., 2014). He *et al.* showed that *Chlamydia* induced the activation of the NLRP3 inflammasome in a TLR2-dependent manner, and that this was required for the cleavage and activation of Caspase-1 and pro-IL-1β (He *et al.*, 2010). In this study, we show that *Chlamydia* induces a sustained increase in the expression of Caspase-1 and increases the levels of cleaved Caspase-1 (10kDa) in the lungs in AAD (*Figure 5.2 H and J*). Thus, *Chlamydia* infection in established AAD may induce the activation of the NLRP3 inflammasome, increase the activation of Caspase-1, and increase the levels of IL-1β through a common pathway prior to steroid treatment and the recapitulation of AAD. We inhibited the NLRP3 inflammasome *in vivo* with a novel and specific inhibitor of NLRP3 (MCC950). In unpublished observations, MCC950 has been shown to reduce the cleavage of pro-Caspase-1 and inhibit the release of IL-1β, but not TNF-α, from macrophages stimulated with LPS and ATP, monosodium urate crystals or nigericin. Furthermore, MCC950 does not inhibit IL-1β secretion mediated via the activation of NLRC4 or AIM2 inflammasomes with *Salmonella* or dsDNA.
In this study, the therapeutic administration of MCC950 at 1mg/kg suppressed neutrophilic airway inflammation in *Chlamydia*-induced, severe, steroid-insensitive AAD in the absence of steroids (*Figure 5.6 D*). Treatment with MCC950 at 1mg/kg also strongly trended towards suppressing total airway inflammation and AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD in the absence of steroids but surprisingly, further increased lung IL-1β levels (*Figure 5.6 A, B, G, and H*). These data show that targeting the NLRP3 inflammasome with MCC950 at 1mg/kg is partially effective in *Chlamydia*-induced, severe, steroid-insensitive AAD and may suggest that the NLRP3 inflammasome promotes neutrophilic airway inflammation via effector pathways that are not suppressed by the anti-inflammatory effects of steroids. However, the strong downward trends of airway inflammation and AHR following treatment with MCC950 at 1mg/kg suggest that targeting the NLRP3 inflammasome, like with Caspase-1, requires optimisation of the treatment strategy to be maximally beneficial in *Chlamydia*-induced, severe, steroid-insensitive AAD. Thus, we increased the dosage concentration of MCC950 to 10mg/kg and reassessed the role of the NLRP3 inflammasome in the pathogenesis of *Chlamydia*-induced, severe, steroid-insensitive AAD. Significantly, the therapeutic administration of MCC950 at 10mg/kg reduced lung IL-1β levels, airway inflammation and AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD in the absence, or presence, of steroids (*Figure 5.7*). Importantly, treatment with MCC950 at 10mg/kg had similar effects to treatment with α-IL-1β and the caspase inhibitors, which suggests that the key features of infection-induced, severe, steroid-insensitive AAD are induced by the NLRP3 inflammasome-dependent, Caspase-1-mediated, activation of IL-1β in the lungs.
To demonstrate a widespread role for the NLRP3 inflammasome/Caspase-1/IL-1β signalling axis in the induction of severe, steroid-insensitive asthma, and that it is not specific to Chlamydia-associated disease, we investigated the effects of inhibition of these factors in Haemophilus-induced, severe, steroid-insensitive AAD. H. influenzae is commonly isolated from the lungs of non-eosinophilic asthmatics. 60% of stable asthmatics with high levels of bacteria in their airways were culture-positive for H. influenzae and, importantly, these individuals were more likely to have non-eosinophilic asthma and be on high doses of inhaled steroids (Wood et al., 2010). Significantly, both Chlamydia and Haemophilus respiratory infections induce T_{H1} and/or T_{H17} responses (Zhou et al., 2009; Horvat et al., 2010a; Essilfie et al., 2011; Essilfie et al., 2012) and Haemophilus infection can up-regulate the NLRP3 inflammasome to induce the Caspase-1-dependent secretion of IL-1β from murine macrophage and human respiratory cells (Rotta Detto Loria et al., 2013). Thus, we hypothesised that Haemophilus, like Chlamydia, may induce severe, steroid-insensitive AAD through an NLRP3 inflammasome/Caspase-1/IL-1β signalling axis in the lungs.

To verify the functional contribution of IL-1β signalling in the pathogenesis of Haemophilus-induced, severe, steroid-insensitive AAD and identify the potential for therapeutic targeting, we inhibited IL-1β in vivo with α-IL-1β, as done previously in Chlamydia-induced, severe, steroid-insensitive AAD (Figure 5.3 and 5.8). Treatment with α-IL-1β in Haemophilus-induced, severe, steroid-insensitive AAD, unlike with steroids, but like in Chlamydia-induced disease, suppressed infection-induced IL-1β back to levels in sham-infected groups, confirming effective inhibition in the lungs (Figure 5.8 A). Inhibition of IL-1β also suppressed Haemophilus-induced, steroid-insensitive neutrophilic airway inflammation and AHR in the absence, and presence,
of steroids (Figure 5.8 D, G and H). This indicates that Haemophilus-induced IL-1β production in the lungs is not effectively reduced with steroid treatment, the key features of Haemophilus-induced, severe, steroid-insensitive AAD are IL-1β-dependent, and that targeting elevated IL-1β in the lungs in lieu of steroids may be relevant. Thus, targeting IL-1β in the lung may have broad therapeutic relevance to respiratory infection-induced, severe, steroid-insensitive asthma and may be a new and effective treatment for this disease. Furthermore, elevated IL-1β occurs in COPD where it is implicated in the induction of neutrophilic airway inflammation, airway remodelling and emphysema, indicating that IL-1β-targeted therapy could also be effective in other steroid-insensitive inflammatory conditions (Churg et al., 2009; Gibson and Simpson, 2009; Wanderer, 2009; Singh et al., 2010; Rotta Detto Loria et al., 2013).

In this study, we assessed the role of Caspase-1 in the pathogenesis of Haemophilus-induced, severe, steroid-insensitive AAD by treating infected mice with YVAD, and compared the effects with those observed following the same treatment in Chlamydia-induced, severe, steroid-insensitive AAD (Figure 5.5 and 5.9). Treatment with YVAD suppressed lung IL-1β levels in Haemophilus-induced, severe, steroid-insensitive AAD (Figure 5.9 A). These data show that treatment with YVAD, like with α-IL-1β, inhibits Haemophilus-induced IL-1β in the lungs independently of the effects of steroids and implicates Caspase-1-mediated activation of IL-1β in the pathogenesis of Haemophilus-induced, severe, steroid-insensitive AAD. Treatment with YVAD also reduced total and neutrophilic airway inflammation (Figure 5.9 B and D), and suppressed AHR (Figure 5.9 G and H). Importantly, these effects were similar to those observed following α-IL-1β treatment and were comparable to the effects of α-IL-1β and YVAD treatments in Chlamydia-induced, severe, steroid-
insensitive AAD. These data suggest that treatment with YVAD affects a common pathogenic pathway in both *Chlamydia*- and *Haemophilus*-induced, severe, steroid-insensitive AAD, that the Caspase-1-mediated activation of IL-1β promotes the key features of *Haemophilus*-induced, severe, steroid-insensitive AAD, and that targeting infection-induced Caspase-1 activity in the lungs *in lieu* of steroids may be therapeutically relevant. Furthermore, these data suggest that the pathogenesis of *Haemophilus*-induced, severe, steroid-insensitive AAD, like with *Chlamydia*-induced, severe, steroid-insensitive AAD, involves an infection-induced NLRP3 inflammasome/Caspase-1 signalling axis that promotes the activation of IL-1β in the lungs.

Thus, we sought to examine the role of the NLRP3 inflammasome in the pathogenesis of *Haemophilus*-induced, severe, steroid-insensitive AAD, by treating mice with MCC950 at 10mg/kg and compared the effects on the phenotype to those observed following treatment with MCC950 at 10mg/kg in *Chlamydia*-induced, severe, steroid-insensitive AAD (Figure 5.7 and 5.10). The therapeutic administration of MCC950 at 10mg/kg reduced airway inflammation and AHR in *Haemophilus*-induced, severe, steroid-insensitive AAD in the absence, or presence, of steroids (Figure 5.10). Importantly, these effects were similar to those observed following α-IL-1β and YVAD treatments and were comparable to the effects of treatment with α-IL-1β, YVAD and MCC950 at 10mg/kg in *Chlamydia*-induced, severe, steroid-insensitive AAD. These data suggest that the key features of both *Chlamydia*- and *Haemophilus*-induced, severe, steroid-insensitive AAD are induced through NLRP3 inflammasome activity. Since treatment with MCC950 at 10mg/kg, unlike with steroids, suppressed the key disease features of *Chlamydia*- and *Haemophilus*-induced, severe, steroid-insensitive AAD back down to sham-infected,
steroid-treated levels it is likely that infection-induced NLRP3 inflammasome activity is required for IL-1β-dependent pathology in this model. Collectively, these data demonstrate that the key features of Chlamydia- and Haemophilus-induced, severe, steroid-insensitive AAD are induced by an NLRP3 inflammasome-dependent, Caspase-1-mediated, activation of IL-1β in the lungs.

