Novel therapeutic approaches for the treatment of allergic airways disease

Adam Michael Collison

B Biomed Sci (Hons)

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School of Biomedical Sciences and Pharmacy

University of Newcastle

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Statement of originality

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

Adam Collison
Acknowledgement of Authorship

I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of my thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publications.

Adam Collison
Contribution to Papers:

Chapter 3:

Joerg Mattes and Paul Foster initially conceptualised and with Adam Collison designed experiments. Joerg Mattes and Adam Collison co-ordinated mouse models and collected samples with Max Plank.

Figure 3.1: Adam Collison conducted miRNA specific qPCR A and B.

Figure 3.2: Adam Collison performed airways resistance measurement and with Joerg Mattes analysed data A, B and C. Together they collected and analysed BALF D, E and F.

Figure 3.3: Adam Collison ran mouse models and with Joerg Mattes collected an analysed data from qPCR A, airways reactivity B, BALF C, and histology D,E and F.

Figure 3.4: Adam Collison with Joerg Mattes collected samples then analysed cytokines by ELISA A. Adam Collison conducted FACS analysis to determine cellular profile. Joerg Mattes analysed array data identifying OBF and Adam Collison ran confirmatory qPCR D.

Joerg Mattes
Chapter 4:

Joerg Mattes and Paul Foster initially conceptualised and with Adam Collison designed experiments. Adam Collison co-ordinated mouse models and collected samples with Joerg Mattes and Max Plank.

Figure 4.1: Adam Collison performed miRNA specific qPCR B, C and D.

Figure 4.2: Adam Collison performed histological analysis A, B, C, D, E, F, and G.

Figure 4.3: Adam Collison conducted airways resistance analysis A, B and C.

Figure 4.4: Adam Collison performed cytokine measurement by ELISA A, B and C and qPCR D and E.

Supplementary Figure 4.1: Adam Collison performed miRNA specific qPCR.

Supplementary Figure 4.2: Adam Collison performed BALF analysis A and B.

Supplementary Figure 4.3: Adam Collison performed histological A and B and resistance analysis C.

Joerg Mattes
Chapter 5:

Chronic aerosolised ovalbumin induced allergic airways disease models were run in the laboratory of Rakesh Kumar at the University of New South Wales. Adam Collison ran and analysed miRNA microarrays with samples obtained from these experiments, designed and tested the antagomir against identified targets. Phenotypic effects of antagomir were quantified by Cristan Herbert.

Table 5.1: Adam Collison performed and analysed miRNA microarray.

Figure 5.1: Adam Collison conducted miRNA specific qPCR to confirm array targets

Figure 5.2: Phenotypic measures post antagomir treatment were conducted by Cristan Herbert A, B, C and D

Figure 5.3: qPCR of miRNA target was conducted by Cristan Herbert.

Joerg Mattes
Chapter 6:

Adam Collison ran the mouse model, extracted RNA and with Joerg Mattes analysed the microarray data that identified Midline 1 as a TRAIL regulated protein. Together they designed the allergic mouse experiments and with Luke Hatchwell extended this work into Rhinovirus models. Together Adam Collison and Luke Hatchwell conducted mouse studies as detailed below.

Peter Wark and Melinda Tooze performed and supervised studies on healthy and asthmatic subjects and performed associated cell culture experiments. Nicole Verrils and Helen Carpenter performed and analysed PP2Ac measurements and immunoprecipitation and contributed to experiment design. Anthony Don synthesised the AAL(s). Nives Zimmerman and Marc Rothenberg conducted the initial transcriptome microarray. Nathan Bartlett and Sebastian Johnson assisted in the design of Rhinovirus experiments and provided RV1B for further propagation. Ana Pereira de Siqueira and Paul Foster assisted in the coordination and supervision of mouse and human studies. Joerg Mattes conceptualized and supervised the studies.

Figure 6.1: Together Adam Collison and Luke Hatchwell ran mouse models conducted qPCR and immunohistochemistry analysis A, C, D and I. Adam Collison conducted resistance analysis E. Luke Hatchwell conducted PP2A activity assays B and H, histological F and cytokine analysis G.

Figure 6.2: Luke Hatchwell conducted PP2a activity assays A, BALF and cytokine analysis D,E and F. Adam Collison conducted resistance analysis C and with Luke Hatchwell co-ordinated and analysed qPCR data B.
Figure 6.3: **Adam Collison** performed resistance measurement and analysis **A and F**. Luke Hatchwell performed BALF analysis **B and G**, PP2a activity assays **C and H**. Together **Adam Collison** and Luke Hatchwell co-ordinated and analysed qPCR **D** and conducted multiplex cytokine analysis **E and I**.

Figure 6.4: A B C D E

Supplementary figure 6.1: **A Adam Collison** performed airways resistance measurement and analysis. Luke Hatchwell conducted **B BALF analysis C histology analysis and cytokine analysis D and E**.

Supplementary figure 6.2: Luke Hatchwell and Helen Carpenter conducted Western Blotts

Supplementary figure 6.3: Luke Hatchwell and Stuart Reeves conducted qPCRs in **A, B, C and D**

Joerg Mattes
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This thesis is the culmination of the most significant body of work I have yet undertaken. Ultimately it represents the long awaited end to my formal education and the beginning of my independent research career. I have only been able to arrive here with the assistance of many others, who have seen fit to reach into my life with something of their own.

Firstly I would like to thank Alice, specifically thankyou for all the times you have so patiently allowed me to put my work ahead of our life. For the weekends and late nights that you have been put off, thankyou – I could never have continued down this path without your honest and freely given support, despite what it costs you.

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My extended family have also been fundamental, the active interest and support offered to me through my life to date has been of greater value to me than you know. Auntie Linda and Wim, Bestmate, Grandma and Grandpa thanks.

I have been fortunate to be part of a vibrant research group who were happy to help with advice and assistance at many many points along the way. Joerg has been a great supervisor through both my honours and PhD, thankyou for the keen interest you have shown in our research projects, your consistent enthusiasm has been a driving force in the completion of this work and it is much valued. I look forward to being part of your research team into the future. Paul also has my thanks, not only for his advice as a supervisor for both my honours and PhD but for creating the environment in his lab with the friendly and co-operative mindset that I have enjoyed for the past 5 years.

Others have been invaluable to me at the coalface. I would particularly like to thank Luke and Max for the various help offered along the way – particularly providing an extra set of skilled hands on lengthy sacrifice days. Thanks to Ana for helping me wade through red tape more often than we would have liked – your support was more valuable than you believe. To Dicky as well for helpful advice and open experience along the way. Also Stuart, Shannon and Fiona for their various technical assistance, particularly with the running of arrays.

The pre-eminent gratitude that I would hope to express in both my life and work is to my Saviour and Lord Christ Jesus. This world is His and everything in it, I hope that my usage of the abilities He has gifted me would always point to and glorify His great name.
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Abstract of thesis

This thesis presents studies on novel therapeutic interventions for the treatment of allergic asthma providing proof of concept through extensive investigations in mouse models of human disease. The current increase in the incidence of asthma worldwide along with inability of current medications to treat the primary causes of the disease indicates that novel therapeutic approaches are required. This will improve the quality of life and disease burden concerns of the community. I investigated two alternate therapeutic approaches in an effort to identify new candidate targets with significant therapeutic potential.

The first research chapter (see chapter 3) presents an initial study on the role of miRNA in the development of allergic airways disease (AAD). This study also demonstrates proof-of-concept for the use of modified, cholesterol conjugated complementary sequences termed antagomirs to specifically inhibit the expression of miR-126 in the airways and to alleviate AAD.

The second research chapter (see chapter 4) presents a comparative study in which treatment of allergic mice with an antagomir suppressing miR-145 is compared to mice treated with the current gold standard therapy, systemic glucocorticoids. Here it is demonstrated that the novel therapeutic approach of selectively inhibiting the upregulation of miR-145 in the airway wall is as potent as treatment with systemic dexamethasone to alleviate AAD.

The third research chapter (see chapter 5) presents a study where miR-126 was inhibited in a chronic model of AAD. The findings of this study confirm an important role of miR-126 in the regulation of allergic airways inflammation but suggest that in this model miR-126-independent mechanisms promote the
development of tissue remodelling, hallmark features of chronic asthma. These results suggest that targeting a single miRNA may not be sufficient to reduce all aspects of AAD.

The fourth research chapter (see chapter 6) presents a study of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induced signalling pathway in AAD and rhinovirus (RV) -induced exacerbation of AAD. Here I identify a novel role for TRAIL induced Midline-1 (Mid1) driven polyubiquination and silencing of the Protein Phosphatase 2a (PP2a). Furthermore, blocking this signalling pathway through either the silencing of Mid1 with siRNA or the synthetic reactivation of PP2a using the small molecule AAL(S) was capable of alleviating AAD and RV-induced exacerbation. This study provides proof-of-concept that modulation of the TRAIL induced signalling pathway may provide therapeutic benefit in the treatment of AAD.

Together these studies have investigated novel and relevant targets for therapeutic intervention in AAD. By targeting immuno-regulatory systems such as miRNAs and TRAIL regulated signalling cascades at the initial site of allergen exposure –the airway surface, these approaches have the potential to successfully modulate the complex aberrant immune response that initiates and underpins allergic asthma.
Publications

The following publications have arisen from data presented in the current thesis:

**Research publications:**


*authors contributed equally to this paper

**Review publications:**


Conference publications:

2011 American Thoracic Society International Conference
“The development of House Dust Mite induced allergic airways disease is regulated by a novel E3 ubiquitin ligase-dependent deactivation of a protein phosphatase”

2011 Thoracic Society of Australia and New Zealand Annual Scientific Meeting
“The development of House Dust Mite induced allergic airways disease is regulated by a Midline-1 dependent deactivation of PP2a”
Adam Collison

2010 AusBiotech Annual Scientific Meeting
“Inhibition of a novel ubiquitin ligase using siRNA as a novel therapeutic approach for asthma”
Adam Collison

2010 Australasian Society of Immunology Annual meeting
“The development of House Dust Mite induced allergic airways disease is dependent on the ubiquitin ligase Midline-1”
Adam Collison, Luke Hatchwell, Ana Pereira de Siqueira, Nicole Verrills, Paul Foster, Joerg Mattes

2009 Australasian Society of Immunology Annual meeting
“The role of MicroRNA 145 in Allergic Airways Disease”
A Collison, J Mattes, M Plank, PS Foster

2009 Australasian Society of Immunology Annual meeting
“Inhibition of MicroRNA-126 suppresses TH2 cell effector function and the development of allergic airways disease”
A Collison, J Mattes, M Plank, PS Foster.

2009 American Thoracic Society International Conference
“The Identification of TRAIL as a Mediator of Airways Remodelling in a Chronic Model of Murine Allergic Airways Disease”
A Collison, M Plank, A Pereira de Siqueira, S Reeves, S Phipps, PS Foster, J Mattes

2009 American Thoracic Society International Conference
“MicroRNAs Are Crucial in the Development of Airways Hyperreactivity” A Collison, J Mattes, M Plank, PS Foster

2008 Australasian Society of Immunology Annual meeting
“TRAIL is a crucial regulator of airways remodelling in a mouse model of chronic allergic airways disease”
A Collison, M Plank, A Pereira de Siqueira, S Reeves, S Phipps, PS Foster, J Mattes
**List of abbreviations**

AAD – allergic airways disease
AEC – airway epithelial cell
AHR – airways hyperreactivity
AP – activator protein
CCL – chemokine ligand
CxCL – chemokine (c-X-c motif) ligand
CMV - cytomegalovirus
DC – dendritic cell
DGCR8 - DiGeorge syndrome critical region gene 8
DISC - death-inducing signalling complex
Drosha - ribonuclease 3
ERK- extra- cellular signal-regulated kinase
FADD - fas-associated death domain
FasL - CD95 / fas ligand / Apo1 ligand
FEV₁ – forced expiratory volume in one second
FLICE - FADD-like interleukin-1b-converting enzyme
FLIP – FLICE-inhibitory protein
FOXp3 - forkhead box P3
GATA3 – GATA binding protein 3
HDM - house dust mite
HIV - human immunodeficiency virus
HPF – high power field
HSUR- Herpes virus saimiri noncoding uridine-rich RNA
HPA - hypothalamic-pituitary-adrenal axis
ICS- inhaled corticosteroids
IFN - interferon
Ig – immunoglobulin
siRNA – short interfering ribonucleic acid
SOCS1 – suppressor of cytokine signalling 1
SOX - sex determining region Y –box
STAT - signal transducer and activator of transcription
TAB2 - mitogen-activated protein kinase kinase kinase 7 binding protein 2
TCR - T-cell receptor
Th - T helper
TLR - toll like receptor
TNF- tumour necrosis factor
TRAIL - tumour necrosis factor – related apoptosis - inducing ligand
TRIF - TRI-domain containing adapter-inducing interferon-β
T-regs - regulatory T-cells
Chapter 1: General Introduction

Some sections of this chapter were published in Clinical and Experimental Pharmacology and Physiology 2009 doi: 10.1111/j.1440-1681.2009.05258.x
1.1 Asthma pathogenesis – a brief overview

Asthma is a chronic disease of the lung that is characterised by airways hyperreactivity (AHR), reversible airflow obstruction and airways inflammation.\(^1-4\) The incidence and morbidity of asthma have been increasing rapidly in the Western world over the past two decades\(^5\) although more recent evidence suggests that incidence may have reached a plateau in the mid to late 1990s\(^6-8\). One out of five children in Australia suffer from asthma making it the most common chronic disease in childhood that persists into adulthood in the majority of cases\(^9\). Current therapies focus on treating symptoms rather than disease aetiology, highlighting the importance to identify disease mechanisms and to develop novel therapeutic approaches\(^3,4,10\). Unfortunately, asthma aetiology and pathogenesis is thought to be extremely complex, involving the interaction of environmental and genetic factors that underpin the development of persistent airway inflammation and obstruction\(^3,11\).

The primary symptoms of asthma are recurrent episodes of wheezing, coughing, breathlessness and chest tightness\(^5\). It is commonly accepted that asthma is related to an inflammation of the airways, with the histopathologic features including multicellular infiltration, injury of the airway epithelium, apoptosis, and subepithelial fibrosis\(^1,2\). Traditionally an influx of eosinophils, lymphocytes and in some cases neutrophils can be observed along with activated degranulating mast cells\(^1,2,12,13\). More recently the predominant inflammatory cell type found in airway secretions of asthmatics has been used to categorise them into different subphenotypes, for example neutrophilic, eosinophilic, or paucigranulocytic\(^14,15\). It has been demonstrated that inflammation correlates
with the development of AHR and has a prominent role in airflow obstruction, suggesting that inflammation is a significant contributor to the diseased state\textsuperscript{1,2}.

The allergic asthmatic response to allergens can be categorised into two phases. The early phase, which initiates within minutes of allergen exposure, is primarily a bronchospasasmic response of the respiratory muscles. This phase is characterised by an increase in smooth muscle tone leading to the narrowing of the airways in conjunction with the rapid onset of mucosal oedema. These immediate symptoms have been associated with mast cell factors such as histamine, bradykinin, and leukotrienes released by degranulating cells\textsuperscript{5,16}. The late phase response occurs hours subsequent to allergen exposure and is dominated by an influx of inflammatory cells. The associated narrowing of the airways is in part due to the accumulation of lymphocytes, neutrophils and or eosinophils within the airway epithelium and lung parenchyma as a result of T-cell initiated chemotaxis\textsuperscript{5,14,17}.

### 1.2 Limitations of current asthma therapies

Current treatments for asthma fall into two broad categories, namely anti-inflammatory mediators and bronchodilators. The mode of action of \(\beta\)-agonists as bronchodilators is rather rudimentary, doing nothing to address the disease process, merely attempting to counter its effects upon airway patency. Through selective binding to \(\beta_2\)-adrenergic receptors, intracellular cyclic adenosine levels are elevated, in turn inducing relaxation of the airway smooth muscle. The reduced tone within the smooth muscle acts as an intervention to relieve the constriction of the airways present in acute asthmatic exacerbations, and is effective against both the early and late phases of the asthmatic response. \(\beta\)-
agonists are classified by the duration of their effect. Short-acting β-agonists remain the frontline treatment as rapid-relief to address the immediate airflow limitation associated with an acute exacerbation. Long acting β-agonists are able to provide symptom control for at least 12 hours and are often used to complement inhaled corticosteroids (ICS) in patients whose symptoms are not adequately controlled by reduced inflammation. However, the safety of extended usage and high dose β-agonists remains controversial, as it has been linked to elevated morbidity.

The most common of the anti-inflammatory drugs are ICS, which act to improve ongoing symptoms and prevent asthmatic exacerbations. ICS are the first-line therapy for persistent asthma and they are considered to be the most effective of the treatments currently available. However, the ability of ICS to inhibit the ongoing airway remodelling of asthmatic disease is controversial. Even when delivered orally at considerably higher doses, steroids are unable to adequately modulate the disease phenotype in up to 10% of asthmatics. These patients are said to have steroid resistant or severe refractory asthma and are beyond the scope of current therapies. The side effect profile of high dose corticosteroids is also of concern. Up to 90% of the dose of ICS may be deposited in the mouth and pharynx meaning that these areas are directly affected at both an immunologic and non-immunological level. The net results of these effects are dysphonia, increased susceptibility to Candida colonization, pharyngitis, persistent cough and bronchospasm. ICS and oral administration of synthetic corticosteroids in high doses dysregulate the hypothalamic-pituitary-adrenal (HPA) axis, the natural regulator of glucocorticoid levels. Even a single dose of ICS has been demonstrated to elicit a measureable suppression of the
HPA axis\textsuperscript{23,32}. In severe cases the HPA repression can be sufficient to induce adrenal crisis with children particularly susceptible\textsuperscript{33}. Bone mineral density is also reduced with extended usage of corticosteroids. While the degree to which this effect is elicited by ICS is somewhat controversial\textsuperscript{34}, the weight of evidence suggests that it is indeed a concern at levels commonly used by moderate to severe asthmatics\textsuperscript{23,35,36}. At high doses ICS has also been demonstrated to reduce the synthesis of skin collagen, which in some cases leads to skin thinning and subsequent ecchymosis\textsuperscript{37,38}. Despite the broad and potentially severe side effects of ICS therapy the benefit has repeatedly been shown to outweigh the cost. At a clinical level one study demonstrated that for elderly patients hospitalised for an asthma-related exacerbation there was a 39\% reduction in the risk of death in the following year if ICS were administered and a 29\% relative reduction in the chance of a hospital admission\textsuperscript{39}. However, chronic remodelling of the asthmatic airways still occurs with constant use of corticosteroids even when the inflammatory profile is significantly reduced by treatment\textsuperscript{3,40,41}.

A further class of asthma therapeutics referred to as leukotrine inhibitors act either upon cysteinyl leukotriene production through inhibiting 5-lipoxygenase or by interfering with the leukotrine-receptor interaction as Cys LT1 receptor antagonists\textsuperscript{42}. The action of these inhibitors is effective against the early phase allergic response but does not reduce late phase symptoms or AHR as effectively as ICS\textsuperscript{42-44}. Their ability to reduce eosinophilia of the airways has been demonstrated to match that of ICS \textsuperscript{45}.