In summary, our study demonstrates for the first time that the NLRP3 inflammasome is pivotal in promoting steroid-insensitive inflammation and AHR in respiratory infection-induced, severe, steroid-insensitive AAD. Importantly, we have identified the potential functional relevance of an infection-induced NLRP3 inflammasome/Caspase-1/IL-1β signalling axis in promoting these disease features. Our data suggest that the therapeutic inhibition of the NLRP3 inflammasome/Caspase-1/IL-1β signalling axis may be effective in lieu of steroid treatment in severe, steroid-insensitive asthma in humans. Thus, our findings highlight the potential and relevance of therapies that target specific disease processes, which is more attractive than non-specifically inhibiting inflammatory responses with steroids. Targeting the NLRP3 inflammasome/Caspase-1/IL-1β signalling axis may also be applicable to the treatment of other steroid-insensitive inflammatory conditions.
Chapter 6:

General Discussion and Conclusions

In this chapter, I discuss the novel observations from my investigations of the mechanisms of neonatal *Chlamydia* respiratory infection-induced chronic lung disease, and adult *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, steroid-insensitive asthma. I will also discuss how these findings highlight the therapeutic potential of targeting key infection-induced molecules and pathways for the treatment of *Chlamydia* and *Haemophilus* respiratory infection-induced sequelae.
6.1 Significance of research

*Chlamydia* respiratory infections have been widely linked with the development and exacerbation of asthma, particularly more severe forms of asthma, in both children and adults. Murine models of *Chlamydia* respiratory infection and AAD were used to investigate the mechanisms that underpin the association between infection and asthma.

I have made important and novel observations that demonstrate how neonatal *Chlamydia* infection results in chronic lung disease. I show that neonatal *Chlamydia* infection induces a group of 5 miRNAs (miR-155, miR-21, miR-223, miR-146b and miR-203) during infection and that the therapeutic inhibition of each miRNA can prevent the development of key disease features, including lung inflammation and histopathology, persistent AHR, emphysema-like alveolar enlargement and increased severity of AAD in later life.

I have also made important and novel observations that further our understanding of the mechanisms that underpin the association between *Chlamydia* respiratory infection and severe, steroid-insensitive asthma. I demonstrate that *Chlamydia* respiratory infection in established AAD induces a miR-21/PI3K/pAkt/HDAC2 signalling axis to promote severe, steroid-insensitive AAD. Importantly, the therapeutic inhibition of *Chlamydia*-induced miR-21 and/or PI3K signalling restores sensitivity to steroid treatment. Additionally, the therapeutic inhibition of miR-21 also suppresses the key features of *Haemophilus* respiratory infection-induced, severe, steroid-insensitive AAD.

I also demonstrate that *Chlamydia* respiratory infection induces an NLRP3 inflammasome/Caspase-1/IL-1β signalling axis to promote severe, steroid-insensitive AAD. Importantly, the therapeutic inhibition of *Chlamydia*-induced NLRP3
inflammasome and/or Caspase-1 and/or IL-1β signalling restores sensitivity to steroid treatment. Furthermore, the therapeutic inhibition of each component of this axis also suppresses the key features of *Haemophilus* respiratory infection-induced, severe, steroid-insensitive AAD.

It is very difficult to model all facets of severe asthma in mouse models including the number and intensity of exacerbations and history of near-fatal attacks and hospitalisations, however, our model does reproduce many of the hallmark features of severe asthma in humans. These include, innate immune activation, presence of respiratory infections, increased T_H1- and T_H17-associated responses, increased neutrophilic inflammation, increased IL-1β and inflammasome responses, and decreased macrophage function (data obtained in our laboratory in separate studies).

My studies further our understanding of the role of neonatal *Chlamydia* respiratory infection in the development of chronic lung disease and severe asthma in later life, and the roles of *Chlamydia* and *Haemophilus* respiratory infections in promoting severe, steroid-insensitive asthma. Importantly, my studies suggest that therapeutically targeting key *Chlamydia* and *Haemophilus* respiratory infection-induced factors in the lung may be effective for the prevention and/or treatment of severe asthma.

### 6.2 *C. muridarum* and *H. influenzae* respiratory infections in mice

In order to examine the mechanisms that are induced by *Chlamydia* and *Haemophilus* respiratory infections it is necessary to develop appropriate mouse models that recapitulate the features of human disease.
We modelled *Chlamydia* respiratory infection using the natural mouse chlamydial pathogen, *C. muridarum* (Nigg and Eaton, 1944; Gogolak, 1953), which is the most appropriate strain to use in the examination of host-pathogen interactions in mice. Several studies have shown that *C. muridarum* generates a productive respiratory infection in mice using low initial numbers of infectious bacteria (Yang et al., 1996; Yang et al., 1999; Horvat et al., 2007; Qiu et al., 2008; Horvat et al., 2010a; Horvat et al., 2010b; Starkey et al., 2012; Starkey et al., 2013a; Starkey et al., 2014). Respiratory infection of mice with *C. muridarum* results in robust monocytic and neutrophilic airway inflammation and the development of regions of lung consolidation, with the peaks of infection and infection-induced inflammation at approximately 10dpi and 14dpi, respectively (Yang et al., 1996; Yang et al., 1999; Horvat et al., 2007). This phenotype closely resembles the immunological features, and pathophysiological time course, of human disease (Horvat et al., 2007). In contrast, *C. pneumoniae*, which is not a natural mouse *Chlamydia* pathogen, does not generate a productive infection and requires high levels of bacteria to induce immune responses in mice, often in the absence of clinical symptoms (Masson et al., 1995; Naiki et al., 2005; Campbell et al., 2008; Penttila et al., 2008).

We modelled *Haemophilus* respiratory infection in mice using a clinical isolate of non-capsulated (non-typeable) *H. influenzae* (NTHi) (Pittman, 1931). NTHi has a high prevalence in the community, causes lower respiratory tract infections and is a significant cause of community-acquired pneumonia (Murphy, 2003). Respiratory infection of mice with NTHi results in a productive infection, the recruitment and activation of neutrophils and is well established as an appropriate strain to use in the examination of host-pathogen interactions in mice (Craig et al., 2001; Murphy, 2003; Essilfie et al., 2011; Essilfie et al., 2012).
Thus, we conclude that \textit{C. muridarum} and NTHi are highly appropriate for studying host-pathogen interactions in mouse models of human disease.


d. A key subset of five miRNAs modulate the severity of neonatal \textit{Chlamydia} respiratory infection

We have previously demonstrated that neonatal \textit{Chlamydia} respiratory infection in mice results in chronic respiratory pathology, including persistent AHR and emphysema-like alveolar enlargement (Horvat et al., 2010b; Starkey et al., 2014) and this has since been confirmed by others (Jupelli et al., 2011). We also demonstrated that neonatal \textit{Chlamydia} respiratory infection increased the severity of Ova-induced AAD in later life by increasing airway-associated MSC numbers and lung IL-13 expression, and further increased Ova-induced AHR. In my PhD studies I have extended these findings and have demonstrated for the first time that miR-155, miR-21, miR-223, miR-146b and miR-203 promote key features of neonatal \textit{Chlamydia} respiratory infection-induced lung disease both during infection and in later life. The therapeutic inhibition of each of these miRNAs \textit{in vivo} using miRNA-specific antagonirs differentially suppressed infection-induced airway inflammation, gross lung histopathology, persistent AHR, emphysema-like alveolar enlargement and the increased severity of AAD in later life.

These are novel observations that broaden our understanding of the mechanisms that underpin the host immune response to neonatal \textit{Chlamydia} respiratory infection. These miRNAs, or combinations of these miRNAs, may also be important in \textit{Chlamydia} genital tract infection-induced disease as well as in other neonatal respiratory infections associated with the development of asthma. Our findings highlight the potential relevance of therapeutically targeting specific
miRNAs during early life respiratory infection in humans to support, or as an alternative to, treatment with antibiotics to suppress infection-induced disease.

6.3.1 miRNAs promote more severe neonatal *Chlamydia* respiratory infection

miRNAs are highly conserved, small, non-coding RNAs that potently regulate gene expression at the post-transcriptional level and can modulate numerous biological responses, including the immune response (Baltimore et al., 2008; Lodish et al., 2008). Increasing evidence shows that miRNAs are involved in both positive and negative feedback mechanisms and their expression patterns can be altered by infections (Cremer et al., 2009; Liu et al., 2009; Xiao et al., 2009; Xiao and Rajewsky, 2009; Schulte et al., 2011; Izar et al., 2012). Importantly, miRNAs have been shown to broadly affect pathways that modulate innate and adaptive immune responses, which highlights the potential pathogenic consequences of altering their expression for prolonged periods. Thus, it is possible that the protracted profile of overexpression of miRNAs during neonatal *Chlamydia* respiratory infection may have permanent influences on the developing lung and immune system.

I show that neonatal *Chlamydia* respiratory infection induces a profile of increased expression of 5 miRNAs (miR-155, miR-21, miR-223, miR-146b and miR-203) in the lungs. Importantly, the increase in the expression of these miRNAs overlaps with the historic peaks of infection (10dpi) and infection-induced airway inflammation and histopathology (14-15dpi) that we have observed in this model (Horvat et al., 2007; Horvat et al., 2010b).

Significantly, miR-155, miR-21, miR-223 and miR-146b are known modulators of immune responses. TLR ligands and pro-inflammatory cytokines can induce miR-155 and miR-146 expression in DCs, macrophages and T cells (Taganov
et al., 2006; O'Connell et al., 2007; O'Connell et al., 2010), and these miRNAs as well as miR-21 are important in the macrophage inflammatory response to infections (Taganov et al., 2006; O'Connell et al., 2007; O'Connell et al., 2010; Sheedy et al., 2010). miRNAs also play important roles in granulocyte development and function. Studies have shown that the sustained overexpression of miR-155 in vivo in mouse bone marrow increased the number of immature granulocytes in a similar manner to injection with LPS (O'Connell et al., 2008; O'Connell et al., 2009). In contrast, several studies have shown that TLR-dependent miR-155, miR-21 and miR-146 expression can also negatively regulate inflammatory pathways in myeloid cells (Taganov et al., 2006; Hou et al., 2009; Ruggiero et al., 2009; O'Connell et al., 2010; Sheedy et al., 2010). For example, several studies have shown that miR-155 negatively regulates the expression and activity of SHIP, which is an important endogenous negative regulator of PI3K and TLR4 signalling (Sly et al., 2004; Costinean et al., 2009; O'Connell et al., 2009). Thus, miR-155 can promote inflammatory responses through the liberation of Akt activation, however, Akt signalling can also suppress macrophage-derived miR-155 expression (Androulidaki et al., 2009). These reciprocal functions suggest the presence of a negative feedback loop. Others have shown that the myeloid transcription factors PU.1 and CCAAT/enhancer-binding protein-β (CEBPB) induce the expression of miR-223 (Fukao et al., 2007) and that miR-223 null mice have increased numbers of hyperactive neutrophils, spontaneously develop inflammatory lung pathology, and have evidence of increased lung tissue destruction following challenge with LPS (Johnnidis et al., 2008). Collectively, these studies suggest that miRNAs are also important in the resolution of inflammatory responses and highlight their role in fine-tuning the magnitude and length of the immune response to infection. Thus, I performed proof-of-principle experiments to assess the potential
pathogenic roles of overexpressed miR-155, miR-21, miR-223, miR-146b and miR-203 in our model of neonatal Chlamydia respiratory infection. Neonatal mice infected with Chlamydia were treated with miRNA-specific inhibitors (antagomirs) in vivo and the effects on weight gain, airway inflammation, lung histopathology, persistent AHR, emphysema-like alveolar enlargement and increased severity of AAD in later life were assessed.

We previously showed that neonatal Chlamydia respiratory infection reduced the rate of weight gain and induced pulmonary inflammation (Horvat et al., 2007). I show that inhibition of miR-155 or miR-223 partially improved the reduced rate of weight gain in infected, neonatal mice, suggesting that lung-expressed miR-155 and miR-223 may be important in generating clinical signs of disease in early life Chlamydia infection. In contrast, inhibition of miR-21, miR-146b or miR-203, had no beneficial effects on infection-induced suppression of rate of weight gain. Surprisingly, the inhibition of each miRNA suppressed Chlamydia-induced airway inflammation, particularly macrophages, and gross lung histopathology at 14dpi back to levels observed in sham-infected mice. These data indicate that these 5 miRNAs play important, individual roles in the recruitment of inflammatory cells, especially macrophages, to the lungs and in the development of lung histopathology during neonatal infection. However, these data may also suggest that reduced weight gain during neonatal Chlamydia infection is not a consequence of miRNA-dependent inflammatory cell influx into the lungs during infection. Therefore, it is possible that miR-155 and miR-223 could have complex interactions with other pathways that regulate weight gain during neonatal development. Furthermore, it will be informative to assess the impact of miRNA inhibition during neonatal Chlamydia infection on
*Chlamydia* numbers at 10dpi (historic peak of infection). Due to time constraints these were not explored in my PhD studies.

### 6.3.1.1 Increased miR-155 expression during neonatal *Chlamydia* infection promotes emphysema-like alveolar enlargement in later life

We have previously shown that neonatal, but not infant or adult, *Chlamydia* respiratory infection results in emphysema-like alveolar enlargement that persists into later life (Horvat et al., 2010b; Starkey et al., 2014). Others have confirmed these observations by showing that *C. muridarum* challenge of 1-day old, but not 7-day old, BALB/c pups significantly reduced radial alveolar counts at 8 weeks of age (Jupelli et al., 2011). Taken together, these studies indicate that respiratory infections may cause permanent damage to lung structure if they occur during the critical phase of postnatal alveolarisation. We recently showed that neonatal *Chlamydia* infection induces the apoptosis of alveolar epithelial cells through a TRAIL-dependent mechanism and that this promotes emphysema-like alveolar enlargement (Starkey et al., 2014). Interestingly, the administration of recombinant-TRAIL or anti-death receptor (DR)5 agonistic antibody to neonatal WT mice, in the absence of infection, did not result in emphysema-like alveolar enlargement, suggesting that both signals need to be induced concurrently by infection to promote this phenotype. These are important findings that highlight the potential role of infection-induced apoptosis in the development of emphysema-like alveolar enlargement. However, they do not exclude the possibility that neonatal *Chlamydia* respiratory infection may interfere with processes that drive alveolarisation and normal lung development during this critical postnatal period.
I demonstrate an alternative neonatal *Chlamydia* infection-induced, miR-155-dependent mechanism underpinning the development of emphysema-like alveolar enlargement. Human term neonates possess approximately 15-20% of their adult alveolar complement (Langston et al., 1984; Burri, 2006), suggesting that alveolarisation is largely a postnatal event in humans. In contrast, rodents are born without alveoli but like humans undergo postnatal development of the lungs and alveoli (Burri, 2006; Padela et al., 2008). This raises several important points for consideration when interpreting my findings in the context of the developing neonatal lung. I have shown that *Chlamydia* infection at all ages rapidly increases the expression of miR-155 in the lungs, suggesting that miR-155 is important in the innate immune response to *Chlamydia* infection irrespective of the age of infection. However, my data demonstrate that *Chlamydia*-induced miR-155 responses are detrimental to the developing lung during neonatal *Chlamydia* infection. Several studies have identified targets and immune functions for miR-155 in a variety of cell types and its induction has been linked with TLR-mediated inflammatory responses to infection and the differentiation of immune cells (Rodriguez et al., 2007; Vigorito et al., 2007; Dorsett et al., 2008; Rai et al., 2008; Teng et al., 2008; Ceppi et al., 2009). However, the putative functions of miR-155 in lung development and tissue regeneration remain poorly understood despite evidence that it is expressed by, and induced in, fibroblasts and the mesenchymal cells that surround the lung epithelium (Martin et al., 2006; Stanczyk et al., 2008; Pottier et al., 2009). Collectively, these data may suggest that *Chlamydia* induces the expression of miR-155 in cells of non-myeloid origin, such as epithelial cells and fibroblasts, in the developing neonatal lung and that this may affect processes outside of immunological decision-making in
response to infection, including those required for successful post-natal
alveolarisation.