Anti-IgE monoclonal antibodies have also been demonstrated to have some effectiveness in the treatment of allergic asthma\textsuperscript{46,47}. However, there have been
several concerns raised relating to possible side effects with this treatment.\textsuperscript{48} Anti-IgE therapy in conjunction with ongoing ICS treatment has been demonstrated to reduce the number of exacerbations, improve asthma symptoms and quality of life in multiple clinical trials\textsuperscript{49-55}. Further to this the required dosage of ICS and bronchodilators was decreased\textsuperscript{48,56}.

Anti-Interleukin (IL) 5 monoclonal antibodies have been trialed in the treatment of asthma, however initial studies showed no significant benefit to patients following treatment despite reduced levels of eosinophils in both the blood and airways\textsuperscript{57,58}. More recent studies focused upon treatment of patients with persistent eosinophilia and asthma in spite of high dose ICS therapy. Within this disease subtype, anti-IL-5 treatment has had considerably more success with a significant reduction in the rates of exacerbation observed. However, while eosinophilia was also reduced other key disease markers such as AHR were unaltered\textsuperscript{58-60}. Similarly humanised monoclonal antibodies targeting IL-13 have also been shown to improve the lung function of patients with poorly controlled asthma. The greatest improvement in the forced expiratory volume in one second (FEV\textsubscript{1}) was seen in patients with elevated periostin prior to the initiation of anti-IL-13 treatment though the number of severe exacerbations was not altered\textsuperscript{61}.

The effectiveness of direct antibody trials in patients with otherwise refractory asthma demonstrates the potential for specific therapies in better managing the diseased airway. However, none of the targets investigated to date have been able to adequately address the symptoms, despite offering benefits beyond conventional ICS and bronchodilator therapies. The narrow scope of the
antibody therapies has meant that they are unable to comprehensively counter established asthmatic disease, which involves the intricate interplay of multiple aberrant innate and adaptive immune pathways. The optimal approach going forward is to focus on the identification of novel targets, which are capable of orchestrating multiple aspects of the immune response. Modulation of these key regulatory systems will allow for the correction of the manifold discords responsible for allergic asthma. The Tumour Necrosis Factor-related apoptosis-inducing ligand (TRAIL) pathway and microRNA (miRNA) are two distinct regulatory frameworks, both of which have been implicated in the development and maintenance of the asthmatic phenotype. This project will focus on elucidating the precise role played by each of these mechanisms within the allergic airways and determine the plausibility of pursuing them as future therapeutic targets.

1.3 Emerging role of TRAIL as a key regulator of inflammatory responses

TRAIL (also called Apo2 ligand) was first cloned by Wiley et al. and later by Pitti et al. and shows sequence homology with CD95 / Fas / Apo1 ligand (FasL) and Tumour Necrosis Factor (TNF). Like other members of the TNF superfamily, TRAIL is a Type II membrane protein, has cysteine-rich pseudo repeats in its extracellular domain and forms a soluble molecule upon protein cleavage. Thus, TRAIL can be bound to the cell surface or secreted as a signalling molecule. It is composed of 281 amino acids and its gene, designated \textit{TNFSF10}, has been located on human chromosome 3 at position 3q26, isolated from other members of the TNF superfamily. The \textit{TNFSF10} gene is 20 kilobases (kb) in length and contains four introns of approximately 8.2, 3.2, 2.3 and 2.3 kb and five exons of
222, 138, 42, 106 and 1245 nucleotides. Unlike TNF-α and FasL, TRAIL does not contain either TATA or CAAT boxes. An apparent binding site for SP1 transcriptional factor, CCAAT / enhancer binding protein, octamer-binding transcription factor(OCT)-1, activator protein (AP)-1 and ets variant 4 has been found in the promoter region\textsuperscript{66}. Although TRAIL induces apoptosis in transformed cells, it leaves most other cells unharmed\textsuperscript{65,67}. In addition, TRAIL may have an important role in the regulation of immune responses, inflammation and allergy. Here, we review our current understanding of the signalling pathways used by TRAIL to regulate apoptosis and inflammation.

1.3.1 Apoptotic TRAIL signalling

Much of the research conducted has focused on the ability of TRAIL to activate the extrinsic apoptotic pathway in immortalized cell lines and primary tumour cells. The apoptotic effects of TRAIL are exerted through the formation of a homotrimer, which interacts with the death receptors DR4 (TRAIL-R1)\textsuperscript{64,68} and DR5 (TRAIL-R2)\textsuperscript{64,69,70}. Binding of TRAIL to DR4 or DR5 recruits Fas-associated death domain (FADD) and procaspase 8 into the death-inducing signalling complex (DISC), which is followed by activation of the caspase cascade (Fig. 1.1)\textsuperscript{71-73}. The TRAIL-activated extrinsic apoptotic pathway and the mitochondrial intrinsic pathway are interconnected via the BH-3 only protein Bid, which is a substrate of caspase 8. Bid is cleaved into its truncated form, which activates Bax and Bak in the mitochondria and leads to the release of cytochrome c and other pro-apoptotic factors.

In addition, TRAIL interacts with the glycol phospholipid-anchored receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4)\textsuperscript{73,74}. The DcR1 receptor is able to bind
TRAIL, but is unable to propagate caspase 8 or apoptotic signalling through displays significant sequence identity with the extracellular domains of the DR4 and DR5 receptors, but has a truncated death domain and is thus also unable to propagate an apoptotic signal. The expression of DcR1 and DcR2 is higher in healthy cells compared with tumour cells. Some studies have reported that over expression of these receptors reduces TRAIL-induced apoptosis\textsuperscript{75}. Thus, DcR1 and DcR2 may act as TRAIL-neutralizing decoy receptors. However, TRAIL signalling through DcR2, as well as through DR4 and DR5, has been shown to activate mitogen-activated protein kinase (MAPK), protein kinase B (PKB) and nuclear factor (NF)-κB, which may promote non-apoptotic or even anti-apoptotic pathways\textsuperscript{76}.

1.3.2 Non-apoptotic TRAIL signalling

Phosphorylation and subsequent degradation of inhibitors of κB (IκB) by IκB kinases (IKK) results in NF-κB activation\textsuperscript{77,78}. Receptor-interacting protein (RIP) is recruited into the TRAIL receptor complex following TRAIL binding and promotes phosphorylation of IKK\textsuperscript{79}, possibly via activation of NF-κB-activating kinase, a member of the MAPK family (Figure. 1.1)\textsuperscript{80}. Accordingly, activation of NF-κB by TRAIL is blocked in IKKc-deficient cells and RIP dominant-negative mutants\textsuperscript{81}. In addition, TRAIL may modify the ubiquitination status of RIP by upregulation of A20, a zinc-finger protein\textsuperscript{82}. Inhibition of apoptosis by caspase inhibitors also promotes NF-κB activation by TRAIL, although the exact signalling pathways have not been identified\textsuperscript{83}. Nuclear factor-κB may have anti-apoptotic functions and this may be determined by the relative amounts of RelA and cRel molecules in the active NF-κB molecule\textsuperscript{84,85}. 
Furthermore, TRAIL can activate other pro-inflammatory intracellular signalling pathways, such as the MAPK and phosphoinositide 3-kinase (PI3-K) pathways. Further investigations into the molecular link between TRAIL and the activation of NF-κB, MAPK and PI3-K signalling pathways may unravel important roles of this cytokine in the regulation of survival and inflammation.

### 1.3.3 Antiviral responses and TRAIL

The expression of TRAIL is activation dependent, with TRAIL expressed by CD11c+ dendritic cells (DCs), airway epithelial cells (AECs), human peripheral blood T lymphocytes, natural killer (NK) cells, eosinophils, neutrophils and monocytes (e.g. in response to cytokine or antigen exposure)\textsuperscript{74,85-89}. Expression of TRAIL is not found at immunologically privileged sites (e.g. brain, testes) and in the liver\textsuperscript{90}. This nearly ubiquitous expression of TRAIL has often been associated with its role in regulating apoptosis, but may also be of relevance for its role in modulating immune responses.

TRAIL is induced by interferon (IFN)-γ and TNF-α after human cytomegalovirus (CMV) infection\textsuperscript{91}, as well as by type I IFN exposure in DCs and activated T cells\textsuperscript{92,93}. Interestingly, DR4/DR5 expression was upregulated by CMV, but only in infected cells, making them more susceptible to TRAIL-induced apoptosis\textsuperscript{91}. Furthermore, CD4+ and CD8+ T cells become susceptible to TRAIL-induced apoptosis following infection with human immunodeficiency virus (HIV)\textsuperscript{94}. During influenza infection of the lungs, TRAIL is highly expressed on the surface of NK cells as well as CD4+ and CD8+ T cells\textsuperscript{95} and TRAIL deficiency is associated with decreased T cell-mediated cytotoxicity and more severe disease\textsuperscript{96}. In contrast, in a lethal i.v. influenza pneumonia model, inhibition of TRAIL protected
mice from severe disease\textsuperscript{97}. Of particular relevance may be TRAIL expression on macrophages in the alveolar space, because they promote apoptosis of both infected and non-infected AECs, leading to alveolar barrier dysfunction in the IV influenza model\textsuperscript{97}. Together, these studies demonstrate that TRAIL is upregulated during the antiviral response and may affect viral infections by promoting apoptosis, which may aid in the resolution of infection but, under certain circumstances, may be detrimental by impairing epithelial function.

1.3.4 Effects of TRAIL on T Cells

Non-activated CD4 positive (+) and CD8+ T cells are resistant to TRAIL-mediated killing \textit{in vitro}, although they express DR4/5\textsuperscript{98,99}. However, activation of T cells with IL-2 resulted in TRAIL susceptibility\textsuperscript{100} and TRAIL caused death of antigen-specific memory CD8+ T cells\textsuperscript{101}. The generation of effective memory CD8+ T cells is thought to be dependent on help from CD4+ T cells, but the underpinning mechanisms are not fully understood. Notably, abrogation of TRAIL restored the function of CD8+ T cells, even if they had developed without CD4+ cell help\textsuperscript{101}. Thus, TRAIL is a negative regulator of cytotoxic CD8+ T cells and, in the absence of TRAIL, memory CD8+ T cells can develop without the help of CD4+ T cells. In addition, TRAIL may be involved in CD4+ T cell responses, because human T helper (Th) 1 cell clones are sensitive to TRAIL-induced apoptosis, whereas Th2 cell lines are not\textsuperscript{102}. Furthermore, inhibition of TRAIL enhanced IFN-\gamma release by resting CD4+ T cells\textsuperscript{103}. Autoimmune diseases are associated with an altered activation of Th and cytotoxic T cells and C57BL/6 mice deficient in TRAIL (\textit{TNFSF10}-/-) have an accelerated development of streptozotocin-induced diabetes and collagen-induced arthritis\textsuperscript{104}. In addition, \textit{TNFSF10}-/- mice may
display a deficit in negative thymocyte selection\textsuperscript{104}, although this finding has not been reported uniformly\textsuperscript{105}.

1.3.5 Emerging role of TRAIL in allergic Inflammation

It has been reported that TRAIL and its receptor systems are upregulated in the respiratory epithelium of asthmatic subjects and that this is correlated with eosinophilic airway inflammation\textsuperscript{106}. Alveolar macrophages and eosinophils from asthmatics also express more TRAIL on their cell surface and TRAIL prolongs the lifespan of eosinophils \textit{in vitro}\textsuperscript{85,106}. Eosinophils are prominent and important effector cells in allergic inflammation, potentially capable of presenting allergen and affecting T cell activation\textsuperscript{107,108}. The link between eosinophils and Th2 cell activation may be crucial in the pathogenesis of asthma and, in the absence of eosinophils (or Th2 cytokines such as IL-13\textsuperscript{109,110}) mice are largely protected from the development of hallmark features of asthma, including AHR, airway epithelial cell mucin accumulation and extracellular matrix changes in the lungs (airway remodelling)\textsuperscript{111-113}. Thus, TRAIL may promote eosinophilia and Th2 cell activation.

In order to determine the role of TRAIL in allergic airway disease, we have recently used BALB/c mice deficient in TRAIL and challenged their lungs with repeated doses of ovalbumin (OVA) after intraperitoneal OVA sensitization\textsuperscript{87}. Although TRAIL- sufficient mice expressed TRAIL in the respiratory epithelium and developed airway inflammation, AHR, mucus hypersecretion and Th2 activation, all hallmark features of asthma were diminished in the genetic absence of TRAIL. Thus, TRAIL expression in the airway wall was both required and sufficient for the development of allergic airway disease, because topical
inhibition by small interfering RNA molecules was therapeutic and instillation of TRAIL into the airways caused experimental asthma. Interestingly, the effect of TRAIL was linked to IL-13 and signal transducer and activator of transcription (STAT) 6 activation, because $\text{IL-13}^{-/-}$-mice were fully protected against the TRAIL-induced effects. In addition, TRAIL directly upregulated chemokine ligand (CCL) 20 release in AECs, promoting recruitment of activated T cells and antigen-presenting DCs into the airways. Thus, TRAIL is a critical regulator of eosinophilic inflammation and allergen-specific T cell activation in the lung. Interestingly, TRAIL also augmented the severity of experimental allergic conjunctivitis\textsuperscript{114}. The receptor systems and the intracellular signalling cascades used by TRAIL to promote inflammation have yet to be identified. In the context of the induction of apoptosis, TRAIL has been shown to activate NF-κB, MAPKs and PI3-K \textit{in vitro}. NF-κB regulates innate and adaptive immune responses and plays a crucial role in GATA binding protein 3 (GATA3) expression, Th2 differentiation and cytokine release in allergic inflammation\textsuperscript{115}. Notably, Tang et al. reported recently that DR4/5 overexpression in immortalized cell lines led to NF-κB-dependent release of CCL20 together with other cytokines (chemokine (c-X-c motif) ligand (CXCL) 8, CXCL2, CCL3 and TNF-α)\textsuperscript{116,117}. Activation of two MAPKs, namely extracellular signal-regulated kinase (ERK) and p38 MAPK, but not C-Jun NH2-terminal kinase (JNK), was found in airway epithelium and smooth muscle cells in biopsy specimens of asthmatics and this activity strongly correlated with disease severity\textsuperscript{118}. Furthermore, inhibition of p38 kinase or ERK1/2 activation precluded the development of hallmark features of experimental asthma\textsuperscript{119,120}. Inhibition or gene targeting of PI3-K and adenovirus-mediated phosphatase and tensin homologue (PTEN) overexpression reduced
Th2 cytokine release, airway inflammation and abolished the development of AHR\textsuperscript{121-123} (for an extensive review of tyrosine kinase signalling in asthma, readers are directed to a recent review by Wong\textsuperscript{124}). Therefore, it is tempting to speculate that TRAIL exploits these pathways to regulate all the downstream hallmark features of allergic airway disease.

\textbf{Figure 1.1}: Binding of Tumour Necrosis Factor-related apoptosis-inducing ligand (TRAIL) to its death receptors (DR)4 (TRAIL-R1) and DR5 (TRAIL-R2) induces apoptosis via recruitment of the Fas-associated death domain (FADD) and procaspase 8 into the death inducing signalling complex (DISC), which is inhibited by FADD-like interleukin-1b-converting enzyme (FLICE)-inhibitory protein (FLIP). Caspase 8 activates the extrinsic and the mitochondrial intrinsic apoptotic pathway. The DcR1 receptor (TRAIL-R3) lacks an intracellular domain and only acts as a decoy receptor for TRAIL. The DcR2 receptor (TRAIL-R4) has a truncated death domain and may also act as a decoy receptor. The DcR2, DR4 and DR5 receptors may also recruit receptor-interacting protein (RIP) into the receptor complex upon TRAIL binding. Receptor-interacting protein phosphorylates (p) I\(\kappa\)B kinase (IKK), which phosphorylates I\(\kappa\)B, leading to its degradation. Degradation of I\(\kappa\)B promotes phosphorylation of nuclear factor (NF)-kappaB. Although TRAIL activates phosphatidylinositol 3-kinase (PI3-K), mitogen-activated protein kinase (MAPK) and protein kinase B (PKB) signalling, the molecular pathways remain poorly defined.
1.3.6 Therapeutic potential of modulating the TRAIL signalling pathway

Recent evidence demonstrating an important role of TRAIL in the regulation of inflammation along with apoptosis (Table 1) suggests that a better understanding of the intracellular signalling pathways downstream of TRAIL activation may lead to novel therapeutic strategies for diseases as diverse as cancer, infections, autoimmunity, asthma and allergies. Of particular relevance is the characterization of the specific receptor systems activated and the pro-inflammatory factors regulated by TRAIL in vivo using specific inhibitors, transgenic mice, transcriptomics and proteomics. I hypothesised that the upregulation of non-apoptotic TRAIL signalling in allergic lung disease presented a potentially novel pathway that could present multiple novel targets for siRNA or small molecule inhibitors to modulate the aberrant inflammatory response that underpins allergic airways disease. The role of TRAIL pathways in the antiviral response is also of interest with recent studies highlighting a significant role for viral infections of the airways and/or the corresponding immune response in exacerbations of asthma\(^{125,126}\).

1.4 Emerging role of microRNAs in disease pathogenesis and strategies for therapeutic modulation

MicroRNA (miRNA) are small noncoding RNAs approximately 22nt in length. They are predicted to exert a regulatory influence over at least half of all human mRNAs at the posttranscriptional level\(^{127}\). Their significance is further highlighted through the stringent conservation of many miRNA sequences from invertebrates through to mammals. The production of mature miRNAs is an intricate process that has many levels of control. miRNA are encoded in the
genomic RNA and are transcribed in an RNA polymerase II dependent manner\textsuperscript{128}. While some genes encode for miRNAs in their introns, others have their own start codons and promoter regions\textsuperscript{129,130}. Some miRNAs are encoded in clusters with one promoter region resulting in the transcription of two or more distinct miRNAs in a continuous transcript\textsuperscript{131}. All miRNAs originate as primary transcripts (pri-miRNA) before being processed within the nucleus by Drosha (ribonuclease 3)\textsuperscript{132}. Recent evidence suggests a role for the DiGeorge syndrome critical region gene 8 (DGCR8) being involved in a co-transcriptional interplay with the Drosha pri-miRNA processing \textsuperscript{133}. Additional regulation over up to 50\% of pri-miRNA transcripts occurs through adenosine-to-inosine editing, a mechanism common with traditional mRNA processing\textsuperscript{134}. The substitution of inosine can exert an influence on both the level of expression or the function of the miRNA\textsuperscript{135-137} and is another level of control with editing having been shown to alter Dicer processing of specific miRNAs\textsuperscript{138} and to differ between cell types\textsuperscript{134,136}. Those miRNAs encoded in introns can bypass the usual Drosha/DGCR8 regulated cleavage. Instead the pre-miRNA is a direct result of splicing and disbranching events within the nucleus\textsuperscript{139,140}. Once the \simul 70 nucleotide pre-miRNA stemloop is formed, it is transported to the cytoplasm through an exportin 5 dependent mechanism\textsuperscript{141}. In the cytoplasm the enzyme Dicer cleaves the loop from the pre-miRNA forming a miRNA duplex\textsuperscript{142-144}. In what currently seems to be an exceptional case it has been shown that miR-451 is not processed by Dicer in the cytoplasm. Instead Argonaute 2 cleaves the mature sequence from the precursor\textsuperscript{145}. This alternative pathway is required in this instance as the miR-451 mature sequence unusually spans the loop of the pre-miRNA. Once the miRNA duplex is formed one or both of the strands are
then integrated into the RNA-induced silencing complex (RISC). Here the miRNA acts as a specific guide strand which allows the Argonaute proteins, namely AGO1-4, QDE2 and RDE-1 to be attached to the target mRNA strand\textsuperscript{129,146}. Although each miRNA precursor can potentially produce two mature miRNAs, these miRNAs are not expressed at the same level. Indeed there is no correlation in cytoplasmic levels of distinct mature miRNA from the same precursor\textsuperscript{147}.