Recent studies have shown that FGF-7 is endogenously expressed in the lung
mesenchyme, is a potent mitogen for type II pneumocytes, and is required for early
lung organogenesis and postnatal alveolarisation (Bottaro et al., 1990; Panos et al.,
1993; Post et al., 1996; Yano et al., 2000; Padela et al., 2008). Importantly, FGF-7
mRNA possesses a functional binding site for miR-155 in its 3’ UTR (Pottier et al.,
2009). Significantly, we show that neonatal Chlamydia infection increases miR-155
expression and concomitantly decreases FGF-7 expression in the lungs at 10dpi. Most
significantly, the inhibition of miR-155 during neonatal infection restores FGF-7
expression and prevents the development of emphysema-like alveolar enlargement in
later life. Taken together, these data suggest that Chlamydia-induced miR-155
expression interferes with postnatal alveolarisation in the lungs of infected neonates
and that this may occur through the targeted disruption of FGF-7.

Another point to consider is that miR-155 has been shown to interfere with
TRAIL and Fas-mediated apoptosis by directly targeting Caspase-3 and/or FADD,
respectively (Ovcharenko et al., 2007; Wang et al., 2011). Thus, our findings with
miR-155 appear to contradict our earlier report on the role of TRAIL in neonatal
Chlamydia infection (Starkey et al., 2014). Importantly, however, we showed that
TRAIL is increased by neonatal infection at 15dpi, which is a time point 10 days after
miR-155 is robustly induced in the lungs. Collectively, these data suggest that
neonatal Chlamydia infection may induce emphysema-like alveolar enlargement via a
number of distinct mechanisms; the first of which involves miR-155-mediated
disruption of alveologenesis from 5dpi onwards in the developing lung, which
precedes TRAIL-mediated apoptosis of alveolar epithelial cells from 15dpi onwards.
Thus, the association between miR-155-mediated disruption of alveologenesis and TRAIL-induced apoptosis of alveolar epithelium necessitates further investigation. Furthermore, it is possible that neonatal *Chlamydia* infection-induced miR-155 is required to trigger responses that indirectly lead to the induction of TRAIL. Due to time constraints this was not explored in my PhD studies.

6.3.1.2 **Increased miR-155, miR-21 and miR-203 in neonatal *Chlamydia* infection are critical mediators of infection-induced AHR and increased severity of AAD in later life**

   Indeed, we have previously shown that early life, but not adult, *Chlamydia* respiratory infection induces persistent AHR and enhances the severity of AAD in later life (Horvat et al., 2007; Horvat et al., 2010b). I show, through targeted antagomir treatment against specific miRNAs, that neonatal *Chlamydia* respiratory infection-induced expression of miR-155, miR-21 and miR-203 promotes the development of persistent AHR, and infection-enhanced AHR in AAD, in later life. Importantly, administration of antagomirs to neonatal mice only occurred during the first 15 days of infection, which suggests that it is the early infection-induced expression of these miRNAs that exerts permanent influences on the immunological milieu in developing mice.

   Emerging evidence suggests that miR-155 and miR-21 play roles in immunological processes that are associated with the development of asthma. In one study Martinez-Nunez *et al.* found that miR-155 regulated IL-13-dependent responses through direct binding and inhibition of IL-13Rα1 in human macrophages, which led to reduced STAT6 activation and decreased expression of IL-13-dependent genes (Martinez-Nunez et al., 2011). Indeed, we previously showed that IL-13 and STAT6
are required for the development of persistent AHR following Chlamydia respiratory infection in infancy, however, this phenotype is induced by decreased decoy receptor IL-13Rα2 rather than increased production of IL-13 (Starkey et al., 2013a). Significantly, Wu et al., found that miR-21 was dose-dependently increased by IL-13 in in vitro cultures of bronchial epidermal cells isolated from asthmatics (Wu et al., 2014), and Lu et al., showed that the IL-13-induced expression of miR-21 is largely dependent on the activation of IL-13Rα1 (Lu et al., 2009). Others have shown that miR-21 directly targets IL-12p35, which is decreased in IL-13 transgenic mice. Importantly, the targeted inhibition of miR-21 in mice increased the levels of IFNγ (Lu et al., 2009; Lu et al., 2011). Collectively, these studies suggest that Chlamydia-induced miR-155 and miR-21 potentially exert many complex and often opposing immunological effects during neonatal infection. This may explain why neonatal Chlamydia infection is associated with mixed T<sub>H1</sub>/T<sub>H2</sub> Ova-specific helper T cell cytokine responses and increased severity of hallmark features of asthma, including mucous hypersecretion and AHR, in AAD in later life (Horvat et al., 2007).

Several studies have identified putative functions for miR-203, however, the understanding of its role in inflammatory processes is limited. Sonkoly et al. were the first to show that miR-203 is enriched in keratinocytes and that the overexpression of miR-203 in human psoriatic plaques is associated with decreased expression of SOCS-3, which may promote inflammatory responses (Sonkoly et al., 2007). Others have shown that SOCS-3 deficiency is associated with the chronic activation of IL-6-induced STAT3 signalling and suggest that this may drive the development of psoriatic plaques (Croker et al., 2003; Lowes et al., 2007; Sonkoly et al., 2007). This miR-203-dependent pro-inflammatory mechanism is potentially applicable for neonatal Chlamydia respiratory infection, however, this was not explored in my
studies. Another consideration is that *Chlamydia*-induced miR-203 expression may interfere with the development of the pseudo-stratified epithelium in the neonatal airways through the targeted disruption of p63, which is critical for the development of stratified epithelium. Significantly, p63 is expressed in bronchial stem cells (Wang et al., 2002a), regulates stem-cell maintenance in stratified epithelium, and is a validated target of miR-203 (Yi et al., 2008). Therefore, it is possible that the *Chlamydia*-induced overexpression of miR-203 in the developing lung may be detrimental to the normal development of the airway epithelium, which may predispose to more severe respiratory infections and the development of inflammatory airway diseases, including asthma.

In conclusion, miR-155-, miR-21- and miR-203-induced responses have been shown to be important in several facets of lung development and immune responses. Thus, in certain infections (such as *Chlamydia* infection) therapeutics that attenuate the expression of each, or combinations of, these miRNAs may be broadly beneficial. Targeted miRNA-specific therapeutics may prevent immediate tissue pathology and attenuate aberrant immune responses, which may protect against severe inflammatory disease in later life. However, this strategy would need to be considered and implemented with caution in order to preserve the function of processes that drive normal tissue development and immune function. The study described in Chapter 3 was a pilot study that aimed to identify the miRNAs that are induced, and are important, in neonatal *Chlamydia* respiratory infection. Through proof-of-principle experiments I have identified functionally important miRNAs that can be targeted during neonatal *Chlamydia* infection to protect against infection-induced chronic lung disease. Characterisation of the mechanisms of action of the identified miRNAs, as
well as the identification of their direct mRNA targets, was not fully explored in my PhD studies due to time constraints, however, this will be a priority of future studies.

6.4 *Chlamydia* and *Haemophilus* respiratory infections induce severe, neutrophilic, steroid-insensitive AAD

We previously showed that *Chlamydia* and *Haemophilus* respiratory infections during sensitisation to Ova results in exaggerated Th1 and/or Th17 immune responses and neutrophilic airway inflammation in Ova-induced AAD (Horvat et al., 2010a; Essilfie et al., 2011; Essilfie et al., 2012). We also showed that the combination of *Haemophilus* infection and AAD promotes bacterial persistence and a phenotype that resembles steroid-insensitive, neutrophilic asthma (Essilfie et al., 2012). These findings further our understanding of numerous clinical studies that associate *Chlamydia* and *Haemophilus*, and other, respiratory infections with non-eosinophilic forms of asthma that are refractory to steroid treatment. Wark *et al.*, found that exacerbating asthmatics with evidence of *Chlamydia* respiratory infection have increased numbers of neutrophils in the lungs (Wark et al., 2002), and others have shown that asthmatics with evidence of *Chlamydia* infection are resistant to steroid treatment (Cho et al., 2005; Patel et al., 2010). In fact, airway neutrophil numbers predicted the presence of *Chlamydia* respiratory infection in a cohort of asthmatics with severe refractory asthma (Patel et al., 2010). Also, *H. influenzae* is one of the most commonly isolated bacterial pathogens from the lungs of non-eosinophilic asthmatics. Wood *et al.*, found that 60% of stable asthmatics with bacterial colonisation in their airways were culture-positive for *H. influenzae* and that these individuals were more likely to have non-eosinophilic asthma and be on high doses of inhaled steroids (Wood et al., 2010).
In Chapters 4 and 5 of this Thesis I expand on these clinical and our previous experimental observations by developing novel models of infection-induced AAD that more accurately reflect the human scenario. We propose that it is respiratory infection in patients with established asthma that drives the development of severe, neutrophilic, steroid-insensitive asthma. Therefore, in order to model infection in established asthma I overlayed respiratory infection with *Chlamydia* or *Haemophilus* in mice with established Ova-induced AAD. The phenotype of AAD in the acute Ova model wains over time (data not shown - part of a separate manuscript not presented in this Thesis) and so I recapitulated the phenotype with two additional Ova challenges 19 days after infection. Importantly, this model enabled us to assess the impact of a resolved respiratory infection on established AAD. Hallmark disease features were assessed on day 35 of the study protocol in the absence, or presence, of treatment with steroid (DEX). Using these models, I have demonstrated, for the first time, novel mechanisms that underpin how *Chlamydia* and *Haemophilus* respiratory infections promote the development of severe, neutrophilic, steroid-insensitive AAD. Using several approaches I have shown that *Chlamydia* and *Haemophilus* infections can induce two distinct signalling axes with previously unrecognised pathogenic roles in steroid-insensitive AHR and airway inflammation in AAD. These include the miR-21/PI3K/pAkt/HDAC2 and NLRP3 inflammasome/Caspase-1/IL-1β signalling axes.

Importantly, my work demonstrates the potential relevance of therapeutically targeting these signalling axes as improved options for treating severe, steroid-insensitive asthma in humans.
6.4.1 Infection-induced miR-21 promotes severe, steroid-insensitive, neutrophilic AAD

In Chapter 2, I show that neonatal, infant and adult *Chlamydia* respiratory infections commonly induce increases in the expression of miR-21 in the lungs, which potentially indicates that miR-21 is important in the general immune response to *Chlamydia* infection but may also play a role in *Chlamydia*-induced lung disease. Thus, I next sought to assess the role of miR-21 in our novel models of *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, neutrophilic, steroid-insensitive AAD. I demonstrate (in Chapter 4) that *Chlamydia* infection-induced miR-21 expression in the lungs is not suppressed by steroid treatment and drives disease by amplifying PI3K activity that suppresses HDAC2. Using a combination of miR-21-specific antagonirs and the pan-PI3K inhibitor, LY29, I show that inhibition of miR-21 or PI3K reversed the suppression of PTEN and HDAC2, and restored steroid sensitivity of airway inflammation and AHR. These data suggest that the sustained up-regulation of miR-21 plays an important role in inducing steroid-insensitive airway inflammation and AHR in *Chlamydia*-induced, severe, steroid-insensitive AAD. I also showed that inhibition of miR-21 in *Haemophilus*-induced, severe, steroid-insensitive AAD, like in *Chlamydia*-induced, severe, steroid-insensitive AAD, restored steroid sensitivity to airway inflammation and AHR. In summary, my study demonstrates for the first time that miR-21 promotes steroid-insensitive airway inflammation and AHR in respiratory infection-induced, severe, steroid-insensitive AAD.

Of the several miRNAs that have been implicated in asthma pathogenesis, miR-21 in particular is consistently shown to be important in mouse models of AAD (Lu et al., 2009; Mattes et al., 2009). miR-21 is robustly up-regulated during
pulmonary inflammation and several studies have shown its role in suppressing T\(_1\) immune responses through the direct inhibition of IL-12p35, and in promoting T\(_2\) responses and eosinophilic inflammation (Lu et al., 2009; Lu et al., 2011; Sawant et al., 2013). IL-13 also plays an important role in the up-regulation of miR-21 expression (Lu et al., 2009; Lu et al., 2011; Wu et al., 2014). Collectively, these studies indicate that miR-21 is important for T\(_2\) immune responses and, consequently, the focus of miR-21 studies in asthma have been in delineating its role in immune polarisation during allergic sensitisation (Lu and Rothenberg, 2013). However, given that miR-21 has multiple mRNA targets, other than IL-12p35, it is likely that the overexpression of this miRNA exerts effects beyond promoting T\(_2\) immune responses. I show (in Chapter 4) that Chlamydia respiratory infection results in an increase in miR-21 expression on day 32 of our model of AAD, which represents the time point immediately prior to recapitulation of disease when steroid administration commenced. Importantly, this is 18 days after Chlamydia infection when infection-induced airway inflammation is at baseline levels prior to re-challenge with Ova, suggesting that the Chlamydia-induced, and sustained, overexpression of miR-21 is likely crucial in driving the severe, neutrophilic, steroid-insensitive phenotype in response to airway allergen exposure. Furthermore, Chlamydia-induced, severe, steroid-insensitive AAD is characterised by exaggerated expression of a broad range of T\(_1\)- and T\(_17\)-associated factors and the suppression of T\(_2\)-associated factors (data not shown - part of a separate manuscript not presented in this Thesis) in the lungs on day 35, which possibly indicates that miR-21 is not strongly inducing, or induced by, T\(_2\)-associated immune responses in severe, steroid-insensitive AAD.

Significantly, I show that Chlamydia-induced miR-21 expression on day 32 is accompanied by concomitant decreases in GR-\(\alpha\), PTEN and HDAC2 mRNA
expression. Several studies have shown that miR-21 directly inhibits PTEN, which normally antagonises PI3K activity (Meng et al., 2007; Yamanaka et al., 2009; Miletic et al., 2010). These data indicate that *Chlamydia*-induced miR-21 expression might potentiate PI3K-dependent signalling, which is strongly associated with inflammation and AHR in AAD and infection-induced neutrophilic inflammation (Newcomb et al., 2008). I show that *Chlamydia*-infected mice have increased levels of nuclear pAkt, a well-established marker of PI3K-dependent signalling (Lee et al., 2006; Newcomb et al., 2008). Importantly, several studies show a PI3K-mediated reduction in HDAC2 activity as a key mechanism of steroid insensitivity in COPD and that the non-selective inhibition of PI3K, using LY29, restores HDAC2 activity and sensitivity to steroids in a cigarette smoke-induced model of COPD (Barnes, 2004; Ito et al., 2006c; Ito et al., 2006b; Barnes and Adcock, 2009; Marwick et al., 2009; To et al., 2010). Also, smoke-exposed PI3Kδ kinase dead knock-in transgenic mice had normal sensitivity to steroids and reduced tyrosine nitration of HDAC2 (Marwick et al., 2009; To et al., 2010). Collectively, these studies suggest that sustained overexpression of miR-21 may result in the inhibition of PTEN and the subsequent amplification of PI3K-dependent signalling, which can decrease HDAC2 activity and lead to steroid insensitivity.

In conclusion, I have defined the functional relevance of infection-induced activation, and maintenance, of a miR-21/PI3K/pAkt/HDAC2 signalling axis in a previously unrecognised pathogenic role in steroid insensitivity. It is possible that sustained activation of this signalling axis plays an important role in severe, steroid-insensitive asthma. Thus, the use of targeted therapeutics for the inhibition of miR-21 may be an effective strategy to reinstate steroid sensitivity in severe, steroid-insensitive asthma, and potentially other steroid-insensitive diseases, including...
COPD, IBDs and arthritis (Barnes, 2004; Barnes et al., 2004; Barnes, 2008b; Barnes, 2009; Barnes and Adcock, 2009; Takagi et al., 2010; Iliopoulos et al., 2011; Paraskevi et al., 2012; Svrcek et al., 2013).

### 6.4.2 Infection-induced increases in NLRP3 inflammasome activity and IL-1β responses promote severe, steroid-insensitive, neutrophilic AAD

In **Chapter 2**, we also show for the first time that neonatal, infant and adult *Chlamydia* respiratory infections commonly induce robust increases in the expression of 15 genes in the lungs at 10dpi (historic peak of infection (Horvat et al., 2007)). I hypothesised that these genes, or combinations of these genes, play important roles in the immune response to *Chlamydia* respiratory infection irrespective of the age of infection, through the signalling pathways that they induce and/or mediate. I also considered the possibility that *Chlamydia*-induced overexpression of these genes can drive exaggerated immune responses to infection that may result in pathology and promote features of *Chlamydia*-induced lung disease. Importantly, this gene list includes a group of 5 genes that are associated with NLRP3 inflammasome activation and/or severe, steroid-insensitive asthma in humans (i.e. Caspase-1, IL-1β, SAA3, TLR2 and TNF-α). Furthermore, in **Chapter 4**, I showed that *Chlamydia* infection-induced, severe, steroid-insensitive AAD is associated with increases in the mRNA expression of IL-1β, TLR2 and TNF-α at day 35.