The exact mechanism through which miRNA act is still being elucidated and at this point remains controversial\textsuperscript{148}. Direct cleavage of the target mRNA has been observed within mammalian cells in a few cases where the miRNA-target interaction is almost entirely complementary\textsuperscript{149,150}. However, this is yet to be demonstrated to be a significant mechanism in target regulation\textsuperscript{145}. In the majority of confirmed miRNA-mRNA interactions occur with the miRNA 5’-proximal region forming an imperfect hybrid with the 3’-untranslated region (3’UTR) of the target mRNA. Recent evidence suggests that miRNA are also capable of binding to mRNA coding regions\textsuperscript{151-154} and that target specificity is not exclusively limited to the 5’ seed region of the miRNA\textsuperscript{155} with the rate of repression influenced by the sequence context immediately around the direct binding site\textsuperscript{156}. The functional significance of these alternative binding mechanisms remains to be explored. Intriguingly it has been shown that multiple miRNA can bind to different sites on a single mRNA transcript. This interplay between miRNA adds yet another layer of complexity to exquisite control exerted over mRNA translation\textsuperscript{157}. miRNA have almost exclusively been seen as a form of RNAi impeding the translation of mRNA to protein through, deadenylation, P-body sequestration and direct degradation of mRNA targets.
**Figure 1.2:** miRNA silencing pathway (adapted from Mattes et al. Am J Respir Cell Mol Biol. 2007 Jan;36(1):8-12). Once transcribed in the nucleus the pri-miRNA has its tails cleaved by Drosha (a) to create the pre-miRNA which is subsequently exported to the cytoplasm via exportin5(b). Within the cytoplasm dicer cleaves the loop revealing the miRNA duplex (c) which proceeds to unwind and one or both strands are subsequently incorporated into a RISC. The seed sequence of the miRNA determines the binding and subsequent silencing targets of the RISC(d).
However, recent reports indicate that target inhibition is not the sole purpose of miRNA by demonstrating the capability of miRNA to activate target translation under certain conditions\textsuperscript{159-162}. Despite our general naivety as to the mechanisms behind miRNAs regulatory role, dysfunction in these pathways has been demonstrated to be significant in numerous diseases. Here I will highlight the roles of miRNA in allergic and inflammatory airways diseases and examine future prospects for miRNA modulation as a therapeutic strategy.

1.4.1 Immune regulation by miRNA

Several miRNA have been implicated in the regulation of both innate and adaptive immunity. It is anticipated that as our understanding of this regulatory system grows so will the number of individual miRNA involved in immune regulation. Here I will focus on the better characterised miRNAs that have been demonstrated to regulate immune processes that underpin the development and maintenance of immune responses with a particular focus upon miRNA that are able to modulate multiple factors implicated in disease pathogenesis.

1.4.1.1 miR-9

miR-9 has been found to be upregulated in response to LPS stimulation within human polymorphonuclear neutrophils (PMN) and monocytes. Indeed in a study of over 360 miRNAs miR-9 was the only microRNA to be upregulated in both cell types in response to TLR4 activation. The upregulation of miR-9 within PMN and monocytes is not limited to the TLR4 signalling pathway with agonists of TLR2, TLR7/8 and several proinflammatory cytokines including TNF-\(\alpha\) and IL-1\(\beta\) also inducing expression within these cell types\textsuperscript{163}. While transcription of miR-9 is
MyD88 NF-κβ dependent its seed sequence also contains a target for the NF-κβ 1 transcript suggesting negative feedback of NF-κβ through miR-9 expression.

Figure 1.3: miRNA are involved in the regulation of multiple inflammatory cell types

1.4.1.2 miR-21

miRNA21 is highly conserved across vertebrates indicating it most likely plays an important regulatory role of crucial functions within these organisms. In addition miR21 has been confirmed as an oncogene and is amongst the most common genes upregulated in solid tumours\textsuperscript{164-167}. Within the immune system miR21 has been demonstrated to play significant roles in homeostasis and disease. Increased expression of miR-21 has been linked to the differentiation of monocytes to DCs\textsuperscript{168}. MiR-21 is also one of the most abundant miRNA expressed
by T-cells with significantly higher expression in effector T-cells than naïve and memory populations\textsuperscript{169}. T-cell receptor stimulation upregulates miR21 expression targeting RAS guanyl releasing protein 1 (RASGRP1). This inhibition of T-cell receptor (TCR) signal transduction impedes TCR signalling strength. This has been demonstrated with synthetic overexpression of miR21 leading to decreased T-cell activation\textsuperscript{170}. In regulatory T-cells (T-regs) miRNA expression has been shown to be necessary for the expression of forkhead box P3 (FOXp3) though it exerts these effects through and unknown, seemingly indirect mechanism\textsuperscript{171}.

1.4.1.3 miR-133a

Within human bronchial smooth muscle cell cultures (hBSMCs) miR-133a has been shown to regulate ras homologue gene family, member A (RhoA) production. IL-13 stimulation of these cells was sufficient to reduce miR-133a expression and also increase levels of its target RhoA. BSMCs isolated from allergic mice showed that RhoA was elevated and miR-133a expression reduced\textsuperscript{172}. Decreased levels of miR-133a have also been linked to myeloproliferative disorders with lower expression found in the neutrophils of patients with this class of disease\textsuperscript{173}. miR-133a has also been associated with the hypertrophy of skeletal muscle\textsuperscript{174}, another significant process in the pathogenesis of asthma.

1.4.1.4 miR-145

miR-145 is most commonly characterised as a tumour suppressing miRNA through its ability to inhibit multiple oncogenic miRNAs (oncomirs). It has also been demonstrated to play a crucial role in the development of smooth
muscle and is required for the differentiation of fibroblasts into smooth muscle cells. miR-145 is one of the most highly expressed miRNA within the human airways and trachea.

1.4.1.5 miR-146

miR-146 remains the most comprehensively studied NF-κB dependent miRNA within the immune system. It is significantly downregulated in the airways of rats following cigarette exposure and has been demonstrated to be up-regulated in both macrophages and monocytes in response to LPS stimulation.

More recently it has been demonstrated that miR-146a and miR-146b are activated via divergent signalling pathways within human alveolar A549 epithelial cells downstream of IL-1β, with miR-146a being down stream of NF-κB and JNK-1/2 pathways and miR146b mediated via MEK-1/2 and JNK1/2. The authors suggest that this goes part-way to explaining the two differing isoforms of this particular miRNA. Synthetic increases in miR-146 expression prior to IL-1β exposure resulted in a reduced CCL5 and IL-8 release by the epithelial cells. These findings implicate both of the miR146 isoforms in the regulation of early immune signalling within the epithelium in both bacterial (LPS) and viral (IL-1β) environments. miR-146a expression is directly regulated by NF-κB activation. This is consistent with findings that TNF-α and IL-1β signalling along with TLR2, 4 or 5 activation have been demonstrated to rapidly induce the expression of miR-146a while TLR3 and 9 activation does not alter its expression. Several of the targets of miR-146 are the downstream signalling molecules of the TLRs and IL-1 receptor system suggesting it may play an immune limiting role as a part of a negative feedback loop on proinflammatory...
signals. This hypothesis extends to recent clinical findings in primary cell cultures from chronic obstructive pulmonary disease (COPD) patients. Persistent inflammation plays a significant role in the pathogenesis of COPD and significantly decreased levels of miR-146a have been identified within human fibroblasts isolated from the airways of COPD patients when compared to those of smokers who had not developed COPD\textsuperscript{183}. This was linked to the overproduction of the prostaglandin E2 within the COPD patient group with miR-146a capable of directly inhibiting the translation of E2 mRNA.

1.4.1.6 miR-147

miR-147 has been demonstrated to be upregulated in vivo within macrophages from the lungs of mice treated with LPS. This upregulation is specific to the TLR4 pathway and not TLR2 or 3. It is also dependent on both myeloid differentiation primary response gene (88) (MyD88) spell out and TRI-domain containing adapter-inducing interferon-β (TRIF) signalling downstream of TLR4\textsuperscript{184}. The promotion of miR-147 was through both STAT1 and NF-κB binding to its promoter sequence. This is another example of an miRNA in a negative feedback loop where TLR4 signalling induces miR-147 expression within the macrophages which in turn acts to inhibit TLR signalling through a yet undiscovered mechanism\textsuperscript{184}.

1.4.1.7 miR-155

miR-155 is one of the miRNA most associated with allergic disease. It is coded for in an exon in a non-coding RNA within the B-cell integration cluster (BCI) on chromosome 21\textsuperscript{185,186} and has been demonstrated to be regulated by both NFκB and AP-1\textsuperscript{187,188}. Early experiments in mice deficient for bic/miR-155
demonstrated that expression was required for normal function of B and T cells as well as DCs\textsuperscript{189} although this was demonstrated to be at least in part due to the cytokine regulation in cellular development\textsuperscript{190}. Functionally miR-155 has been shown to be induced in monocytes, macrophages and DCs in response to LPS, IFN, poly IC and TNF-\textgreek{a}\textsuperscript{188,187,191,192}. Sustained expression of miR-155 is capable of elevating the numbers of immature granulocytes \textit{in vivo}\textsuperscript{193}. The upregulation of miR-155 in macrophages is not direct but rather a result of a secondary signal through the TNF autocrine or paracrine signalling pathways, indeed pharmacological inhibition of JNK inhibits miR-155 induction\textsuperscript{187}. miR-155 expression is low in alveolar epithelial 549 cells\textsuperscript{194} and knockdown of miR-155 within human mDCs increased the production of IL-1\textgreek{b}\textsuperscript{192}. In addition an miR-155 mimic has been shown to decrease the expression of NF-\textkappa\textgreek{B} reporter assays in human cell lines\textsuperscript{192,195} and inhibit the expression of the pro-inflammatory TGF\textgreek{b} activated kinase 1 (TAK-1) also known as mitogen-activated protein kinase kinase kinase 7 binding protein 2 (TAB 2). In chronic rheumatoid arthritis (RA) miR-155 has been found to be significantly upregulated and responsible for the downregulation of matrix metalloproteinase (MMP) 1 and 3 within RA fibroblasts possibly limiting the local tissue damage caused by these inflammatory markers\textsuperscript{196}. However, in other situations miR-155 has been shown to have a positive regulatory role in inflammation. When expression of miR-155 is enforced in the bone marrow the phenotype exhibited mimics that of myeloproliferative disorder which can also be transiently induced by LPS injections\textsuperscript{197}. Indeed miR-155 itself is expressed within bone marrow derived macrophages (BMDMs) following stimulation with LPS\textsuperscript{198} and other TLR ligands\textsuperscript{187} targeting inositol polyphosphate-5-phosphatase (SHIP1) which itself
is a negative regulator of TLR4 signalling in a negative feedback loop\textsuperscript{199}. Suppressor of cytokine signalling (SOCS) 1 is another protein critical for LPS induced TLR signalling\textsuperscript{198}. Direct LPS stimulation of BMDMs results in altered processing of pre-miR-155 by the KH-type splicing regulatory protein binding to the terminal loop promoting maturation. This mechanism effectively upregulates the level of mature miR-155 within the cell allowing discrete control of inflammatory mediators including IL1\(\beta\), IL10, IL12, CCL 5, CXCL10, CXCL11 and SOCS3\textsuperscript{200}. Interestingly miR-155 is negatively regulated by the protein kinase AKT-1 which limits the extent to which it is upregulated following LPS stimulation at a transcriptional level \textsuperscript{198}. These data suggest that upregulation of miR-155 in response to LPS may be due to an increased processing rate of previously transcribed pre-miRNA. The transcription of pre-miR-155 would seem to be limited through the action of AKT-1, however, the combined effect of these two pathways is yet to be studied simultaneously and the balance of these pathways remains speculation until they are examined simultaneously.

\textbf{1.4.1.8 miR-223}

Unlike most of the previously discussed miRNA the action of miR-223 in relation to allergy and inflammation is to inhibit its development by specifically restricting the expression of granulocytic progenitors and dampening neutrophil activation\textsuperscript{201}. Fazi \textit{et al.} showed upregulation of miR-223 in the differentiation of granulocytic cells under the induction of C/EBP\(\alpha\) - a transcription factor crucial for differentiation\textsuperscript{202}. Our more recent understanding of miRNA mechanisms, namely the ability of individual miRNA to alter effects within the same cells at different phases of the cell cycle\textsuperscript{160,203} may well reconcile the apparent
discrepancies between these two reports. Given that Fazi et al. conducted experiments in a leukemic cell line while Johnnidis et al. report was from experiments in mouse models, it is more likely that the latter findings are representative of the actual role of miR-223 in the complex in-vivo environment in which differentiation must take place. The expression of miR-223 is also different between monocyte derived DCs and monocytes pointing towards a role for miR-223 in the differentiation of other critical inflammatory cell types. However, this remains poorly defined and requires further research\textsuperscript{168}. The role of miR-223 regulation in inflammation is not limited to granulocytes as it has been demonstrated to increase IFN\textgreek{g} production in response to LPS within estrogen stimulated splenic lymphocytes\textsuperscript{204}. miR-223 has also been linked to the ischemic damage of smooth muscle in mouse models. However, expression levels directly correlated with infiltrate of inflammatory cells and it is possible that the expression of miR-223 is confined to these infiltrated granulocytes\textsuperscript{205}. The absence of miR-223 expression in regenerating tissue, muscle fibres and fibrotic lesions, supports the concept that expression is limited to inflammatory cells.

1.4.1.9 The let-7 family

Lethal 7 (let-7) was one of the first miRNA identified in \textit{C. elegans}\textsuperscript{206} and is highly conserved across species\textsuperscript{207}. Within the human genome there are multiple isoforms denoted, namely from let-7a to let-7i and miR-98\textsuperscript{208}. Of these isoforms let 7a and let 7f also have multiple precursors that, in the case of let-7f, occur in different sections of the genome\textsuperscript{208} allowing exquisite and independent control over the expression of these miRNAs. This family of miRNA while differing with several point mutations in their sequence share the same 5’ seed sequence and
are thus predicted to share the same target profile based on our current understanding of miRNA/target interactions. They are of particular interest as several members of this miRNA family have been implicated in the regulation of immune factors and remodelling pathways.

1.4.1.9.1 Let-7b

Let-7b has been shown to play a regulatory role in the innate immune system. Let-7b has target sites within the 3’ UTR of the TGF\(\beta\) sequence and has been implicated within primary primate macrophages to be involved in a negative feedback loop as it is downregulated in response to TGF\(\beta\) exposure\(^{209}\).

1.4.1.9.2 Let-7d

Let-7d is abundantly expressed within the epithelial cells of healthy human lungs. It has been shown to be downregulated by TGF\(\beta\), and decreased expression of let-7d causes mesenchymal transition within epithelial cells \textit{in vitro} and \textit{in vivo}. This process has been demonstrated to increase collagen deposition within the lungs of mouse models. Similarly, repressed levels of let-7d have been found within the airways of human idiopathic pulmonary fibrosis (IPF) patients, suggesting that this miRNA may play a role in the pathogenesis of the diseased lung phenotype\(^{210}\).

1.4.1.9.3 Let -7f

Let-7f has been shown to be one of 3 miRNA sequences downregulated in the lung during pulmonary hypertension induced by chronic hypoxia\(^{211}\). Let-7f has also been shown to promote sprout formation in cultured human endothelial cells indicating an important role in angiogenesis, a crucial feature of airways
remodelling\textsuperscript{212}. Subsequent \textit{in silico} analysis predicted that let-7f targets the endogenous angiogenesis inhibitor thrombospondin-1, however this is yet to be confirmed \textit{in vivo}.

\textbf{1.4.1.9.4 Let-7g}

Expression of let-7g has been inversely correlated to type I collagen alpha2 levels in hepatocellular carcinoma patients and later confirmed as a target \textit{in vivo}\textsuperscript{213}. While the authors suggest that this may be a mechanism through which let-7g acts to inhibit the progression of the carcinoma, collagen deposition also plays a significant role in several chronic respiratory diseases and this is another avenue through which the let-7 family may exert a regulatory influence on multiple aspects of asthma disease pathogenesis.

\textbf{1.4.1.9.5 Let-7i}

Let-7i is a member of the let-7 family of miRNA which all share a seed region of near perfect homology. Let-7i is regulated in a MyD88/NF-κB dependent manner and has been shown to be capable of targeting TLR-4 leading directly to decreased protein expression. Interestingly, the expression of let-7i was downregulated upon infection of the small intestine by the parasite \textit{Clostridium parvum} which requires TLR-4 signalling to induce a cholangiocyte response through NF-κb signalling\textsuperscript{214}. Moerover, upon microbe infection of cultured human epithelial cells the p50 subunit of NF-κB in complex with C/EBPβ acts to suppress the expression of Let-7i through chromatin deacetylation\textsuperscript{215}.

This summary of miRNA involved in immune responses is by no means exhaustive and it is anticipated that their number will expand rapidly into the
future as more comprehensive miRNA array technologies become accessible, and our understanding of their mechanisms of action will further develop.

**Figure 1.4:** miRNA have been demonstrated to play a role in the development of several processes key to the development of allergic airways disease.

1.4.2 miRNA in Asthma

While there is increasing evidence that miRNA play a significant role in the regulation of inflammation and allergy, to date relatively little work has been conducted *in vivo*. Such work, however, is critical to establish the importance of these regulatory systems in disease. A recent profiling study with a limited panel of 227 miRNA and a small cohort of 8 patients found that mild asthmatics did not have any alteration in miRNA expression within biopsied airway cells, nor did treatment with the corticosteroid budesonide illicit any changes over a 4 week period[216]. The limitations of the study design prevent any firm hypothesis being formed, however, it does highlight the need for a more comprehensive study to
establish the importance of these recently discovered pathways within human disease. The highly conserved nature of miRNA sequences suggests that findings in vivo are more likely to accurately represent the human disease than other regulatory mechanisms that are not as similar in animal models.

miR-21 has been shown to play a key role in the homeostasis of the lung and airways. It is frequently identified as one of the most upregulated miRNA in lung carcinomas where it has been linked to direct inhibition of the tumor suppressor PTEN. Lu et al. used a doxycycline-induce lung-specific IL-13 transgenic mouse to identify the upregulation of miR-21 downstream of IL-13. This was one of the first in vivo reports linking miRNA dysregulation with factors that play a key role in the development of allergic lung disease. Lu et al went on to demonstrate the ability of miR-21 to directly target the 3’ untranslated region of IL-12p35 enabling an influence over IL-12 which in turn regulates Th cell differentiation, a process thought to be instrumental in the development the Th2 bias observed in allergic asthma.

1.4.3 Therapeutic Potential of miRNA modulation

Several alternatives have been proposed for the synthetic manipulation of miRNA levels, with some degree of success in vitro and even in vivo animal models. Current technologies downregulating miRNA expression levels are far more common than methods to either induce greater expression or deliver synthetically derived miRNA to the target cell cytoplasm though this may change into the future.

Antagomirs are cholesterol-conjugated single-stranded RNA molecules of 21 to 23 nucleotides in length and entirely complementary to the functional miRNA
strand. They were initially reported to be capable of knockdown of target miRNA in the liver, lung, intestine, heart, skin, bone marrow for more than a week after one intravenous injection \(^{219,220}\). In our hands intranasal administration alone was sufficient to induce complete knockdown of target miRNA within the airways\(^{221}\). Recent developments in our understanding of miRNA metabolism suggest that the complete complementarity with which antagomirs bind to their miRNA target lead to the degradation of the miRNA sequence within the cell\(^{222}\).

An alternative method is based on the principle of competitive inhibition known as miRNA sponges. This technique uses a mRNA sequence with strong promoters expressing multiple tandem binding sites for the miRNA target of interest\(^ {223}\). More recently long non-coding RNA sequences have been suggested to act as endogenous miRNA sponges and have been implicated in the pathogenesis of liver cancer\(^ {224}\). The effectiveness of synthetic sponges in vivo has also recently been demonstrated with the effective knockdown of miR-31 in mice\(^ {225}\) able to elicit a significant phenotypic effect in breast cancer models. However, this model required cells with stable expression of the sponge induced through a retroviral vector to be orthotopically implanted into the mice. Thus this technique still requires further development before it will be of use as a direct therapeutic intervention strategy.

Another possible strategy for therapeutic influence over miRNA is through manipulation of miRNA promoter transcription. It is currently estimated that up to 10% of miRNA are under epigenetic control\(^ {140}\) and it has been demonstrated that the transcription of some miRNAs are dependent on the DNA methylation status under the influence of DNA methyltransferase 1 and 3b\(^ {226}\). Recently
synthetic small molecules have been developed to inhibit miR-21 in both a specific and non-specific manner\textsuperscript{227}. Modification of the diazobenzene structure allowed for improved potency and increased specificity and the authors postulate that it targets the transcription of the miR-21 gene while allowing the downstream miRNA machinery to function normally although the precise mechanism of action remains to be elucidated\textsuperscript{227}. Interventions targeting the body's own miRNA regulatory mechanisms are likely to become more attractive as our understanding of the regulation of miRNA transcription develops and more drugs capable of manipulating these pathways are identified.

Adeno-, Lenti- and Retro-viral vectors have also been used to insert both miRNA inhibitors in the form of anti-miRs, sponges and miRNA sequences with promoters to artificially up regulate miRNA expression \textit{in vivo}. However, as these models require the infection of the subject to confer treatment, the use of these technologies in patients is unlikely in the immediate future.

\textbf{1.5 Conclusion}

The TRAIL inflammatory signalling pathway and miRNA regulation networks are two novel mechanisms that have the potential to play significant roles in the development and maintenance of AAD within the airway wall itself. Modulation of these pathways may be an attractive method to treat the underlying causes of disease at the site where the abnormal immune response occurs, rather than merely addressing the consequences as is typical of current therapeutic approaches.
Chapter 2: Methods
2.1 Mice

WT, Tnfsf10−/−, Tlr4−/−, Myd88−/−, and Stat6−/− BALB/c mice (6-14 weeks) were obtained from the University of Newcastle’s special pathogen-free facility and housed in individually ventilated cages in approved containment facilities within the David Maddison Clinical Sciences Building, University of Newcastle (Newcastle, Australia). All experiments were approved by the Animal Ethics Committee of the University of Newcastle. Mouse studies in Chapter 5 performed at the University of New South Wales were complied with the requirements of the Animal Care and Ethics Committee of the University of New South Wales.

2.2 HDM induced allergic airways disease (AAD) (chapters 3, 4 and 6)

House dust mite (HDM)(Dermatophagoides pteronyssinus) extract was obtained from Greer Laboratories (Lenoir, NC) as a lyophilized preparation of milled mites. HDM was resuspended in sterile saline (SAL) and mice were treated through the nose with 50µg protein/50µL (Der p 1 constituted ;10% of protein weight) or vehicle sterile endotoxin-free saline on days 1, 2, and 3 for sensitization. Sensitization was followed by daily HDM exposure (5µg/50µL) on days 14, 15, 16, and 17 to induce allergic airways disease (Figure 4.1). Nonsensitized mice (vehicle-treated) received sterile endotoxin-free saline only. Experiments were conducted on day 18, 24 hours after the last HDM exposure.

2.3 Rhinovirus (RV) induced exacerbation of HDM induced AAD (chapter 6)

In some experiments, we inoculated allergic - (day 18, one day after HDM extract challenge) or non-allergic mice intranasally with RV1B (2.5x10⁶ TCID₅₀) or UV-inactivated RV1B in 50µl of saline. Mice were euthanized 24hrs after the
last allergen/virus challenge.

2.4 Chronic OVA induced AAD (chapter 5)

The protocols employed for sensitisation and inhalational challenge have previously been described. Briefly, specific pathogen-free female BALB/c mice aged 7-8 weeks (Animal Resources Centre, Perth, Western Australia) were systemically sensitised by intraperitoneal injection of 50μg of alum-precipitated chicken egg Ovalbumin (OVA) (Grade V, ≥98% pure, Sigma Australia) 21 and 7 days before inhalational challenge, then exposed to aerosolised OVA in a whole body inhalation exposure chamber (Unifab Corporation, Kalamazoo, MI) [12]. Chronic low-level challenge involved exposure to ≈3 mg/m³ aerosolised OVA for 30 minutes/day on 3 days/week for up to 6 weeks. Particle concentration within the chamber was continuously monitored using a DustTrak 8520 instrument (TSI, St Paul, MN). All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the University of New South Wales (reference numbers: 06/119B and 08/09B). Data were collected from 6 animals per group for microRNA (miRNA) profiling and 8 animals per group for treatment with antagomirs. Control groups included naïve mice and mice that were not sensitised but were challenged for 6 weeks with aerosolised OVA. For miRNA profiling, chronic challenge was performed for 1, 2, 4 or 6 weeks. Animals were sacrificed 48 hours after the final challenge.

2.5 Airways hyperreactivity (AHR) measurement

We assessed AHR invasively in separate groups of anesthetized mice by measurement of total lung resistance and dynamic compliance. Percentage increase over baseline (PBS) in response to nebulized methacholine was
2.6 Antagomirs

Target miRNA sequences were downloaded from miRBase, Wellcome Trust, Sanger Institute, Cambridge, UK (Sanger database; http://microrna.sanger.ac.uk/sequences/). We ordered scrambled antagomir from Dharmacon (nonspecific RNA VIII, blasted against the mouse genome)\textsuperscript{220}. The sequence of antagomirs used are as follows:

ant-miR-126:

\[ \text{5'}\text{mG.*.mC.*.mA.mU.mU.mA.mU.mA.mC.mU.mC.mA.mC.mG.mG.mU.mA.*.mC.} \]
\[ \text{.mG.*.mA*.} \text{3'-Chl,} \]

ant–miR-145:

\[ \text{5'}\text{mA.*.mA.*.mG.mG.mG.mA.mU.mC.mU.mG.mG.mG.mA.mA.mA.mA.mC.} \]
\[ \text{mU. mG.*.mA.*.mC.*.3'-Chl;} \]

ant–let–7b:

\[ \text{5'}\text{mA.*.mA.*.mC.mC.mA.mA.mC.mA.mC.mC.mU.mA.mC.mU.mA.mC.mC.*.m} \]
\[ \text{U.*.mC.*.mA.*.3'-Chl;} \]

and ant–miR-21:

\[ \text{5'}\text{mU.*.mC.*.mA.mA.mC.mA.mU.mC.mA.mG.mU. mC.mU.mG.mA.mU.mA.mA.mG.} \]
\[ \text{*.mC.*.mU.*.mA.*.3'-Chl} \]

where “m” were 2'-OMe modified phosphoramidites, “*” were phosphorothioate phosphorothioate linkages, and “-Chl” was hydroxyprolinol-linked cholesterol.

We administered 50 μg antagomir/50 μL sterile saline intranasally (i.n.) at day 13 (24 h before the first out of four daily HDM rechallenges) and then every second day until mice were sacrificed at day 18. Within the chronic OVA model
(chapter 5) mice were treated with antagonir each 7 days through the challenge period.

2.7 Administration of dexamethasone

Dexamethasone (3 mg/kg; Sigma-Aldrich, New South Wales, Australia) or vehicle control was administered by intraperitoneal injection on day 13 (24 hours before the first HDM re-exposure) and then every second day until mice were killed on day 18.

2.8 Short interfering (si) RNA

We ordered siRNAs with no similarities to other sequences from Ambion (Applied Biosystems). Sequence of the antisense strand of siRNA-Midline-1 was: 5'-UUAGGUAAUCCAGACAUUCta-3'. We ordered the Nonsense siRNA (chosen to have an equivalent CG content) with no similarities to other sequence from Ambion (Option 2). We administered 3.75nmol siRNA/25ml of sterile saline i.n. at day 13 (after HDM sensitization and 24hrs before first HDM challenge) and then every second day until mice were sacrificed. In the asthma exacerbation studies, mice were treated 24hrs before RV1B challenge.

2.9 Isolation of mRNA and miRNA

Total RNA including miRNA was isolated with mirVana miRNA Isolation kit (Ambion) from lower airway tissue. Briefly, the trachea and lungs were isolated, and using two pairs of forceps, the parenchyma was separated from the larger airways by blunt dissection. This facilitated effective separation of the airway wall from the parenchyma leaving several generations of airway for analyses. The preparation consists of resident airway cells, such as epithelial
cells, fibroblasts, smooth muscle cells, and the basement membrane as well as infiltrating inflammatory cells.

### 2.10 Quantitative RT-PCR

qRT-PCRs were performed with the TaqMan Gene Expression Assays for the respective miRNA (Applied Biosystems). miRNA expression was normalized to 18S or sno-202 RNA.

### 2.11 miRNA microarraying

Mice received HDM 50 µg intratracheally or saline (SAL) and were sacrificed at 2, 8, and 24 h post-challenge. Total RNA was extracted from bluntly dissected airways employing the Ambion mirVANA kit according to the manufacturer's protocol. miRNAs were enriched with the Ambion flashPAGE system. The Ambion 1564V1 probeset was printed on microarray epoxy slides by the Australian Genome Research Facility, Parkville, Australia. miRNA was polyadenylated and labelled with Cy3 using the mirVana miRNA labelling kit and arrays were hybridised and washed as described by Ambion. Slides were scanned using a GenePix4000B (Molecular Devices) and GenePix 6.0 software was used to quantify raw signal intensities. Analysis of microarray data was conducted using Genespring GX software (Agilent). Percentile shift normalisation (75th percentile) was performed with subsequent fold change calculations conducted against mean normalised naïve expression levels.

### 2.12 mRNA microarraying

Total RNA was extracted from the airways using Ambion mirVANA kits according to the manufacturer's protocol. Whole genome Illumina microarrays
(SM6V2) were conducted at the SRC Microarray Facility, Brisbane, Australia. Analysis of microarray was conducted using Genespring GX 10.

2.13 Flow cytometry analysis

We stained cells with anti-CD4-FITC, anti-CD11b-PerCP, anti-CD11c-PE, biotinylated anti-major histocompatibility complex (MHC)II conjugated to Streptavidin-FITC, respective isotype controls (BD Biosciences PharMingen), and anti-mouse plasmacytoid dendritic cell (mPDCA)-1-PE (Miltenyi). Numbers of positive cells were quantified by flow cytometry (FACS Calibur).

2.14 Airway morphology studies

Lung tissue was stained, cells identified by morphological criteria, and quantitated by counting 10 high power fields (HPF)s in each slide.

2.15 Cytokine analysis

Peribronchial lymph node cells were excised, filtered, and cultured in the presence or absence of 50 µg/µL HDM (optimal concentration) for 6 days. We determined interleukin (IL)-13, IL-5, and interferon (IFN)-γ by ELISA (BD Biosciences PharMingen).

2.16 Isolation of proximal airway tissue

Airway tissue was isolated by blunt dissection, using two pairs of forceps to separate lung parenchyma from the larger airways and leaving several generations of airway attached to the trachea. Airway tissue was frozen in liquid nitrogen until RNA extraction was performed.
2.17 Assessment of airway inflammation and remodelling (Chapter 5)

In the antagonir and control-treated mice, allergic airways inflammation and airway wall remodelling were quantified in longitudinally oriented sections of formalin-fixed, paraffin-embedded tracheas, or horizontally oriented sections from the mid-zone of the single lobed left lung, as previously described. Assessment included numbers of intraepithelial eosinophils and of chronic inflammatory cells in the lamina propria of the airway, extent of subepithelial fibrosis and grading of mucous cell change. The validity and reliability of the morphometric techniques we employed have been established in previous reports.

2.18 Immunostaining (Chapter 5)

Expression of eotaxin in the airway epithelium was demonstrated using a goat polyclonal antibody to a 19-amino acid peptide corresponding to an epitope at the carboxy terminus of mouse eotaxin (Santa Cruz Biotechnology, Santa Cruz, California) (sc-6182). Immunoperoxidase staining of formalin-fixed, paraffin-embedded sections was performed following antigen retrieval in 0.01M citrate buffer (pH 6.0), as previously described. Intensity of immunoreactivity was semi-quantitatively scored as grade 0 = no staining, grade 1 = weak staining, grade 2 = moderate staining and grade 3 = strong staining.

2.19 Protein Phosphatase (PP)2a activity assay (Chapter 6)

PP2a activity was measured by R&D Systems PP2A DuoSet IC activity assay kit according to the manufacturer’s instructions. This was performed on homogenized mouse lungs and harvested BEAS-2B cells.
2.20 Immunofluorescent detection

We fixed and incubated formalin-preserved lung sections with either anti-Midline-1 antibody followed by a secondary PE-conjugated antibody (Santa Cruz Biotechnology), or an AlexaFluor®488-conjugated antibody against phosphorylated-p38 (Cell Signaling Technology). Nuclei were counterstained with DAPI (Sigma). Microscopic analysis was performed with an Olympus BX51 UV microscope using DP Controller 3.1.1.267 software (Olympus).

2.21 Immunoblotting

We separated protein derived from lung homogenates on 12% SDS-PAGE and transferred to nitrocellulose membranes. We probed membranes with an antibody against PP2Ac or beta-actin (Sigma), followed by HRP-linked antirabbit secondary antibodies. Bands were visualised with a Fujifilm LAS-3000 Intelligent Dark Box (Fujifilm) and quantitated using the Multi Gauge software package (Fujifilm). The relative level of PP2Ac was determined by dividing the densitometric volume of the PP2Ac band by the beta-actin band.

2.22 Immunoprecipitation

BEAS-2B cells were lysed in the presence of protease inhibitors (Pepstatin, Leupeptin, Aprotinin, and PMSF, Sigma, Australia). Protein lysate (500mg) was incubated with 4mg PP2A-C mAb (clone 1D6, Millipore, Australia) and protein A agarose beads (Millipore, Australia) at 4°C overnight followed by 3 x washes in lysis buffer. Immunoprecipitated proteins were separated on 12% polyacrylamide gels and transferred to nitrocellulose. Immunoblots were probed with primary polyclonal antibodies PP2Ac, alpha4 (Novus Biologicals) or
Midline-1 (Santa Cruz Biotechnology), and appropriate secondary antibodies as described above.

2.23 Airway epithelium cell cultures

We used BEAS-2B cells and primary bronchial epithelial cells (BECs) that were obtained from patients with stable persistent asthma and healthy controls by bronchoscopy using a single sheathed nylon cytology brush\textsuperscript{231}. We cultured the BEAS-2B cells in DMEM (Thermo Scientific) with 5% FCS and BECs in bronchial epithelial cell growth medium (Clonetics) as previously described\textsuperscript{231}. After one passage we seeded BEAS-2B cells onto 12-well trays, cultured them until 80% confluent, and incubated them with house dust mite (50mcg/mL) or TRAIL (1000ng/ml) for 24h. BECs were also infected with RV1B and harvested 6hrs or 24hrs later as described earlier\textsuperscript{231}. TRAIL, Midline-1 and CCL20 expression were quantified employing rtPCR. All studies were approved by the local human research ethics committee and written informed consent was obtained.

2.24 Statistical analysis.

Results are presented as mean ± SEM, or as medians (interquartile range) for grading. Differences between groups were assessed using a Student t test, the Mann-Whitney test, one-way ANOVA or Kruskal- Wallis test, followed by a Newman-Keuls or Dunn's post test as appropriate. Comparison of airways resistance between groups was performed by using ANOVA, and significance was assessed for the entire dose-response curve. The software package GraphPad Prism  (GraphPad Software, San Diego, CA) was used for data analysis and preparation of graphs.
Chapter 3: Antagonism of microRNA-126 suppresses the effector function of Th2 cells and the development of allergic airways disease

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3.1 Abstract

Allergic asthma is an inflammatory disease of the lung characterized by abnormal T helper-2 (Th2) lymphocyte responses to inhaled antigens. The molecular mechanisms leading to the generation of Th2 responses remain unclear, although toll-like receptors (TLRs) present on innate immune cells play a pivotal role in sensing molecular patterns and in programming adaptive T cell responses. Here we show that in vivo activation of TLR4 by house dust mite antigens leads to the induction of allergic disease, a process that is associated with expression of a unique subset of small, noncoding microRNAs. Selective blockade of miR-126 suppressed the asthmatic phenotype, resulting in diminished Th2 responses, inflammation, airways hyperresponsiveness, eosinophil recruitment, and mucus hypersecretion. miR-126 blockade resulted in augmented expression of POU domain class 2 associating factor 1, which activates the transcription factor PU.1 that alters Th2 cell function via negative regulation of GATA3 expression. In summary, this study presents a functional connection between miRNA expression and asthma pathogenesis, and our data suggest that targeting miRNA in the airways may lead to anti-inflammatory treatments for allergic asthma.
3.2 Introduction

Asthma is a chronic inflammatory disease of the airways that has been described as an epidemic in developed countries \(^{234,235}\). The clinical condition is characterized by episodic breathlessness and wheezing, together with enhanced airways hyperresponsiveness (AHR) to a variety of stimuli that is associated with aberrant allergen-specific CD4+ T helper-2 lymphocyte (Th2 cells) responses\(^5,234\). These findings have lead to the paradigm that activation of Th2 cells by inhalation of allergens plays a pivotal role in disease development and progression through the actions of their secreted cytokines. Indeed, the hallmark features of allergic asthma, such as elevated serum immunoglobulin (Ig)E, airway eosinophil infiltration, mucus hypersecretion and AHR have all been linked to the effector functions of the Th2 cytokines interleukin (IL)-4, -5, -9, and -13\(^5,236\). An emerging concept for a mechanism potentially causing asthma is that the innate immune system inappropriately senses allergens as foreign and dangerous and responds with a programmed adaptive Th2 immune response \(^{237,238}\).