Inflammasome activation and the excess production of IL-1β are strongly implicated in the pathogenesis of severe, steroid-insensitive asthma (Konno et al., 1996; Wanderer, 2009; Hastie et al., 2010; Baines et al., 2011; Besnard et al., 2012; Kim et al., 2014; Simpson et al., 2014). Inflammasomes are comprised of several signalling complexes that induce the maturation of IL-1β and IL-18 (Martinon et al.,
Thus, inflammasome activation is likely to be important in diseases that are associated with elevated IL-1β responses. Of the recognised inflammasomes, the NLRP3 inflammasome is the best characterised and widely implicated in inflammatory diseases, including severe, steroid-insensitive asthma (Simpson et al., 2014). Importantly, however, it is unclear as to how these responses are induced in severe, steroid-insensitive asthma and whether these responses drive the development of this phenotype of asthma. We propose that it is respiratory infection in patients with established asthma that promotes the development of this form of the disease. TLR ligation in response to stimulation with PAMPs is important in initiating the expression and assembly of inflammasome components (Schroder and Tschopp, 2010). Thus, many studies use TLR agonists and/or pro-inflammatory cytokines, such as LPS and TNF-α, respectively, to prime for inflammasome activation (Schroder and Tschopp, 2010). Importantly, both TLR2 and TLR4 are required in the recognition of, and response to, Chlamydia and Haemophilus respiratory infections (Prebeck et al., 2001; Wang et al., 2002b; Wieland et al., 2005; Rodriguez et al., 2006; He et al., 2011). We previously showed that TLR2 is important in the response to early life Chlamydia respiratory infection (Beckett et al., 2012). Others have shown that TLR4 is critical for effective innate immune responses to Haemophilus respiratory infection (Wang et al., 2002b; Wieland et al., 2005). Significantly, both Chlamydia and Haemophilus respiratory infections are linked with severe, steroid-insensitive asthma and have been shown to induce NLRP3 inflammasome-dependent, Caspase-1-mediated IL-1β responses (Wark et al., 2002; He et al., 2010; Patel et al., 2010; Wood et al., 2010; Rotta Detto Loria et al., 2013). Thus, in our models, Chlamydia and Haemophilus respiratory infections serve as the priming agents for inflammasome assembly. Importantly, these are non-
synthetic, biologically relevant stimuli and are more representative of what is likely to occur in humans. Following its assembly the NLRP3 inflammasome is activated by DAMPs, including extracellular ATP, which activates the P2X7R (Burnstock and Knight, 2004; Ferrari et al., 2006; Lich et al., 2006; Mariathasan et al., 2006; Sutterwala et al., 2007). Importantly, ATP and the P2X7R are increased in asthma and the inhibition of ATP-mediated P2X7 signalling suppresses disease in experimental asthma (Idzko et al., 2007; Muller et al., 2011). Furthermore, these factors are important in host defence against infections, including Chlamydia, and potentially Haemophilus, infections (Darville et al., 2007; Rotta Detto Loria et al., 2013). Thus, in Chapter 5, I investigated the role of NLRP3 inflammasome responses in our novel models of Chlamydia and Haemophilus respiratory infection-induced, severe, steroid-insensitive asthma. I demonstrate for the first time that Chlamydia and Haemophilus respiratory infections induce an NLRP3 inflammasome/Caspase-1/IL-1β signalling axis in the lungs to promote severe, steroid-insensitive AAD. Initially, I showed that inhibition of lung IL-1β with α-IL-1β suppressed Chlamydia and Haemophilus infection-induced, steroid-insensitive airway inflammation and AHR in AAD in the absence, and presence of steroids. Next, using a combination of Caspase-1 (ZVAD and YVAD) and NLRP3 inhibitors (MCC950) in vivo, I demonstrate that infection-induced Caspase-1- and NLRP3-dependent responses play important and connected roles, and are required for infection-induced IL-1β responses in, severe, steroid-insensitive AAD. In summary, this study demonstrates for the first time that respiratory infection-induced NLRP3 inflammasome activity critically promotes steroid-insensitive airway inflammation and AHR in AAD and that this occurs through the activation of Caspase-1, which in turn activates IL-1β. These data suggest that Chlamydia and Haemophilus respiratory infections in asthma induce aberrant
priming and activation of the NLRP3 inflammasome, which then potently induces IL-1β responses through activation of Caspase-1. Significantly, and to the best of our knowledge, this study is the first to show a potential causal relationship between respiratory infection-induced activation of an NLRP3 inflammasome/Caspase-1/IL-1β signalling axis and severe, steroid-insensitive asthma.

Most significantly, these data suggest that therapeutic strategies that target this axis may be effective in lieu of steroid treatment in severe forms of asthma that are associated with aberrant NLRP3 inflammasome activity and/or IL-1β responses. Furthermore, targeting the NLRP3 inflammasome/Caspase-1/IL-1β signalling axis may also be an alternative approach to the treatment of other steroid-insensitive inflammatory diseases, including COPD, IBD and arthritis (Barnes, 2004; Barnes et al., 2004; Barnes, 2008b; Barnes, 2009; Barnes and Adcock, 2009; Takagi et al., 2010; Iliopoulos et al., 2011; Paraskevi et al., 2012; Svrcek et al., 2013).

6.5 Future directions

6.5.1 Elucidating the mechanisms that underpin the effects of miR-155, miR-21, miR-223, miR-146b and miR-203 in neonatal Chlamydia respiratory infection-induced chronic lung disease

I have shown novel roles for 5 miRNAs (i.e. miR-155, miR-21, miR-223, miR-146b, miR-203) in promoting the key features of neonatal Chlamydia respiratory infection-induced chronic lung disease. I am now pursuing experiments to further these novel findings.

The studies outlined in Chapter 3 did not determine the effects of inhibiting each miRNA on Chlamydia load in the lungs at 10dpi, which is the time point that represents the historic peak of infection in this model (Horvat et al., 2007). To address
this I will isolate DNA from the lungs of \textit{Chlamydia}-infected neonatal mice that have been treated with miRNA-specific antagonirs and determine \textit{Chlamydia} numbers by real-time qPCR, as previously described (Horvat et al., 2010a; Horvat et al., 2010b; Asquith et al., 2011). This analysis will potentially identify the specific miRNAs that are required for the survival or resolution of \textit{Chlamydia} infection in the lungs and may be informative for characterising the miRNA-dependent mechanism(s) that underpin infection.

I have demonstrated that all five of these miRNAs are important in neonatal \textit{Chlamydia} infection-induced airway inflammation and gross lung histopathology at 14dpi. Initially, this suggests that targeting any of these miRNAs may be effective in treating neonatal infection. However, I show that the \textit{Chlamydia}-induced overexpression of each of these miRNAs has unique effects on the development of long-term disease features. This is unsurprising since each miRNA possesses a unique array of gene targets with minimal overlap and suggests that they regulate different biological pathways during infection. To address this, I will perform a bioinformatics-based, cross-examination of my miRNA and gene expression microarray data to search for validated and \textit{in silico}-predicted mRNA targets of miR-155, miR-21, miR-223, miR-146b and miR-203. I will also pursue separate \textit{in silico} analyses using several computational miRNA target prediction algorithms. Initially, the TargetScan program will be used to identify potential mRNA targets based on perfect seed sequence complementarity to the 3’ UTR of mRNAs, as described previously (Lewis et al., 2003; Krek et al., 2005). However, this approach is restrictive and does not account for imperfect complementarity and/or seed mismatches and/or binding sites outside of the 3’ UTR. Thus, I will also perform analyses using Microcosm and PITA, as previously described (John et al., 2004; Kertesz et al., 2007; Forman and Coller,
2010; Moretti et al., 2010). As mentioned in Chapter 3, my data suggest that neonatal infection-enhanced AHR in AAD in later life may be a summative consequence of infection-induced persistent AHR and Ova-induced AHR and that this effect is dependent on *Chlamydia*-induced miR-155, miR-21 and miR-203. Thus, these new studies will be fundamental for the delineation of the effects of *Chlamydia*-induced overexpression of miR-155, miR-21 and miR-203 on the development of persistent AHR and increased severity of AAD in later life.

We previously showed that *Chlamydia* infection of neonatal mice suppresses airway-associated eosinophilic inflammation and increases airway-associated MSC numbers in AAD in later life (Horvat et al., 2010b). Thus, to expand the findings of the current study, I will assess the impact of inhibiting miR-155, miR-21, miR-223, miR-146b and miR-203 during neonatal *Chlamydia* infection on inflammatory cell populations (by performing differential leukocyte counts on BAL) and airway-associated MSC hyperplasia in AAD in later life. I will also assess specific cell populations via flow cytometry to interrogate the changes in inflammation, and the cytokines and chemokines being produced, in BAL and homogenised lung tissue. Additionally, I will perform experiments to determine whether neonatal *Chlamydia* respiratory infection promotes features of airway remodelling in later life and investigate the potential role(s) of these miRNAs in the development of these features.