Toll-like receptors (TLRs) differentially sense microbial and viral bioproducts and act as sentinels for the activation of innate host defense pathways\(^{239,240}\). Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, activates cells through TLR4 and the common TLR adaptor protein myeloid-differentiation-primary-response-gene-88 (MyD88) resulting in activation of transcription and proinflammatory pathways\(^{239,241,242}\). LPS is also a prominent constituent of asthma-inducing house dust mite (HDM) allergens and can instruct the immune response to inhaled antigen to generate Th2 responses\(^{237}\). Indeed, it is likely that signals derived from early activation of the innate
immune system play an important role in the generation of aberrant Th2 responses. Furthermore, clinical studies have shown a direct correlation with the levels of LPS in HDM and the severity of asthma, supporting the concept that the innate immune system plays as significant role in disease pathogenesis.243,244. MicroRNAs (miRNA) are emerging as important regulators of gene expression in the immune system by functioning as endogenous inhibitors of translational processes.245. A single miRNA can target hundreds of messenger (m) RNAs and thereby modulate protein output from their respective genes.246. Therefore, a single or specific set of miRNAs may control discrete physiological processes by regulating the production of a few critical proteins that coordinate single or interrelated cellular events (e.g., cell proliferation).245,246. Very recently, TLR signalling and miRNA expression have been linked to one another, and it has been proposed that the coupling of these pathways form elemental regulatory signals of the innate immune system, which lead to activation of inflammatory pathways.180,187,247. Thus, TLRs and miRNAs may collaborate to promote host defense responses and the programming of the adaptive immune response.245. Although promising, the role of miRNA in the regulation of immunological processes is in its infancy, and their contribution to the pathogenesis of diseases of the immune system, of which many have chronic inflammatory components, remains unknown.245,248. However, there are several pioneering studies, which indicate that manipulation of miRNA expression has the potential for therapeutic efficacy.189,219,220,249,250. Currently there are no functional data that relate specifically to the role of miRNA in the regulation of asthma and allergen-induced Th2 responses. However, given that miRNA expression can be controlled through activation of
TLRs by microbial and viral bioproducts, we hypothesized that TLR regulation of miRNA expression in the airways may be an early and fundamental step in the host response to allergen exposure, and that under certain conditions, expression of unique miRNAs may lead to programming of aberrant Th2 responses.

3.3 Results
3.3.1 Exposure of the airways to house dust mite increases the expression of specific miRNAs in the airways through TLR4- or MyD88-dependent mechanisms

Initially we determined if miRNAs were expressed in the airways in response to allergen exposure by employing a well characterized and clinically relevant BALB/c mouse model of HDM-induced allergic asthma. In this model, allergen exposure within the airways results in direct sensitization and the development of allergen-specific Th2 cells. We modified exposure times to allow a temporal analysis of the effect of HDM on the regulation of miRNA expression in the airway wall. The airway wall compartment was separated from the parenchymal tissue by blunt microscopic dissection.

Naive wild-type (WT) BALB/c mice were exposed to a single dose of HDM by directly instilling the allergen into the airways, characterising the expression of miRNAs in the airway wall at 6, 12, and 24 h after challenge. At 24 h, we observed a significant increase in miRNA-16, -21 and -126 (expression increased by 2-fold). Ten days later, we rechallenged the airways with the single dose of HDM and observed a further increase in the expression levels of these miRNA (expression increased 2-fold). miRNAs were not upregulated by challenge with
the control vehicle (saline). The increased expression of miR-16, -21 and -126 was then validated by quantitative PCR (Figure 3.1 A). The potential role of miR-126 in regulating the asthma phenotype was then investigated. The rapid upregulation of miR-126 in the airway wall suggested that pathways linked to innate host defense might be activated by components such as LPS, which are found in HDM extracts. Consistent with this hypothesis miR-126 was not upregulated in the airways of TLR4- or MyD88-deficient mice after HDM sensitization and repeated challenges (Figure. 3.1 B). These data are consistent with our hypothesis that specific miRNA may be expressed as an early component of the innate host response to allergens to potentially modulate gene
expression and to promote Th2-mediated allergic inflammation.

### 3.3.2 TLR4- or MyD88- pathways regulate miR-126 expression and hallmark features of allergic inflammation

As the TLR4- or MyD88- pathways regulated miR-126 expression we next evaluated their role in the development of pathophysiological features of allergic disease. WT, TLR4, or MyD88 deficient (-/-) mice were sensitized with HDM daily for 3 days, and 11 days later, the airways were rechallenged with allergen for 4 consecutive days to induce fulminate allergic inflammation and characteristic lesions (Figure. 3.2). The severity of disease was determined quantitatively 24 h after the last HDM exposure (day 18). Only in WT mice did exposure of the airways to HDM induce hallmark features of allergic asthma (Figure. 3.2.). HDM challenge of WT but not TLR4 -/- or MyD88 -/- mice induced AHR to methacholine (Figure. 3.2 A, B, C, respectively). Suppression of AHR in allergen-challenged TLR4 -/- or MyD88 -/- mice was associated with a significant reduction in the number of eosinophils recruited to the airways (Figure 3.2 D–F).

In allergic MyD88 -/- mice, mucus-producing cells (mean/SEM: 1.9/0.5 cells), IL-5 (2.64/0.32 ng/mL), IL-4 (less than 0.5 pg/mL) levels, and IgG1 serum levels (353/97 ng/mL) were also significantly reduced (P< 0.05) when compared to allergic WT mice (Figure 3.3, Figure 3.4, and below). Thus, activation of the MyD88 signalling pathway by HDM plays a critical role in the induction of miR-126 expression and critical features of allergic inflammation.
3.3.3 Inhibition of miR-126 function suppresses HDM-Induced AHR and hallmark features of allergic airways inflammation

To demonstrate a regulatory role for miR-126 in the development of the phenotypic features of allergic asthma, we designed antagomirs (cholesterol-linked single-stranded antisense RNA) to block miR-126 function. HDM-sensitized WT mice were treated intranasally (i.n.) with antagomirs (ant-miR-126) or with scrambled antagomir (ant-scrambled) as a control. Antagomirs were administered 24 h before the first HDM rechallenge (10 days after HDM sensitization) and then given every 48 h. Disease severity was determined at day 18, 24 h after the last exposure to both HDM and antagomirs. HDM induced miR-126 expression was completely inhibited in the airway wall after treatment with ant-miR-126 but not with scrambled control antagomir (Figure 3.3 A). Inhibition
Figure 3.3: Silencing miR-126 function by antagonim abolishes HDM-induced airways hyperreactivity and reduces allergic inflammation. (A) HDM-induced miR-126 expression after no treatment (no ant), treatment with a scrambled control sequence (ant-scrambled), or treatment with antagonim specific for the miR-126 sequence (ant-miR-126) in allergic mice. Results are mean ± SEM of the fold change from nonallergic mice treated with saline (SAL) only (baseline) (n = 3–4 mice/group). *, P ≤ 0.05. (B) Total lung resistance as percentage change of baseline measurement (water) in response to inhaled methacholine in HDM challenged allergic mice treated with ant-miR-126 or ant-scrambled versus nonallergic (SAL) mice. Results are mean ± SEM (n = 6–10 mice/group). *, P ≤ 0.05. (C) Number of inflammatory cells in BALF. Results are mean ± SEM (n = 3–4 mice/group). *, P ≤ 0.05. (D) Number of peribronchial eosinophils (1000) per high-power field (HPF), (E) representative H&E stained histological section showing decreased eosinophil numbers in tissue (arrows), and (F) number of mucus-producing cells (1000) per HPF. Results are mean ± SEM (n = 3–4 mice/group). **, P ≤ 0.01. Data are representative of two independent experiments.

of miR-126 function in the airway wall abolished HDM-induced AHR to methacholine (Figure 3.3 B). The number of eosinophils and neutrophils infiltrating the bronchoalveolar lavage fluid (BALF) (Figure 3.3 C) and lung tissue (Figure 3.3 D and E) were significantly diminished. Histological examination of ant-miR-126-treated lungs also showed decreased mucus
hypersecretion (Figure. 3.3 F). Mice treated with antagomirs did not display any ill effects, identifying the potential for modulation of miRNA function in the lung as a therapeutic approach.

3.3.4 miR-126 suppresses the effector function of lung Th2 cells

We and others have previously shown that Th2 cells and their effector cytokines IL-4, IL-5, and IL-13 play critical roles in the regulation of antibody production, AHR, mucus cell hypersecretion, and eosinophil accumulation in the lung. In HDM sensitized and challenged mice, exposure of lung to ant-miR-126 suppressed the secretion of IL-5 and IL-13 from allergen-activated peribronchial lymph (PBLN) node Th2 cells (Figure. 3.4 A), which was associated with inhibition of the crucial pathophysiological responses they induce (Figure. 3.3). IL-4 was also significantly reduced in mice treated with ant-miR-126 as compared to those treated with scrambled antagomir (mean/SEM: 16.1/3.5 versus 313.3/7.8 pg/mL, P ≤0.01) although IL-4 release by PBLN cells was altogether markedly lower as compared to IL-13 and IL-5 levels. By contrast, T cell IFN-secretion was not altered significantly after exposure to ant-miR-126 (Figure. 3.4 A). The percentage (Figure. 3.4 B and C) and total numbers of CD4+ T cells (Th2 cells) and myeloid dendritic cells (DCs) (CD11b+ CD11c+ MHC class II high) infiltrating the lung were also suppressed by ant-miR-126 treatment in HDM sensitized and challenged mice. No effect of miR-126 inhibition in the lung on serum levels of allergen-specific IgG1 and IgG2a levels as well as total IgE levels were observed (mean/SEM in ant-miR-126 treated versus un-treated mice for IgG1: 593/70 versus 973/156 ng/mL, P ≤0.07; IgG2a: below 0.4 ng/mL in all samples; total IgE: 33/9 versus 38/5 ng/mL, P ≤0.63).
3.3.5 Inhibition of miR-126 Function Alters Gene Expression in the Airways of Allergic Mice

To gain insights into the downstream inflammatory pathways regulated by miR-126 we performed a gene array analysis. We compared the transcriptomes of the airway walls isolated from HDM sensitized and challenged WT mice that had been treated with ant-miR-126 or scrambled control. Interestingly, the expression of only a limited number of RNA transcripts was affected by ant-miR-126 treatment, and most of these differentially regulated genes that encode for regions in the Ig (Ig) kappa (n =15), lambda (n = 3), or heavy (n= 11) chains.

Moreover, the only non-Ig gene significantly upregulated by miR-126 inhibition in vivo was POU domain class 2 associating factor 1, also named Oct binding factor 1 (OBF.1) or B-cell Oct binding protein 1 (BOB.1) (Figure 3.4 D). Importantly, OBF.1/BOB.1 is not only a critical regulator of antibody production but also the transcriptional factor PU.1, which negatively regulates TLR4 expression and Th2 responses by suppressing GATA3 expression. Indeed, quantitative analysis of PU.1 and GATA3 expression after ant-miR-126 treatment showed increased and decreased expression, respectively (Figure 3.4 D).
Figure 3.4: Silencing miR-126 function by antagomir impairs Th2 responses in the lung. (A) IL-5, IL-13, and IFN-γ release from in vitro HDM-stimulated peribronchial lymphnode cells after no treatment (SAL) or treatment with a scrambled control sequence (ant-scrambled) or treatment with antagomir specific for the miR-126 sequence (ant-miR-126) in allergic mice and in nonallergic mice (SAL). Results are mean ±SEM (n = 6–10 mice per group). *, P ≤ 0.05, ant-scrambled versus SAL; **, P ≤ 0.01, ant-miR-126 versus SAL. (B) Percentages of CD4+ cells in lung homogenates and (C) percentages of CD11b+CD11c− MHC class II low (myeloid DCs) and pDC+ MHC class II high cells (plasmacytoid DCs) in lung homogenates. Results are mean ± SEM of three experiments (n = 4–6 mice/group per experiment). (D) Fold change in OBF.1/BOB.1 and PU.1 expression in the airway wall and GATA3 expression in the parenchyma of HDM-treated allergic mice that were exposed to ant-miR-126 as compared to those mice treated with ant-scrambled (baseline). **, P ≤ 0.01, ant-miR-126 versus ant-scrambled. Results are mean ±SEM (n = 4 mice/group). Data are representative of two independent experiments.
3.4 Discussion

The role of miRNAs in the regulation of immunological processes is beginning to emerge\textsuperscript{158,180,189,219,245,247,248,258-260}, however, their contribution to inflammatory diseases such as asthma remains unknown. In this investigation, we employ a well characterized model of HDM induced allergic respiratory inflammation to demonstrate that a select set of miRNAs are rapidly upregulated in the airway wall after allergen exposure. Increased expression of miRNAs and the development of HDM-induced Th2-mediated allergic inflammation were dependent on the TLR4/MyD88 pathway. This data indicated that miRNA expression is intimately linked to the innate host defense response in the lung and could potentially contribute to the regulation of the subsequent immune response. This study also supports emerging data that miRNA expression and function are linked to innate immune responses\textsuperscript{163,192,259}

Of the miRNAs upregulated by HDM exposure, we were able to demonstrate a specific role for miR-126 in the regulation of critical features of allergic disease. By exposing the airways to a specific antisense inhibitor, ant-miR-126, we abolished HDM induced expression of miR-126, which resulted in complete suppression of AHR, attenuation of mucus hypersecretion, and inhibition of eosinophil accumulation in the airways and lung tissue. AHR, eosinophil migration, and mucus production are regulated by the Th2 cytokines IL-5 and IL-13\textsuperscript{113,252-255}, and production of these cytokines were inhibited by ant-miR-126 treatment. Antagomir treatment also inhibited the recruitment of CD4+ T cells (predominately Th2 cells in this model) and Th2 promoting myeloid DCs into the airways, which are critical for the expression of allergic airways disease\textsuperscript{87,261,262}. 
Importantly, overall suppression of the Th2 response by ant-miR-126 occurred without evidence of a switch to a Th1 response, as T cell IFN-secretion and neutrophil recruitment were not promoted by antagonir treatment. Collectively, this data demonstrates that miR-126 is a potent and specific activator of the Th2 regulated allergic inflammatory response and that targeting miRNA expression in the respiratory epithelium may represent an important anti-inflammatory strategy to treat disease.

The way in which miRNAs function has not been fully characterized and identification of the putative transcript targets are currently based on bioinformatics analyses. Examination of databases available in the public domain (e.g., DIANA-microT, miRanda, PicTar, TargetScan) indicates that miR-126 has the potential to modulate hundreds of mRNA transcripts, some of which encode inflammatory mediators and transcriptional factors that modulate their expression. However, the biological relevance of most putative targets has not yet been demonstrated. Indeed, the physiological importance of miRNAs are being primarily ascertained by gain- and loss-of-function experiments. In the context of immune responses, studies on miRNA function suggest critical involvement in activation of transcriptional programs within feedback loops247. In this context, we demonstrate that inhibition of miRNA-126 function results in the increased expression of two factors whose functional activity is integrated to modulate Th2 responses and TLR4 expression. Comparisons of expression patterns of the genes in the airway walls treated with ant-miR-126 or scrambled control showed that the only non-Ig gene upregulated was POU domain class 2 associating factor 1 or OBF.1/BOB.1. OBF.1/BOB.1 was originally described as a B-cell-specific transcriptional coactivator and enhancer of octamer-dependent
transcription when recruited to DNA via protein-protein interactions with octamer binding transcription factor (Oct) -1 or -2 (42, 43). OBF.1/BOB.1 is essential for V(D)J recombination by directly enhancing Ig kappa gene transcription, and in OBF.1/BOB.1 / mice, the production of Ig isotypes is profoundly reduced\(^\text{263}\). The increase in OBF.1/BOB.1 expression by ant-miR-126 treatment resulted in high expression of transcripts that code for Ig chains and are likely to be derived from plasma cells that infiltrate the airways wall during allergic airways inflammation\(^\text{232}\). Recently, a critical role of OBF.1/ BOB.1 in activation of the transcriptional factor PU.1 and for Th2 cell function has been shown \textit{in vivo}. Th2 cells from OBF.1/BOB.1 / mice had reduced levels of PU.1, which resulted in higher GATA3 activity and consequently increased Th2 cytokine release and susceptibility to Leishmania major infection\(^\text{257}\). Conversely, we show that increased expression of OBF.1/ BOB.1 results in increased levels of PU.1 and a suppression of Th2 cell function (cytokine production) and GATA3 expression. Notably, PU.1 is also involved in directing the transcription initiation complex to the proximal TLR4 promoter, thereby representing a major regulator of TLR4 expression\(^\text{256}\), and is a direct target of miR-155\(^\text{260}\). Thus, higher expression of OBF.1/BOB.1 and PU.1 observed after the loss of miR-126 function may be directly linked to the enhanced expression of Ig kappa genes and the impaired Th2/GATA3 regulated allergic responses observed in this study. Furthermore, TLR4-induced miR-126 expression may be integrated in a negative feedback loop, whereby lower OBF.1/BOB.1 levels repress transcriptional activity at the TLR4 promoter through impaired PU.1 binding. Of note, although upregulated after miR-126 inhibition, OBF.1/ BOB.1 does not contain putative binding elements for miR-126 in the 3’ - or 5’ -untranslated region, precluding
further investigations of a direct effect of miR-126 on OBF-1/BOB.1 translation by employing reporter assays. This indicates the complexity of miRNA function \textit{in vivo} and of how miR-126 modulates gene translation to bridge TLR4 signalling and Th2 responses to regulate hallmark features of allergic airways disease. However, the data provides important molecular insight into the anti-inflammatory potential of targeting miRNA-126 and its subsequent impact on key downstream mechanisms that modulate allergic inflammation.

By inducing loss-of-function, we describe a physiological role for miR-126 in linking the innate and adaptive immune responses that respond to HDM allergen and result in allergic disease of the lung. The relevance of our observations to human asthma remains to be determined and will require detailed spatial and temporal analyses of miRNA expression patterns in airway wall cells in response to factors that trigger asthma.

The recognition of the contribution of airway inflammation to pathogenesis of asthma has lead to the employment of broad-spectrum anti-inflammatory agents, mostly glucocorticoids, for the treatment of disease. Steroid therapies primarily focus on the management of disease and function by suppressing a range of known and unknown components of the inflammatory response. However, to the best of our knowledge, they do not target factors that initiate inflammatory cascades that perpetuate progression of disease. Our study not only highlights the potential of miRNAs as targets for anti-inflammatory treatments for allergic airways disease, but also draws attention to the importance of identifying the gene regulatory elements that are pivotal switches for the induction of immunological networks that propagate inflammatory cascades and disease.
Chapter 4: Inhibition of House Dust Mite Induced Allergic Airways Disease by Antagonism of MicroRNA-145 is Comparable to Glucocorticoid Treatment

4.1 Abstract

Glucocorticoids are used as mainstay therapy for asthma but some patients remain resistant to therapy. MicroRNA (miRNA) are important regulators of the immune system by promoting the catabolism of their target transcripts as well as attenuating their translation. The role of miRNA in regulating allergic inflammation remains largely unknown. Blocking miRNA function may provide a new non-steroidal anti-inflammatory approach to treatment. In this chapter I 1) determine the role of specific miRNA in the regulation of hallmark features of allergic airways inflammation and 2) compare the efficacy of antagonising miRNA function with that of steroid treatment. Mice were sensitized and then aeroallergen challenged with house dust mite (HDM) to induce allergic airways disease and alterations in the expression of miRNAs characterised. Next mice were treated with antagonirs that inhibited the function of specific miRNAs (miR) in the lung or treated with dexamethasone and inflammatory lesions and airway hyperresponsiveness (AHR) measured. miR-145 (MIR145), miR-21 and let-7b have been implicated in airway smooth muscle function, inflammation and airways epithelial cell function, respectively. Inhibition of miR-145, but not miR-21 or let-7b, inhibited eosinophilic inflammation, mucus hypersecretion, T helper type 2 cytokine production and AHR. The anti-inflammatory effects of miR-145 antagonism were comparable to steroid treatment. Our study highlights the importance of understanding the contribution of miRNAs to pathogenesis of human allergic disease and their potential as novel anti-inflammatory targets.
4.2 Introduction

Glucocorticoids and long acting β-agonists\textsuperscript{264-266} suppresses inflammation and airways hyperreactivity (AHR) that is associated with the clinical expression of asthma. Indeed, the degree of inflammation and AHR often reflects the activity and severity of disease, and titration of therapy based on the control of AHR may yield superior therapeutic outcomes\textsuperscript{267,268}. Thus, modulation of the factors that regulate the development and persistence of these clinical features are thought to be of central importance in the perpetuation of asthma\textsuperscript{269}. Steroid therapy is effective but primarily focuses on the management of disease and functions by suppressing a range of known and unknown components of the inflammatory response. In particular, glucocorticoids may be effective because they suppress the activation and recruitment of key inflammatory cells in the airways such as T helper type 2 (Th2) cells, eosinophils and mast cells. These cells play central roles in pathogenesis through the release of well characterised pro-inflammatory factors (e.g. cytokines such as interleukin (IL) -13 and -5)\textsuperscript{264}. However, steroid therapy is not always effective, is non-specific and a subset of patients remain resistant to treatment\textsuperscript{270,271}. Long-term treatments with steroids can also have numerous adverse effects and this is of particular concern in affected children\textsuperscript{264,268}. Thus, identification of more effective and specific anti-inflammatory agents for the treatment of asthma would be clinically important\textsuperscript{264,268}.

MicroRNAs (miRNA) are short non-coding RNAs that function as post-transcriptional regulators of gene regulatory programs, by promoting messenger (m)RNA degradation or by directly blocking protein translation\textsuperscript{219}. Because miRNAs are not perfectly complementary to their targets, each is capable of
regulating a large number of mRNAs, and thus miRNAs can modulate entire transcriptional programs, although key individual targets may also be critically important\textsuperscript{248}. Specific miRNAs participate in regulating a range of cellular activities (e.g. differentiation, proliferation and programmed cell death) and dysregulation of function is now implicated in some diseased states (e.g. cancer)\textsuperscript{199}. There is also emerging evidence for an important role of miRNAs in the regulation of hematopoiesis and controlling inflammation\textsuperscript{189,218,219,221,272-274}. Thus, targeting miRNA function may provide a new way to treat diseases of the immune system and aberrant inflammation\textsuperscript{201,221,275}.

Currently functional data on the role of miRNA in the regulation of allergic airways inflammation is very limited\textsuperscript{199}. Furthermore, whether targeting miRNA activity is as effective as steroid treatment remains unknown. In this study we identify and characterise the function of miRNA that are specifically expressed in the airway wall after allergen provocation of mice sensitised to house dust mite (HDM) and the induction of allergic airways inflammation. We also conduct a comparative study with dexamethasone (DEX) to determine if our novel antisense antagonirs designed to functionally block specific miRNA are as effective as steroid treatment.

4.3 Results
4.3.1 HDM increases the expression of miR-145, -21 and let-7b in the airways

24 hrs after the first re-exposure to HDM, miRNA were isolated from the airway wall and expression levels determined by microarray. Of the miRNA expressed miR-145, -21 and let-7b where increased greater than 5 fold (P<0.05, n=4-6
mice) by contrast to control vehicle and their increased expression could be confirmed by qPCR (not shown). Furthermore miR-145, miR-21 let-7b have been implicated in airway smooth muscle function, allergic inflammation and airways epithelial cell function, respectively, suggesting that these miRNA may regulate aspects of the host response to HDM. The level of expression of miR-145, -21 and let-7b were not altered by exposure to saline. The increased expression of these miRNAs was also validated by qPCR on day 18 after repeated re-exposure to HDM (Figure 4.1 B) and the induction of hallmark features of allergic inflammation and AHR (Figures 4.2, 4.3 and 4.4).

**4.3.2 The effect of specific antagonirs on miRNA levels after HDM exposure**

Next we designed antagonirs to block the function of miR-145, -21 or let-7b or scrambled controls. Antagonirs or scrambled controls were administered to HDM-sensitised mice i.n. 24 hr prior to re-exposure and then given every 48hrs (Figure 4.1 A). Expression of target miRNA were then quantified 24 hr after the last re-exposure to HDM (day 18) by qPCR (Figure 4.1 B-D). HDM exposure significantly increased the expression of miR-145, -21 and let-7b and levels of transcripts were not affected in the presence of scrambled controls (Figure 4.1 B-D). By contrast, tissue levels of these miRNAs after HDM exposure were significantly decreased by treatment with the respective antagonir (Figure 4.1 B) (antagonirs were highly specific and did not alter the expression of non-target miRNA, see supplementary figure 4.1).
Figure 4.1: miRNA expression is increased in the airway wall of HDM sensitised an aeroallergen challenged mice and tissue levels are decreased by specific antagonirs. Mice were sensitised to HDM and treated with 12 days later they were exposed to 4 consecutive low dose HDM challenges and sacrificed 24hrs after the final challenge for analysis (A). Control non-sensitised mice received SAL. Mice were also treated with antagonirs (ant-miR) i.n. (50ug/50ul saline) or scrambled control (ant-scrambled) or dexamethasone (3mg/kg) or vehicle control I.P. (A) miRNA-specific qPCR for miR-145, (B) miR-21, (C) and let-7b (D) was conducted on RNA isolated from the airways and normalised to the expression of ribosomal RNA 18s. Data is expressed as fold change from expression levels of SAL controls ± SEM (n=4-8). *p ≤ 0.05.
4.3.3 Inhibition of the function of miR-145 but not miR-21 or let-7b suppresses HDM-induced mucus hypersecretion in airway epithelial cells and eosinophilic inflammation as effectively as dexamethasone treatment

Next we determined the regulatory role for miR-145, miR-21 or let-7b in the development of phenotypic features of allergic asthma and compared effects with those of dexamethasone. Disease severity in HDM sensitized and rechallenged mice was determined on day 18 when cellular levels of miR-145, miR-21 or let-7b were shown to be significantly reduced by antagomir treatment (Figure 4.1 B). HDM induced significant mucus hypersecretion in airway epithelial cells and promoted the recruitment of eosinophils to the airways and these inflammatory responses were not attenuated by treatment with scrambled controls (Figure 4.2). Notably, ant-miR-145 significantly reduced the number of both mucus-producing cells and eosinophils present in the airways of HDM challenged mice (Figure 4.2 A, D and G). By contrast, although ant-miR-21 and ant-let-7b both effectively reduced the levels of their target miRNA to pre-challenge levels, treatment did not inhibit mucus hypersecretion or the accumulation of eosinophils in airway tissue after HDM exposures (Figure 4.2 B and E). Notably, the suppressive effects of ant-miR-145 were comparable to the anti-inflammatory effects of dexamethasone (Figure 4.2 C and F) with an approximately 50% reduction in the number of mucus secreting cells and eosinophils in airway tissue (Figure 4.2: A and D ant-miR-145; C and F DEX; and G tissue histology). These trends were also mirrored in the bronchoalveolar lavage fluid where ant-miR-145 and dexamethasone treated mice showed significantly reduced eosinophil numbers (see supplementary figure 4.2), while
Figure 4.2: Inhibition of miR-145 function by antagomir reduced inflammation in the airways in a manner comparable with to dexamethasone treatment. Paraffin embedded airway tissues from mice treated with specific antagomirs (ant-miR), scrambled control sequence (ant-scrambled), dexamethasone (DEX) or vehicle control (veh) were stained with Periodic Acid Schiff (PAS) or Congo Red (CR) for the detection of mucus producing cells (A-C) or peri-bronchial eosinophils (D-F), respectively. Cells were identified morphologically and enumerated from 10 high-powered fields at 1000x magnification. (G) Representative photographs of cells stained for mucus expression (PAS) and eosinophil infiltrates (CR) in each group with amplified sections showing eosinophil morphology. Results are mean ± SEM (n=4-8). *p ≤ 0.05.
numbers of eosinophils in ant-miR-21 and ant-let-7b treated mice were equivalent to those treated with scramble controls (data not shown).

4.3.4 The effect of antagonim or dexamethasone treatment on the development of AHR

Next we determined the effect of blocking miR-145, miR-21 or let-7b on the development of HDM induced AHR (Figure 4.3). AHR in HDM sensitized and rechallenged mice was determined on day 18 after treatment with antagonims, scrambled controls or dexamethasone. HDM challenge induced significant AHR (Figure 4.3) in mice treated with scrambled controls and this correlated with the development of the allergic inflammatory lesions (Figure 4.2). Notably, treatment with ant-miR-145 or dexamethasone, but not with ant-miR-21 and ant-let-7b, resulted in marked attenuation of bronchial reactivity to methacholine (Figure 4.3). Inhibition of AHR by ant-miR-145 or dexamethasone treatments was directly associated with a reduction in eosinophil infiltrates and suppression of mucus production in the airways (Figure 4.2). We administered a single dose of ant-miR-145 or dexamethasone during established inflammation 12 hrs before final HDM exposure. Inhibition of miR-145 significantly inhibited AHR while DEX treatment was not effective. Ant-miR-145 and dexamethasone both reduced mucus production. However, there was no effect on the accumulation of eosinophils into the BALF or tissues as their influx had already peaked (see supplementary figure 4.3).
Figure 4.3: Inhibition of miR-145 function by antagonir reduced AHR in a magnitude comparable to dexamethasone treatment. (A-C) Lung resistance is presented as a percentage change over baseline measurement (SAL) in response to inhaled methacholine. Mice were treated with specific antagonirs (ant-miR), scrambled control sequence (ant-scrambled) or dexamethasone (DEX) or vehicle control (veh). Results are mean ± SEM, (n=6-10). *p ≤ 0.05 between dose response curves.
4.3.5 The effect of antagonir or dexamethasone treatment on the production of Th2 cell cytokines

We and others have previously shown that Th2 cells through the production of IL-5 and IL-13 play pivotal roles in the induction of hallmark features of allergic airways disease such as eosinophil recruitment, mucus hypersecretion and the development of AHR. Thus, we next investigated whether the secretion of these key cytokines was suppressed by ant-miR-145 treatment and compared responses to dexamethasone treatment after HDM challenge. HDM sensitised and challenged mice were treated with antagonirs, scrambled controls or dexamethasone, and on day 18 Th2 cells from the draining lymph nodes were cultured and stimulated with HDM or without HDM (saline vehicle control) and the levels of IL-5 and IL-13 measured in supernatants (Figure 4.4). Ant-miR-145 and dexamethasone treatments significantly reduced the production of IL-5 and IL-13 from antigen specific Th2 cells. The effect of dexamethasone on Th2 cell cytokine production appeared to be more potent as the levels of IL-5 and IL-13 were reduce to almost non-stimulated levels in these cultures (Figure 4.4 B). Notably inhibition of cytokine production by ant-miR-145 or dexamethasone correlated with suppression of hallmark features allergic airways disease (Figure 4.2 and 4.3). By contrast antagonism of miR-21 or let-7b did not significantly inhibit Th2 cell cytokine production (Figure 4.4 C) or mucus production, eosinophil infiltrates or AHR (Figures 4.2 and 4.3).

4.4 Discussion

In this investigation we have employed a well characterised model of HDM induced allergic airways disease that mimics hallmark features of allergic asthma
Figure 4.4: **Inhibition of miR-145 function by antagonir reduced inflammatory cytokine production within the draining lymph nodes to a level comparable to dexamethasone treatment** (A-C). Peribronchial lymph nodes of the lung were collected from mice treated with (A,C) specific antagonirs (ant-miR), scrambled control sequence (ant-scrambled), (B) dexamethasone (DEX) or vehicle control (veh). Cells were cultured for 6 days prior to supernatant collection. Elisa was conducted for IL-5, IL-13, and IFN-γ and levels are shown from *in vitro* HDM-stimulated peri-bronchial lymph node supernatants above unstimulated levels. Results are mean ± SEM (*n = 6–10*). *p ≤ 0.05.
to demonstrate that the expression of specific miRNAs are induced in the airways in response to allergen provocation. The expression of miR-145, miR-21 and let-7b were significantly increased in the airway wall and up-regulation directly correlated with the development of inflammation and AHR. However, of the miRNA up-regulated only miR-145 played a proinflammatory role for the onset of allergic airways disease. Expression of miR-145 is regulated by a highly conserved genomic sequence 3’ to the pre-miRNA277. miR-145 is one of the most highly expressed miRNA within the human airway and trachea177. Furthermore, miR-145 regulates smooth muscle development and the differentiation of fibroblasts into smooth muscle cells175. miR-145 also functions as a tumor suppressor where it targets transcripts for sex determining region Y –box (SOX)2, octamer-binding transcription factor (OCT)4 and Krueppel-like factor (KLF)4, Mucin 1, junction adhesion molecule (JAM)-A and fascin as well as insulin receptor substrate (IRS)-1278-282. The role of miR-145 in immune responses and inflammation has not yet been described. In this study selective inhibition of miR-145 function but not that of miR-21 or let-7b with antagomirs inhibited the production IL-5 and IL-13 by Th2 cells, the recruitment of eosinophils to the airways, mucus hypersecretion and the development of AHR. Notably, the anti-inflammatory effects of blocking the specific function of miR-145 were equivalent to that of steroid treatment with dexamethasone.

Th2 cells through the secretion of cytokines are thought to play key roles in the pathogenesis of allergic asthma and glucocorticoid therapy may be effective, in part, because they suppress the activity of these cells. In models of allergic asthma IL-5 and IL-13 derived from activated Th2 cells have been shown to play central roles in the induction of allergic inflammatory lesions and AHR. IL-5
regulates the induction of eosinophilia and the recruitment of this granulocyte into tissues\textsuperscript{276,283}. IL-13 by activating the IL-4 receptor type-2 complex and STAT6 pathway induces mucus hypersecretion, tissue accumulation of eosinophils and development of AHR\textsuperscript{109,113,284}. Notably targeting miR-145 suppressed antigen induced production of IL-5 and IL-13 from Th2 cells, which correlated with attenuated eosinophil infiltration into the airways, reduced mucus hypersecretion and abrogated AHR in response to methacholine inhalation. Thus, miR-145 acts a critical proinflammatory molecule in the regulation of allergic inflammation of the airways. Furthermore, antagomirs enter all cells suggesting a global effect of suppressing miR-145 function may have contributed to the anti-inflammatory effects. Although we do not know the precise molecular targets of miR-145 it is emerging that the primary function of miRNA are to inhibit translation of specific mRNA transcripts to alter cellular function, and in the immune system the targets are often factors that regulate key transcription axis\textsuperscript{219,246}. As miR-145 expression was highly induced by HDM exposure it is thus likely to function by inhibiting mRNA transcripts that are negative regulators of inflammatory pathways and provide a pro-inflammatory transcriptional environment. Blockade of miR-145 function with antagomirs prevents subsequent activation of these pro-inflammatory transcriptional circuits. Interestingly miR-145 is expressed in the human respiratory tract\textsuperscript{177} and has also been linked to regulation of innate host defence signalling by inhibiting IFN-β production\textsuperscript{209}. Recently miR-126 was also shown to exert pro-inflammatory effects by altering the levels of mRNA transcripts for factors that negatively regulate the production of the Th2 differentiation factor GATA3\textsuperscript{221}. Inhibition of miR-126 significantly resulted in attenuation of HDM induced
allergic airways inflammation and AHR\textsuperscript{221}. Our studies provide further proof-of-principle data that targeting specific miRNA may provide a new approach to inhibiting allergic inflammation.
4.5 Chapter 4: Supplementary repository

Glucocorticoids are used as mainstay therapy for asthma, however, treatment is not always effective and there are side effects\textsuperscript{285,286}. Thus, the development of more targeted anti-inflammatory therapies may be beneficial for the treatment of asthma. Although modes of delivery of our interventions are not directly comparable and dexamethasone is currently the most relevant approach to therapy, our study provides proof-of-principle data that targeting miRNA has the potential to suppress allergic inflammation. Here we demonstrate that antagonism of miR-145 function was as effective as glucocorticoid therapy. In a side-by-side analysis mice treated with ant-miR-145 or dexamethasone displayed significant reduction in the severity of the inflammatory lesions induced by HDM challenge. Dexamethasone has previously been shown to inhibit inflammation in ovalbumin and chronic HDM exposure models of allergic asthma\textsuperscript{287}. Here we confirm the anti-inflammatory actions of dexamethasone after acute exposure to HDM. Dexamethasone suppressed eosinophil recruitment, mucus hypersecretion, AHR and Th2 production of IL-5 and IL-13 in the lung after HDM exposure. The anti-inflammatory effect of ant-miR-145 was similar to dexamethasone, however, its effect on Th2 cytokine production were not as pronounced. Dexamethasone treatment completely suppressed the production of both IL-5 and IL-13 from antigen reactive Th2 cells, while ant-miR-145 was only able to reduce expression by approximately 50%. However, this partial reduction in Th2 cytokine production by ant-miR-145 treatment was clearly therapeutically beneficial. Interestingly, blocking the function of miR-126 with antagonirs also only partially reduced Th2 cytokine levels despite pronounced inhibition of inflammatory lesions and AHR\textsuperscript{221}. This difference may
also reflect the broader actions of steroids on other pathways not under the influence of these miRNA.