I also showed that *Chlamydia* respiratory infection-induced, emphysema-like alveolar enlargement was dependent on miR-155, and that the *Chlamydia*-induced overexpression of miR-155 was associated with a decrease in mRNA expression of FGF-7 in the lungs. I will perform FGF-7 immunoblot analyses at 10dpi to assess the lung protein levels of FGF-7 during neonatal *Chlamydia* infection. I will also perform immunohistochemical analyses at 10dpi on histological sections from lungs to assess
the tissue localisation of FGF-7 during normal development as well as during neonatal *Chlamydia* infection. I will also assess the impact of reinstating FGF-7 levels in the lungs during neonatal infection on the development of the key features of *Chlamydia*-induced chronic lung disease through the i.n. administration of recombinant mouse FGF-7. Additionally, I will assess the impact of selectively depleting FGF-7 levels in the lungs of neonatal mice through the i.n. administration of FGF-7-specific siRNA.

An additional experiment will also be performed in order to investigate the effects of increased miR-155, miR-21, miR-223, miR-146b and miR-203 in sham-infected WT BALB/c mice to determine the effects of these miRNAs on lung structure and function and severity of AAD in later life in the absence of infection. These studies will be carried out by i.n. administration of miRNA-specific mimetics to neonatal mice and assessing the effects on lung histology, inflammation and function in later life in the presence and absence of AAD. These will be informative experiments that may define the role(s) of specific miRNAs in promoting key disease processes that are also potentially relevant in other early life respiratory infections.

It will also be informative to identify the cellular source(s) of neonatal *Chlamydia* respiratory infection-induced increases in miR-155, miR-21, miR-223, miR-146b and miR-203. To address this I will perform miRNA *in situ* hybridisation on histological sections from lungs collected at 5 and 10 days post neonatal *Chlamydia* infection. Performing these experiments at 5dpi and 10dpi will allow for the delineation of whether it is resident lung cells and/or infiltrating inflammatory leukocytes that express these miRNAs. This will assist in characterising the important cell types and miRNA-mediated mechanisms that underpin neonatal *Chlamydia* respiratory infection-induced chronic lung disease and severe asthma.
6.5.2 Further investigation into the role of miR-21/PI3K/pAkt/HDAC2 signalling in *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, steroid-insensitive AAD

I developed novel mouse models of steroid-insensitive, neutrophilic (i.e. non-eosinophilic) asthma by combining *Chlamydia* or *Haemophilus* respiratory infections and Ova-induced AAD. Additional experiments that I will perform include conducting protein assays to complement the qPCR data showing that *Chlamydia* infection-induced, severe, steroid-insensitive AAD is characterised by exaggerated expression of a broad range of T\(_H\)1- and T\(_H\)17-associated factors and the suppression of T\(_H\)2-associated factors (data not shown - part of a separate manuscript not presented in this Thesis) in the lungs on day 35.

Based on the outcomes of my miRNA and gene expression microarray analyses, I next assessed the role of a novel *Chlamydia* respiratory infection-induced miR-21/PI3K/pAkt/HDAC2 signalling axis in severe, steroid-insensitive AAD. I demonstrate that infection induces a miR-21-dependent, PI3K-mediated signalling pathway that suppresses nuclear HDAC2 to promote steroid-insensitive airway inflammation and AHR in AAD.

I then demonstrated a widespread role for miR-21 in the induction of severe, steroid-insensitive asthma by showing that treatment with Ant-21 also restored steroid sensitivity in *Haemophilus*-induced, severe, steroid-insensitive AAD. Additional experiments that I will perform include the administration of LY29 during *Haemophilus*-induced, severe, steroid-insensitive AAD and examination of the effects of both Ant-21 and LY29 treatments on nuclear levels of pAkt and HDAC2.

My study did not assess HDAC2 activity in *Chlamydia*- and *Haemophilus*-induced, severe, steroid-insensitive AAD. To address this I will immuno-precipitate...
HDAC2 from nuclear and cytoplasmic protein fractions isolated from the lungs of Chlamydia- and Haemophilus-infected mice in AAD and measure HDAC2 activity, as described previously (Ito et al., 2005; To et al., 2010). I will also assess the impact of Ant-21 and LY29 treatment on HDAC2 activity in infection-induced, severe, steroid-insensitive AAD.

The next step will be to identify the lung compartment and/or cellular source(s) of Chlamydia or Haemophilus infection-induced miR-21 expression in severe, steroid-insensitive AAD. To address this I will perform miRNA in situ hybridisation on histological sections from lungs collected on days 24 and 35 of the study protocol. Day 24 of the protocol is 10 days after Chlamydia infection, which is the historic peak of infection in our mouse model (Horvat et al., 2007) and therefore the most appropriate time point to assess Chlamydia-induced expression of miR-21. Furthermore, performing these assays at day 35 will demonstrate whether the source of miR-21 expression during Chlamydia-induced, severe, steroid-insensitive AAD differs from AAD. I will then compare the findings to miR-21 in situ hybridisations performed on lung histological sections from Haemophilus-induced, severe, steroid-insensitive AAD.

I am also investigating the broader effects of treatment with Ant-21 and LY29 on the expression of a range of chemokines and Th1-, Th2- and Th17-associated cytokines in Chlamydia and Haemophilus respiratory infection-induced, severe, steroid-insensitive AAD. I will perform multiplex cytokine arrays on protein isolated from whole lung homogenates at day 35 of the study protocol. These will be informative experiments that will characterise any similarities, or differences, in chemokine and/or cytokine responses that are associated with the restoration of steroid sensitivity and suppression of disease in infection-induced, severe, steroid-
insensitive AAD following Ant-21 and LY29 treatments. Importantly, these analyses may identify other factors that may be therapeutically targeted in infection-induced, severe, steroid-insensitive AAD.

I am currently investigating whether *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, steroid-insensitive AAD is associated with increased airway remodelling, specifically α-smooth muscle actin and collagen deposition in the basement membrane and *lamina reticularis*. Thus, to expand the findings of the current study, I will assess the role of miR-21/PI3K/pAkt/HDAC2 signalling in airway remodelling events in infection-induced, severe, steroid-insensitive AAD.

An additional experiment will also be performed in order to assess the impact of inhibiting *Chlamydia*-induced miR-21 expression during infection, rather than on day 32 at the time point of steroid administration. This will determine whether targeting infection-induced miR-21 expression during ongoing infection can prevent the development of severe, steroid-insensitive AAD. These are relevant experiments that may highlight a strategy whereby asthmatics with evidence of respiratory infection can be pre-treated to prevent the induction of steroid insensitivity.

Furthermore, we will assess the role of miR-21/PI3K/pAkt/HDAC2 signalling in new experiments that omit the systemic route of allergic sensitisation and use a more clinically relevant allergen such as HDM. These studies, whilst not feasible within the timeframe of this Thesis, will be informative and performed in the near future.

I will also investigate the key factors in the miR-21/PI3K/pAkt/HDAC2 axis in clinical samples collected by collaborators in the clinical respiratory team at the University of Newcastle/John Hunter Hospital/Hunter Medical Research Institute, Newcastle, Australia.
6.5.3 Further investigation into the role of NLRP3 inflammasome/Caspase-1/IL-1β signalling in *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, steroid-insensitive AAD

I showed through gene expression microarray analyses that *Chlamydia* respiratory infection increases the expression of Caspase-1, IL-1β, SAA3, TLR2 and TNF-α at 10dpi, irrespective of the age of infection. Additional *in silico* analyses of these genes, using the DAVID functional annotation tool, revealed that these factors are associated with NLRP3 inflammasome activation and severe, steroid-insensitive asthma in humans.

Importantly, respiratory infections with *Chlamydia* and *Haemophilus* are also associated with severe, steroid-insensitive asthma (Wark et al., 2002; Cho et al., 2005; Patel et al., 2010; Wood et al., 2010) and induce NLRP3 inflammasome-dependent, Caspase-1-mediated IL-1β responses (Wark et al., 2002; He et al., 2010; Patel et al., 2010; Wood et al., 2010; Rotta Detto Loria et al., 2013). Thus, I investigated the role of the NLRP3 inflammasome in our models of *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, steroid-insensitive AAD. I showed that both *Chlamydia* and *Haemophilus* respiratory infections induce steroid-insensitive airway inflammation and AHR in AAD through an NLRP3 inflammasome/Caspase-1/IL-1β signalling axis.