Interestingly although miR-21 and let-7b were highly expressed during allergic inflammation blockade of their function was ineffective at modulating the expression of disease. Very recent studies have also shown that these miRNA are expressed in the lung in various models of allergic airways disease\textsuperscript{218,288}. However, the role for these two miRNA in regulating allergic airways disease is unclear. miR-21 is activated by STAT3 signalling\textsuperscript{289} and is frequently identified as one of the most upregulated miRNA in lung carcinoma. In part this is thought to be due to the ability of miR-21 to directly inhibit the tumour suppressor PTEN\textsuperscript{217}. miR-21 has been postulated to induce allergic inflammation by promoting the degradation of IL-12 transcripts and promoting a pro-Th2 type immune environment\textsuperscript{218}. let-7b has previously been implicated as a regulator of the innate immune response through the modulation of IFN\(\beta\). It is able to exert this effect through a seed sequence binding site in the 3’ untranslated region of the IFN\(\beta\) mRNA sequence\textsuperscript{209}. Systemic administration locked nucleic acids (LNA) targeting let 7 family function (antisense oligonucleotides) inhibited Th2 cytokine production, eosinophil accumulation, normalized Muc5AC transcripts and suppressed AHR in an ovalbumin model of allergic asthma\textsuperscript{288}. However, in this study \textit{in vitro} analysis showed that Th2 cell IL-13 mRNA was a direct target.
Supplementary Figure 4.1: miRNA-specific qPCR for miR-145, was conducted on RNA isolated from the airways and normalised to the expression of ribosomal RNA 18s. Data is expressed as fold change from expression levels of SAL controls ± SEM (n=4-8). *p ≤ 0.05.

of let 7 family members indicating that LNA treatment (blockade of function) should increase T-cell production of this cytokine that would potentially exacerbate disease. The difference in observations between this investigation and our study may reflect the mode of administration of the antagonists (systemic Vs i.n.) and/or the models used and the contribution of potential
targets of the miRNA in the inflammatory response. Alternatively redundant signalling between multiple members of the let-7 family may have masked the role of let-7b when its function was specifically inhibited by antagonir treatment.

**Supplementary Figure 4.2:** Total number of cells in bronco-alveolar lavage fluid of (A) mice treated with specific antagonir 145 (ant-miR-145) scrambled control sequence (ant-scrambled), no treatment (HDM) and non-allergic saline controls (SAL) or (B) dexamethasone (DEX) or vehicle control (veh). (n=4-8) *p ≤ 0.05.
in our study. The more general targeting of the let-7 common seed sequence may also be required to disclose a proinflammatory role for this miRNA cluster in response to HDM.

Abnormal expression of miRNA is beginning to be associated with inflammatory diseases of the lung, skin and joints\textsuperscript{199,274}. However, there is very limited functional data on the contribution of miRNA to pathogenesis and whether targeting these molecules is a potential new approach to treatment. Here we demonstrate that miR-145 plays a central pro-inflammatory role in the development allergic airways inflammation to HDM and that blocking its function significantly reduces the severity of inflammatory lesions and AHR. Importantly, the efficacy of targeting one miRNA was equivalent to the therapeutic effect of steroid treatment. Thus targeting miRNA or their key downstream protein targets may provide a more specific way to deliver anti-inflammatory therapy. Our study highlights the importance of understanding the contribution of miRNAs to pathogenesis of human allergic disease and chronic inflammatory conditions.
Supplementary Figure 4.3: Both antagomir targeting miR-145 and dexamethsone display limited ability to reverse established allergic inflammation within the lung. Lung sections from mice treated with a single dose of antagomir targeting miR-145 (HDM + ant-miR-145), scrambled control sequence (HDM-ant-scrambled), dexamethasone (HDM + DEX) or vehicle control (veh) were stained with Periodic Acid Schiff (PAS) or Congo Red (CR) for the detection of mucus producing cells (A) or peri-bronchial eosinophils (B), respectively. Cells were identified morphologically and enumerated from 10 high-powered fields at 1000x magnification. (C) Lung resistance is presented as a percentage change over baseline measurement (SAL) in response to inhaled methacholine (n=4-8) *p ≤ 0.05.
Chapter 5: Altered expression of microRNA in the airway wall in chronic asthma: miR-126 as a potential therapeutic target.

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5.1 Abstract

The role of microRNAs (miRNAs) in regulating gene expression is currently an area of intense interest. Relatively little is known, however, about the role of miRNAs in inflammatory and immunologically-driven disorders. In a mouse model, we have previously shown that miRNAs are potentially important therapeutic targets in allergic asthma, because inhibition of miR-126, one of a small subset of miRNAs upregulated in the airway wall, effectively suppressed Th2-driven airway inflammation and other features of asthma. In the present study, we extended investigation of the therapeutic potential of miRNA inhibition to our well-established model of chronic asthma. Female BALB/c mice were systemically sensitised with ovalbumin (OVA) and chronically challenged with low mass concentrations of aerosolised OVA for up to 6 weeks. Airway tissue was obtained by blunt dissection and RNA was isolated for miRNA profiling. On the basis of the results obtained, animals were subsequently treated with either an antagonir to miR-126 (ant-miR-126) or a scrambled control antagonir once weekly during the 6 weeks of chronic challenge, and the effects on airway inflammation and remodelling were assessed using established morphometric techniques. Compared to naïve mice, there was selective upregulation of a modest number of miRNAs, notably miR-126, in the airway wall tissue of chronically challenged animals. The relative increase was maximal after 2 weeks of inhalational challenge and subsequently declined to baseline levels. Compared to treatment with the scrambled control, ant-miR-126 significantly reduced recruitment of intraepithelial eosinophils, but had no effect on the chronic inflammatory response, or on changes of airway remodelling. In this model of chronic asthma, there was an initial increase in expression of a
small number of miRNAs in the airway wall, notably miR-126. However, this later declined to baseline levels, suggesting that sustained changes in miRNA may not be essential for perpetuation of chronic asthma. Moreover, inhibition of miR-126 by administration of an antagonir suppressed eosinophil recruitment into the airways but had no effect on chronic inflammation in the airway wall, or on changes of remodelling, suggesting that multiple miRNAs are likely to regulate the development of these lesions.

5.2 Introduction
The role of non-coding RNA species in the regulation of mammalian gene expression is becoming increasingly apparent. Among non-coding RNAs, the microRNAs (miRNAs) are of particular interest. These are small non-coding RNAs of approximately 17-24 nucleotides, each of which is predicted to regulate hundreds of genes (both coding and non-coding) by post-transcriptional (and possibly also translational) silencing. There is currently an intense focus on the role of miRNAs in a variety of human diseases, ranging from cardiovascular disorders to malignant neoplasms, with active investigation of the potential of inhibiting miRNAs as a novel approach to treatment.

The role of miRNAs in inflammatory and immunologically-driven disorders is slowly being elucidated. Studies from our group have identified miRNAs as potentially important therapeutic targets in allergic asthma. In a mouse model of acute allergic bronchopulmonary inflammation induced by intranasal challenge with house dust mite (HDM) extract, we demonstrated selective upregulation of a small subset of miRNAs in airway tissues. Furthermore, we showed that inhibition of microRNA-126 (miR-126) by delivery of an antagonir (a cholesterol-linked single-stranded anti- sense RNA that selectively binds to
this miRNA) effectively suppressed Th2-driven airway inflammation, mucus hypersecretion and airway hyper-responsiveness\textsuperscript{221}. We therefore sought to extend investigation of the therapeutic potential of miRNA inhibition in asthma to a study in our well-established model of chronic asthma based on long-term low-level challenge with ovalbumin (OVA)\textsuperscript{293,294}. This more closely replicates several key features of this disease, including acute-on-chronic inflammation of the airway wall, subepithelial and epithelial changes of remodelling, airway-specific hyper-responsiveness, and a spatial distribution of lesions corresponding to that observed in human asthma\textsuperscript{295}. In this report, we describe the time course of altered expression of miRNAs in the airway wall in our model of chronic asthma and assess the potential of using an antagomir to inhibit miR-126 (the most highly-upregulated miRNA) as a therapeutic intervention.

5.3 Results and Discussion

5.3.1 Altered expression of miRNAs in the airway wall in chronic asthma

Compared to naïve mice, there was selective upregulation of a modest number of miRNAs in the airway wall tissue of sensitised, chronically challenged animals. Only 11 miRNAs exhibited a 2-fold or greater increase: these were miR-126, -197, -341, -145, -30c, -23b, -199a, -29a, -129-3p, -16 and -495 (Table 1). The complete dataset is deposited at ArrayExpress (http://www.ebi.ac.uk/arrayexpress) (accession number pending).

Notably, levels of expression of miR-126 were increased to a much greater extent than those of any other upregulated miRNA, and this was confirmed by qRT-PCR
There was clear evidence of regulation of changes in miRNA expression over time: the relative increase was maximal after 2 weeks of inhalational challenge and subsequently declined, so that except for miR-126 all had returned to baseline levels by 6 weeks (Table 5.1).

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<td>2.02</td>
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**Table 5.1: Fold change in expression of miRNAs in airway wall tissues during chronic challenge.** Changes in relative expression of individual miRNAs relative to levels in naïve animals, as assessed by microarray profiling. All miRNAs with >2-fold upregulation at 2 weeks of challenge are listed. Downregulation at other time points is shown in *italics.*

In the control group of animals that were not sensitised but were challenged for 6 weeks with aerosolised OVA, miR-126, -341 and -380-3p were found to be
upregulated by 2-fold or greater. Of these, miR-126 was again the most highly upregulated, with a 5-fold increase compared to naïve mice. This is of interest because we have previously shown that although these animals are not systemically sensitised, there is low-level sensitisation via the respiratory tract as a consequence of the chronic challenge, leading to a specific humoral immune response\textsuperscript{296}. Some of the current findings with respect to altered expression of miRNA in the airway wall are congruent with our previous report\textsuperscript{221} which similarly detected upregulation of a limited number of miRNAs following induction of allergic inflammation\textsuperscript{221}. In that previous study, which was based on a short-term model of HDM-induced asthmatic airway inflammation, miR-126 was the most highly upregulated miRNA in the airway wall. In the present study, we have confirmed that miR-126 is potentially a very important miRNA in asthmatic inflammation, because it was also the most highly upregulated miRNA in the chronic OVA challenge model. Interestingly, in the previous study miR-16 was also upregulated in the HDM model and this was again observed in the chronic OVA model. However, there were some important differences in the model of chronic asthma, notably the absence of demonstrable upregulation of miR-21, and evidence of upregulation of 10 additional miRNAs.

Because we assessed expression of miRNA in airway wall tissues, we do not have information about changes in specific cell types. Airway wall tissues include structural cells (such as airway epithelium, fibroblasts, smooth muscle, cartilage, vascular tissues) and recruited inflammatory cells. Importantly, unlike in short-term models of allergic inflammation of the airways, in our chronic challenge model there is a substantial change in the inflammatory cellular profile in the
airway wall over time. Notably there is progressive accumulation of cells associated with chronic inflammation, especially CD3+ T-lymphocytes and plasma cells. Whether cell-specific changes account for the different profile of expression of miRNA in the chronic challenge model is at present unclear.

Whereas in the HDM model we assessed changes in expression of miRNA following two challenges 10 days apart, in the present study we were able to examine the time course of changes in expression of upregulated miRNAs. Remarkably, this revealed that upregulation was not sustained with continuing challenge, which could imply that expression of these miRNAs plays a more important role in the initiation rather than the perpetuation of asthmatic lesions.

Figure 5.1: Relative expression of miR-126. qRT-PCR confirmation of upregulation of miR-126 in airway wall tissue of animals following chronic challenge. Significant differences compared to naïve controls are shown as * (p<0.05), *** (p<0.001); significant differences compared to mice challenged for 2 weeks are shown as # (p<0.05), ### (p<0.001)(n=6).
5.3.2 Effects of treatment with antagonir to miR-126 on changes of chronic asthma

Animals that were treated with the scrambled control antagonir developed airway wall changes that were indistinguishable from those observed in the chronic challenge model without any treatment \(^{293}\), including recruitment of significant numbers of intraepithelial eosinophils, accumulation of chronic inflammatory cells in the lamina propria, subepithelial fibrosis and widespread goblet cell hyperplasia/metaplasia (Figure 5.2). Long-term administration of ant-miR-126 significantly reduced the numbers of intraepithelial eosinophils in the conducting airways (Figure 5.2 A). However, treatment with ant-miR-126 had no effect on the chronic inflammatory response (Figure 5.2 B). Similarly, changes of remodelling were essentially identical to those in mice treated with ant-scrambled (Figure 5.2 C, D).

To verify that delivery of ant-miR-126 was effective, we assessed the expression of TOM1 (target of Myb1) which is a negative regulator of IL-1\(\beta\) and TNF-\(\alpha\) - induced signalling pathways. TOM1 has been defined as a target of miR-126 and is downregulated by it \(^{297}\). While there was no change in the expression of TOM1 in animals treated with ant-scrambled when compared to naïve mice, TOM1 was markedly and significantly upregulated in animals treated with ant-miR-126 (Figure 5.3). Whether TOM1 has any function in this model is unknown; however, this finding confirmed that ant-miR-126 was biologically active in the airway wall of these animals. Because samples from the antagonir-treated animals were processed for assessment of expression of mRNA, not miRNA, we were unable to directly confirm the effects of treatment with antagonirs on the levels of miR-126.
We have previously shown that accumulation of intraepithelial eosinophils is related to upregulation of expression of eotaxin in the airway epithelium in response to inhalational challenge \(^\text{294}\). To investigate whether the mechanism of the reduction in numbers of eosinophils in antagomir-treated mice was related to inhibition of expression of eotaxin, we performed immunostaining on sections of tracheas. As expected, there was increased immunoreactivity for eotaxin in the epithelium of animals treated with ant-scrambled (median grade 2.0, range 1-3). There was a reduction in animals treated with ant-miR-126 (median grade 1.0, range 0-2), although this difference was not statistically significant. Whether ant-miR-126 had any other cell-specific effects in this model is unknown.

In our previous study in an HDM-induced model of asthmatic airway inflammation, we showed that selective inhibition of miR-126 using a specific antagomir inhibited eosinophil recruitment and AHR \(^\text{221}\). The evidence that treatment with ant-miR-126 was also effective in suppressing eosinophil recruitment into the airways in the model of chronic asthma is encouraging in terms of the potential of antagomir therapy. However, in the short-term HDM model it was not possible to assess the effects of ant-miR-126 on chronic inflammation in the airway wall, or on the development of airway remodelling. Unfortunately, we found that treatment with ant-miR-126 did not inhibit the progression of these lesions. While the frequency of delivery of antagomirs was lower in the present study (once weekly rather than alternate days) it is not altogether surprising that targeting a single miRNA would not suppress all of the changes in a complex disorder such as chronic asthma. We have previously
Figure 5.2: Morphometric assessment of the effects of antagomir treatment. Quantification of (A) profile density of intraepithelial eosinophils (B) profile density of chronic inflammatory cells in the lamina propria (C) thickness of subepithelial collagenisation (all assessed in the trachea) and (D) grade of mucous cell change (assessed in intrapulmonary airways) after 6 weeks of chronic challenge and antagomir treatment. Values are expressed as mean ± SEM (A-C) or median ± interquartile range (D); 8 animals were assessed per group. Significant differences compared to naïve controls are shown as * (p<0.05), ** (p<0.01), *** (p<0.001); significant difference compared to mice treated with ant-scrambled is shown as # (p<0.05)(n=6-8).

demonstrated that in many respects, the lesions of asthma are expressed differently in our chronic challenge model as compared to a short-term model of allergic pulmonary inflammation. Thus our data suggest that ant-miR-126 alone has limited therapeutic potential in asthma. Nevertheless, they emphasise the importance of elucidating the role of other regulatory miRNAs in asthmatic airway inflammation, such as those we have identified in this study, which might allow the development of appropriate combination therapy.
5.4 Conclusions
In this model of chronic asthma, inhalational challenge with OVA initially increased the expression of a small number of miRNAs in the airway wall, notably miR-126. By 6 weeks of challenge, however, this enhanced expression has largely declined to baseline levels, suggesting that sustained changes in miRNA may not be essential for perpetuation of chronic asthma. Inhibition of miR-126 by long-term administration of an antagonir suppressed eosinophil recruitment into the airways. However, this treatment had no effect on chronic inflammation in the airway wall, or on changes of remodelling, reinforcing the complexity of chronic asthma and the likelihood that multiple miRNAs regulate the development of these lesions.
Figure 5.3: Relative expression of mRNA for TOM1. qRT-PCR confirmation of upregulation of *TOM1* in airway wall tissue of animals that received 6 weeks of chronic challenge and treatment with ant-miR-126. Significant difference compared to naive controls is shown as ** (p<0.01), compared to mice treated with ant-scrambled is shown as ## (p<0.01)(n=6-8).
Chapter 6: The E3 ubiquitin ligase Midline-1 links allergen and rhinovirus exposure to asthma via targeting PP2A

Manuscript submitted Nature Medicine 2011
6.1 Abstract:

Asthma is one of the most widespread chronic health problems worldwide. The majority of asthma exacerbations are caused by rhinovirus infections, which contribute enormously to asthma mortality and morbidity. Employing mouse models of house dust mite-induced and rhinovirus-exacerbated airways disease, we show a critical role of the TRAIL-induced E3 ubiquitin ligase Midline-1 in the development of airways inflammation, obstruction, and allergen induced exacerbations. Upon sensing allergen and rhinovirus, Midline-1 was expressed by airway epithelial cells in a TLR4-dependent manner and impaired Protein Phosphatase 2A (PP2A) activity through direct interaction with its catalytic subunit, PP2Ac. Raising PP2A activity in the airways with a non-phosphorylatable FTY720 analogue or with siRNA-mediated targeting of Midline-1 abolished airways obstruction and reduced inflammation, mucus hypersecretion, IL-5, IL-13, CCL20 production, and levels of phosphorylated p38 MAPK in the lung. Inhibition of Midline-1 with siRNA also protected mice from the development of rhinovirus-induced inflammation, airways obstruction, as well as the release of CCL2, 3, 4, 11, 20, CXCL1, 9, and 10 release. Midline-1 was upregulated in airway epithelial cells from asthmatics and closely correlated with TRAIL expression upon ex-vivo rhinovirus infection. Thus, Midline-1 links innate immune pathways activated by allergen and rhinovirus to T helper 2 immunity by impairing PP2A-mediated dephosphorylation of pro-inflammatory signalling cascades such as p38 MAPK.
6.2 Introduction

Asthma is characterized as an obstructive airways disease with reversible episodes of airflow limitation. It is most often associated with allergic inflammation of the lung, which consists of eosinophils, neutrophils, and lymphocytes and occurs concurrently with episodic airway obstruction. Innate and adaptive immune pathways act in concert to orchestrate airways inflammation that is thought to promote airways obstruction. Specifically, upon allergen sensing through Toll-like receptor 4 (TLR4) the respiratory epithelium and underlying mesenchyma release proinflammatory and growth factors into the airways that attract circulating white blood cells. The production of innate proallergic cytokines such as thymic stromal lymphopoietin, granulocyte-macrophage colony-stimulating factor, interleukin(IL)-25, and IL-33 by airway epithelial cells promotes allergic lung inflammation and T cell maturation in part via activation of mucosal dendritic cells. IL-13 production by Th2 cells induces AHR and mucus production in a STAT6-dependent manner resulting in airway obstruction. Importantly activation of both TLR4 and STAT6 signalling pathways in the airway wall are essential for the development of all salient features of allergic asthma. Thus, a bidirectional interaction between structural and immune cells is thought to underpin asthma expression and chronicity.