I showed that *Chlamydia* and *Haemophilus* respiratory infections elevate total IL-1β levels in the lungs during AAD. However, these data do not prove that *Chlamydia* and *Haemophilus* infections increase the cleavage and maturation of pro-IL-1β in AAD. Thus, I am currently optimising immunoblot assays for IL-1β to assess lung protein levels of pro-IL-1β versus mature IL-1β in *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, steroid-insensitive AAD. I will also assess the
impact of treatment with α-IL-1β, YVAD and MCC950 on pro-IL-1β versus mature IL-1β levels in infection-induced, severe, steroid-insensitive AAD.

In future studies I will identify the cellular source(s) of infection-induced NLRP3 inflammasome activity in our models of severe, steroid-insensitive AAD. I am currently optimising immunofluorescence-based NLRP3 assays on BAL cytopsins taken from Chlamydia- and Haemophilus-infected mice in AAD. Interestingly, a recent study identified macrophages as a major source of NLRP3 in the sputum of severe asthmatics (Simpson et al., 2014). Thus, I am performing in vitro cell culture experiments involving Chlamydia (live and UV-inactivated) infection of several immortalised macrophage cell lines (i.e. WT, TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, NLRP3<sup>−/−</sup> and Caspase-1<sup>−/−</sup>). Initially, I will assess whether Chlamydia infection induces IL-1β production and/or activation in macrophages. I will then use the knockout lines (TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, NLRP3<sup>−/−</sup> and Caspase-1<sup>−/−</sup>) to determine the roles of these factors in Chlamydia infection-induced IL-1β production and/or activation in macrophages. I will also assess the impact of MCC950 treatment of Chlamydia-infected WT macrophages on IL-1β production and/or activation in macrophages.

I am also investigating the broader effects of treatment with α-IL-1β, YVAD and MCC950 on the expression of a range of chemokines and Th1-, Th2- and Th17-associated cytokines in Chlamydia and Haemophilus respiratory infection-induced, severe, steroid-insensitive AAD. Initially, I will examine the impact of treatment with MCC950 at 10mg/kg (in the presence of DEX) on total IL-1β levels in the lungs during Chlamydia infection-induced, severe, steroid-insensitive AAD. I will then perform multiplex cytokine arrays on protein isolated from whole lung homogenates at day 35 of the study protocol. These will be informative experiments that will characterise any similarities, or differences, in chemokine and/or cytokine responses
that are associated with the restoration of steroid sensitivity and suppression of
disease in infection-induced, severe, steroid-insensitive AAD following α-IL-1β,
YVAD and MCC950 treatments. Importantly, these analyses may identify other
factors that may be therapeutically targeted in infection-induced, severe, steroid-
insensitive AAD.

Additionally, I will examine the potential immunological interplay between
the miR-21/PI3K/pAkt/HDAC2 and NLRP3/Caspase-1/IL-1β axes in our models.
These will be informative experiments that may identify novel signalling pathways
and/or potential redundancies between the two signalling axes.

As mentioned earlier, I am currently investigating whether Chlamydia and
Haemophilus respiratory infection-induced, severe, steroid-insensitive AAD is
associated with increased α-smooth muscle actin and collagen deposition in the
airway basement membrane and lamina reticularis. Thus, to expand the findings of
the current study, I will assess the role of NLRP3 inflammasome/Caspase-1/IL-1β
signalling in airway remodelling events in infection-induced, severe, steroid-
insensitive AAD.

I will also investigate the key factors in the NLRP3/Caspase-1/IL-1β axis in
clinical samples collected by collaborators in the clinical respiratory team at the
University of Newcastle/John Hunter Hospital/Hunter Medical Research Institute,
Newcastle, Australia.

6.5.4 Targeting Chlamydia and Haemophilus infections in order to prevent severe
lung disease

Currently, no human Chlamydia or NTHi vaccines are available despite the
prevalence of these infections in the community. Thus, treatment of infection with
antibiotics represents one possible strategy for protection against infection-associated sequelae. In unpublished mouse studies we have shown that treatment with macrolide antibiotics is effective for suppressing inflammation and AHR in Chlamydia and Haemophilus respiratory infection-induced, severe, steroid-insensitive AAD without the need for steroids, and treatment with amoxicillin restores steroid sensitivity (original research article currently in peer review). However, the development and administration of anti-Chlamydia and anti-Haemophilus vaccines would be the most effective strategy for the prevention of Chlamydia and Haemophilus respiratory infection-induced lung diseases, respectively.

In other unpublished mouse studies we have shown that immunisation of naïve mothers with an experimental Chlamydia vaccine decreases the severity of features of neonatal Chlamydia infection, including Chlamydia load and lung inflammation at 10dpi, and persistent AHR and increased severity of AAD in later life. Therefore, it is possible that a pro-active therapeutic strategy involving maternal immunisation with an anti-Chlamydia vaccine may provide protection for progeny against Chlamydia-induced lung disease.

We previously showed in a mouse model that an i.n. delivered pneumococcal 7-valent polysaccharide conjugate vaccine (derived from components of Streptococcus pneumoniae) suppressed the hallmark features of Ova-induced AAD, including Th2 responses, mucous hypersecretion, eosinophilic airway inflammation and AHR (Thorburn et al., 2010). Importantly, given that NTHi does not possess a polysaccharide capsule it is necessary to explore other compartments of NTHi to identify relevant immunogens. NTHi outer membrane proteins are a potentially important source of immunogens for the development of a vaccine. However,
progress in this area is hampered due to the low conservation of these proteins across strains (Kyd and Cripps, 1999).

6.5.5 Investigation of the role of miR-155 in the pathogenesis of Chlamydia respiratory infection-induced, severe, steroid-insensitive AAD and investigation of other infections

My miRNA microarray analyses also showed that Chlamydia respiratory infection robustly increases the expression of miR-155, irrespective of the age of infection. Importantly, miR-155 potentiates PI3K activity by suppressing the activity of SHIP (Yamanaka et al., 2009), which normally antagonises PI3K activity via PI3K dephosphorylation. Thus, I am also investigating the role of miR-155 in Chlamydia respiratory infection-induced, severe, steroid-insensitive AAD.

My gene expression microarray analyses showed that neonatal, infant and adult Chlamydia respiratory infections commonly induce increases in the expression of IFN-γ, STAT1, CXCL-9 and CXCL-10. I also showed that Chlamydia infection-induced, severe, steroid-insensitive AAD is associated with exaggerated expression of these factors. Indeed, others have demonstrated that Chlamydia respiratory infections induce strong Th1 responses and increase STAT1 signalling (Geng et al., 2000; Yang et al., 2008). Importantly, STAT1 signalling is induced by IFN-γ and is important for the expression of several pro-inflammatory genes, including CXCL-9 and CXCL-10 in AAD (Fulkerson et al., 2004). Thus, I am also investigating the role of miR-155 in the context of STAT1-dependent signalling in Chlamydia respiratory infection-induced, severe, steroid-insensitive AAD.

Numerous studies have shown that steroid treatment has limited efficacy in controlling asthma that is associated with viral infections, including influenza
infections (Wark et al., 2001; Hansbro et al., 2008). Thus, we have also established a mouse model of influenza respiratory infection-induced, severe, steroid-insensitive asthma. I will extend our studies by assessing the role of a miR-21/PI3K/pAkt/HDAC2 signalling axis in our novel mouse model of influenza respiratory infection-induced, severe, steroid-insensitive asthma.

6.6 Conclusion

My studies indicate that neonatal *Chlamydia* respiratory infections, as well as adult *Chlamydia* and *Haemophilus* respiratory infections in established asthma, may play roles in the development of chronic lung diseases, including severe asthma. Using representative mouse models, I have identified and validated key roles for 5 miRNAs in neonatal *Chlamydia* respiratory infection-induced inflammation and chronic lung disease and infection-induced severe AAD in later life. I also developed novel mouse models of *Chlamydia* and *Haemophilus* respiratory infection-induced, severe, steroid-insensitive asthma. Using these models I identified and validated the role of a novel miR-21/PI3K/pAkt/HDAC2 signalling axis in infection-induced, severe, steroid-insensitive AAD. Additionally, I have also identified and validated the role of a novel NLRP3 inflammasome/Caspase-1/IL-1β signalling axis in infection-induced, severe, steroid-insensitive AAD. Importantly, I demonstrated that the factors I have identified can also be targeted therapeutically for the prevention/treatment of infection-induced severe, steroid-insensitive AAD and my findings highlight that these factors may be applicable targets for novel therapeutic strategies in human disease.
7. References


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