Asthma exacerbations contribute enormously to disease burden and healthcare costs, particular in infancy and childhood. Rhinovirus infection leads to common cold symptoms in healthy subjects, but in asthmatics this virus causes the majority of exacerbations. Rhinovirus is also a major cause of wheezing
illness in infants and infection is linked to the development of asthma in later life\textsuperscript{307, 308}. Notably we have shown that asthmatics have deficiencies in their antiviral responses, possibly predisposing them to exaggerated inflammatory responses\textsuperscript{231, 309, 310}.

Recently we have also identified tumor necrosis factor TRAIL, which is a member of the TNF superfamily of cytokines, as another innate immune signal released by the respiratory epithelium upon allergen exposure\textsuperscript{87}. TRAIL was essential for the expression of all hallmark features of ovalbumin-induced Th2 cell mediated AAD by promoting CCL20-mediated recruitment of T cells and myeloid dendritic cells to the airways in a STAT6-dependent manner\textsuperscript{87}. Combinations of single nucleotide polymorphisms in the gene that codes for TRAIL (TNFSF10) were also associated with asthma in a large population cohort\textsuperscript{311}. In contrast to ovalbumin, HDM allergy and exposure is clinically relevant in a large proportion of asthmatics and is sensed by airway epithelial cells via TLR4. Here we also show a crucial role for TRAIL in TLR4-dependent HDM-induced AAD, including AHR, inflammatory cell accumulation in bronchoalveolar lavage fluid, airways inflammation and goblet cell metaplasia/airway epithelial cell mucin accumulation, release of Th2 cytokines from \textit{in-vitro} allergen-stimulated peribronchial lymphnode cells, and CCL20 release (Supplementary Fig 6.1). This confirms a crucial and pivotal role of TRAIL in the pathogenesis of allergic inflammation.

\textbf{6.3 Results and Discussion}

We blunt dissected airway tissue from lung parenchyma and determined the gene transcripts that were differentially expressed in the airway tissue of allergic and non-allergic WT mice by employing a genome-wide gene array analysis.
(Array Express accession number # E-MEXP-2960). We found that the microtubule-associated E3 ubiquitin ligase Midline-1 was upregulated in allergic as compared to non-allergic WT mice. However, midline-1 was not upregulated in the respiratory epithelium of house dust mite allergic Tnfsf10−/− mice (Figure. 6.1 A). Crude house dust mite extract is sensed by TLR4 and this signal, similar to TRAIL, is required for the development of AAD. Notably, mice deficient for TLR4 (Tlr4−/−) also failed to upregulate Midline-1 in response to allergen exposure (Figure. 6.1 A). The intracellular TLR4 receptor domain (Toll/Interleukin-1 receptor (TIR) domain) binds to the adapter molecule MyD88 for signal transduction. As expected, we also found reduced Midline-1 expression in allergen-exposed Myd88−/− as compared to WT mice (Figure. 6.1 A). By contrast however, allergen-sensitized and challenged Stat6−/− mice showed increased Midline-1 expression as compared to non-allergic Stat6−/− mice (Figure. 6.1 A) even though this mouse strain -like Tlr4−/−, Myd88−/−, and Tnfsf10−/− mice- was protected from the development of AAD. This suggests that exposure to allergen and TRAIL promotes Midline-1 expression upstream of STAT6. Midline-1 may therefore link TLR4/MyD88 and TRAIL signalling to induce AAD.

The MID1 gene is located at locus Xp22.3 and mutations in MID1 have been associated with X-linked Opitz syndrome, an inherited malformation characterized by midline defects such as clefts. Mutations found in Opitz syndrome patients are thought to lead to a dysfunctional transport of Midline-1 in migrating neural crest cells. Interestingly, linkage between the Xp22 locus and both allergy and asthma has also been observed. More relevant for a role of Midline-1 beyond embryonic development and in the pathogenesis of
Figure 6.1: Midline-1 inhibition abolishes airways hyperreactivity, reduces airway inflammation and increases PP2A activity. (A) Midline-1 mRNA and protein expression in the airway wall. Scale Bar, 10 µm. (B) PP2A activity and (C) PP2Ac levels in lung homogenates. (n=3-4 mice per group). *, P < 0.05 and **, P < 0.01. (D) Midline-1 mRNA and protein expression in the airway wall of non-allergic (SAL) versus allergic (HDM) mice treated with a scrambled siRNA (Nonsense siRNA) or a Midline-1 targeting siRNA (MID-1 siRNA) every second day during the allergen challenge period intranasally. Results are mean±s.e.m. (n=3-4 mice per group). (E) Total lung resistance as percentage change of baseline measurement (water) in response to inhaled methacholine. Results are mean±s.e.m. (n=6-10 mice per group). (F) Number of peribronchial perivascular eosinophils (x1000) and mucus-producing cells (x400) per high-power field (HPF). Results are mean±s.e.m. (n=2-3 mice per group). (G) Cytokine release from in-vitro house dust mite stimulated peribronchial lymphnode cells and CCL20 expression in the airway wall. Results are mean±s.e.m. (n=4-6 mice per group). (H) PP2A activity in lung homogenates. (n=3 mice per group). (I) Phosphorylated p38 MAPK protein expression in the airway wall. Results are mean±s.e.m. (n=2-3 mice per group). Scale Bar, 10 µm. *, P < 0.05 and **, P < 0.01.
inflammatory diseases is the observation that MID1 mutations also lead to hypophosphorylation of microtubule-associated proteins. Specifically Midline-1 is required for the ubiquitin-specific modification and proteasomal degradation of the catalytic subunit of PP2A (PP2Ac) via interaction with a regulatory subunit of PP2A, the α4 subunit. In accordance with these in-vitro studies, PP2A activity and PP2Ac protein were inversely related to Midline-1 expression in-vivo and impaired in allergic WT mice. Nonetheless, they remained unchanged in allergic Th17f10−/− as compared to non-allergic WT mice (Figure. 6.1 B and C; and Supplementary Figure. 6. 2). The PP2A holoenzyme is composed of three subunits but only the PP2A-B subunit is highly variable and confers substrate specificity while PP2A-A and PP2Ac are the highly conserved scaffolding and catalytic proportions, respectively. PP2A is the most abundantly expressed protein phosphatase and regulates protein function by dephosphorylating transcriptional factors such as the MAPK kinase (MAPKK) MKK3/6, which is critically involved in limiting p38 MAPK activation. Notably, the p38 MAPK signalling pathway is activated in the airway wall of severe but not mild asthmatics and promotes AHR, inflammation, mucus hypersecretion, subepithelial fibrosis, smooth muscle hypertrophy and cytokine/chemokine release.

To determine the role of Midline-1 in AAD, we employed small interfering (si)RNA technique to inhibit function. Allergic mice were treated with a commercially available (Ambion) in-vitro validated siRNA directed against Midline-1 (MID-1 siRNA) or a sequence scrambled non-sense siRNA (control) following previously established protocols in our laboratory. Inhibition of Midline-1 (Figure. 6.1 D) during allergen challenge of sensitized WT mice
abolished AHR (Figure. 6.1 E), suppressed airways inflammation and mucus production (Figure. 6.1 F). In addition, this reduced Th2 cytokine release in allergen-stimulated peribronchial lymphnode cultures and CCL20 expression in blunt-dissected airway wall samples (Figure. 6.1 G). Importantly, Midline-1

![Graphs and images](https://example.com/graphs)

**Figure 6.2:** PP2A activation abolishes airways hyperreactivity and reduces airway inflammation and Th2 cytokine release. (A) PP2A activity in lung homogenates from non-allergic (SAL) versus allergic (HDM) mice treated with 2% 2-hydroxypropyl- cyclodextrin (vehicle) or AAL(S) each day during the allergen challenge period intranasally. Results are mean ±s.e.m. (n=4 mice per group). (B) Total lung resistance as percentage change of baseline measurement (water) in response to inhaled methacholine. Results are mean s.e.m. (n=5-8 mice per group). (C) Number of cells in bronchoalveolar lavage fluid (BALF). Results are mean ± s.e.m. (n=3-4 mice per group). (D) Cytokine release from *in-vitro* house dust mite stimulated peribronchial lymphnode cells and (E) CCL20 levels in lung homogenates. Results are mean ±s.e.m. (n=4 mice per group). *, $P < 0.05$ and **, $P < 0.01$.

inhibition raised the activity of its substrate PP2A (Figure. 6.1 H), which also resulted in reduced levels of phosphorylated p38 MAPK (Figure. 6.1 I).
Therefore, Midline-1 expression downstream of TLR4 signalling cascades is essential for the development of AAD and inversely correlated to PP2A activation levels. The decreased PP2A activity observed in the lung tissue of allergic mice, and its reciprocal expression with Midline-1, suggests that targeting PP2A for re-activation may be a useful strategy for anti-asthma therapies. Pharmacological activation of PP2A has recently been demonstrated by the immunosuppressant FTY720. FTY720 is a synthetic sphingosine analogue that causes transient immunosuppression by sequestering lymphocytes in the secondary lymphoid organs (thereby keeping them out of circulation) and has recently been approved by the US Food and Drug Administration (FDA) for treatment of relapsing multiple sclerosis. In order for FTY720 to induce lymphopenia in vivo, the compound must be phosphorylated by the enzyme sphingosine kinase 2. Phosphorylated FTY720 acts as an agonist for the sphingosine 1-phosphate (S1P) receptor, and its induction of lymphopenia is dependent on its binding to the sphingosine-1-phosphate receptor. These effects are all attributed to the phosphorylated form of FTY720, but phosphorylation of the compound is not required for induction of PP2A activity. To confirm a causal relationship between PP2A levels in the lung and asthma pathogenesis, we treated allergic WT mice with the non-phosphorylatable FTY720 analogue, 2-amino-4-(4-heptylophenol)-2-methylbutanol (AAL(S)), that does not bind to S1P receptor like FTY720, but can reactivate PP2A when given into the lungs of allergic mice (Figure. 6.2 A). Under these treatment conditions PP2A activation was also associated with decreased Midline-1 gene expression (Figure. 6.2 B). This in-vivo finding is supported by a previous in-vitro report suggesting cross-regulation of both Midline-1 and PP2A activity by the adaptor protein alpha4. It was shown
that Midline-1 regulates PP2Ac via ubiquitination and PP2A regulates Midline-1 via dephosphorylation \(^{317}\). Nonetheless in this report we provide evidence for an interaction between PP2A and Midline-1 on a transcriptional or post-transcriptional level. Notably, daily intranasal treatment with AAL(S) during repeated house dust mite challenges to sensitized mice precluded the development of AHR (Figure. 6.2 C), reduced airways inflammation (Figure. 6.2 D), Th2 cytokine (Figure. 6.2 E) and CCL20 production (Figure. 6.2 F). This suggests that increasing PP2A activity in the lungs downregulates Midline-1 activity, prevents airways obstruction and attenuates allergic lung inflammation. This indicates that pharmacological activation of PP2A may be a useful therapeutic strategy for allergic asthma.

No effective treatment for rhinovirus-induced lung diseases and asthma exacerbations is currently available. To investigate the therapeutic potential of Midline-1 inhibition, we employed a recently established mouse model of rhinovirus-induced airways inflammation and obstruction \(^{327}\). We challenged non-allergic mice with rhinovirus (RV1B, minor group) or UV inactivated virus (UV-RV1B) along with siRNA therapy directed against Midline-1 or a nonsense sequence. 24hrs after RV1B exposure, naïve mice treated with the control siRNA developed a neutrophil-dominated lung inflammation along with airways hyperreactivity, which coincided with reduced PP2A activity and elevated Midline-1 and CCL20 expression (Figure. 6.3 A to D). RV1B infection also led to the upregulation of a range of chemokines that promote lung inflammation (Figure. 6.3 E). Inhibition of Midline-1 fully protected mice from RV1B-induced
airways inflammation and obstruction (Figure. 6.3 A to E) but did not affect virus replication or increased anti-viral interferon responses (Supplementary Figure. 6.3).

Figure 6.3: Midline-1 inhibition ameliorates rhinovirus-induced airways inflammation, obstruction, and asthma exacerbation. (A) Total lung resistance as percentage change of baseline measurement (water) in response to inhaled methacholine. Results are mean ±s.e.m. (n=6-10 mice per group). (B) Number of cells in bronchoalveolar lavage fluid (BALF). (C) PP2A activity and (D) Midline-1 and CCL20 mRNA and (E) chemokine levels in the airways of naïve mice treated with a scrambled siRNA (Nonsense siRNA) or a Midline-1 targeting siRNA (MID-1 siRNA) 24hrs before RV1B challenge intranasally. Results are mean s.e.m. (n=3-4 mice per group). (F) Total lung resistance as percentage change of baseline measurement (water) in response to inhaled methacholine. Results are mean ±s.e.m. (n=6-10 mice per group). (G) Number of cells in bronchoalveolar lavage fluid (BALF). (H) PP2A activity and (I) chemokine levels in the airways of house dust mite allergic mice treated with a scrambled siRNA (Nonsense siRNA) or a Midline-1 targeting siRNA (MID-1 siRNA) 24hrs before RV1B challenge intranasally. Results are mean ±s.e.m. (n=3-4 mice per group). *, $P < 0.05$ and **, $P < 0.01$.

We infected allergic mice with RV1B 24hrs after their final house dust mite allergen challenge to define the relationship between Midline-1 expression and viral infection. Importantly treatment with a siRNA directed against Midline-1 ameliorated rhinovirus-induced exacerbation of eosinophilic airways
inflammation and obstruction as compared to those treated with a nonsense siRNA (Figure. 6.3 F and G). Midline-1 inhibition also raised PP2A activity (Figure. 6.3 H) and impaired rhinovirus-induced chemokine (Figure. 6.3 I) and IL-5 production (Mean [SE]: 8.6ng/ml [0.6] versus 3.2ng/ml [0.2]; p<0.01). As observed in naïve mice, inhibition of Midline-1 did not affect RV1B replication or stimulate anti-viral IFN responses (Supplementary Figure. 6.3). Therefore inhibition of Midline-1 protects mice from rhinovirus-induced airways inflammation and obstruction independent of affecting viral replication. Our data suggest that anti-inflammatory treatment without boosting anti-viral responses may be a therapeutic strategy for rhinovirus-induced asthma exacerbation potentially by suppressing the severity of the “inflammatory storm”.

To confirm a direct link between TRAIL, Midline-1, and PP2A activity, we stimulated immortalized human airway epithelial cells (BEAS-2B) with TRAIL or house dust mite extract for 24hrs. We found an increase in TRAIL expression after stimulation with house dust mite extract but not after TRAIL stimulation (Figure. 6.4 A). Midline-1 and CCL20 (Figure. 6.4 A) were also expressed upon house dust mite and TRAIL exposure, which inversely correlated with PP2A activity in these cells (Figure. 6.4 B). We lysed BEAS-B2 cells and western blotted for Midline-1, the alpha4 subunit of PP2A, and PP2Ac to understand the details of these interactions (Figure. 6.4 C: lane one). We also performed immunoprecipitations for the PP2Ac subunit and as hypothesized, both Midline-1 and alpha4 were detected in the PP2Ac immunoprecipitant (Figure. 6.4 C). This confirms the direct interaction of Midline-1, PP2Ac, and the alpha4 subunit.
Figure 6.4: Midline-1 in allergen and rhinovirus exposed human airway epithelial cells. (A) TRAIL, Midline-1, CCL20 expression and (B) PP2A activity in BEAS-B2 cells stimulated with house dust mite extract or recombinant TRAIL (1000ng/ml). Results are mean ±s.e.m. and representative for n=3 experiments. (C) Immunoprecipitation for PP2Ac in unstimulated BEAS-B2 cell lysates. Total lysate (lane one), PP2Ac precipitant (lane two), PP2Ac-depleted lysate (lane three). (D) TRAIL, Midline-1, and CCL20 expression in primary airway epithelial cells after RV1B or UV-RV1B infection. Results are mean s.e.m. (n=5 asthmatics and n=5 non-asthmatic controls). (E) Correlation between Midline-1 and TRAIL in primary airway epithelial cells after RV1B infection (n=5 asthmatics and n=5 non-asthmatic controls). *, P < 0.05.

To translate our studies into a clinically significant finding, we exposed primary airway epithelial cells collected from asthmatics and healthy subjects to infective or UV-inactivated RV1B. We found that exposures upregulated Midline-1, CCL-20, and TRAIL 24hrs after rhinovirus infection (Figure. 6.4 D). A correlative relationship appears to exist between Midline-1 and TRAIL expression (Figure. 6.4 E). In independent experiments, infecting primary airway epithelial cells with RV1B for only 6hrs confirmed a strong correlation between TRAIL and Midline-1.
expression (n=18; r=0.8; p<0.01). Thus, rhinovirus induces Midline-1, which is an essential molecule in the establishment of airways inflammation, obstruction, and asthma exacerbation in our experimental model.

About 500 or more human E3 ubiquitin ligases have been identified to date supporting the concept of a broad role in regulating diverse cellular processes through targeting specific substrates for degradation by the proteasome.

The ubiquitin system has been linked to cancer, neurodegenerative and muscle wasting disorders, diabetes, infection and inflammation. In regards to allergic disease, the E3 ligase itch was identified by genetic analysis of a mutant mouse with constant scratching of the skin \(^{328}\). High IgE titers and exaggerated Th2 responses were observed in the absence of itch \(^{329}\). Itch was found to regulate airways inflammation by induction of T cell anergy by targeting ubiquitination of the transcription factor TGF-beta-inducible early gene 1 product \(^{330} \)\(^{331}\). Here we have identified the E3 ubiquitin ligase Midline-1 as a master switch in the development of hallmark features of Th2 mediated allergic asthma, including airways obstruction, allergic inflammation, Th2 responses, chemokine release, and virus-induced disease exacerbation by modulating PP2A. Our results suggest that targeting Midline-1 or increasing activity of its target substrate PP2A may be of therapeutic benefit for patients with stable and rhinovirus-exacerbated asthma.
Supplementary repository for chapter 6

Supplementary Figure 6.1: TRAIL regulates house dust mite induced allergic airways disease, Midline-1, and PP2A activity. (A) Total lung resistance as percentage change of baseline measurement (water) in response to inhaled methacholine in allergic (HDM) versus non-allergic (SAL) wild-type (WT) and Tnfsf10−/− mice. Results are mean±s.e.m. (n=6-10 mice per group). (B) Number of cells in bronchoalveolar lavage fluid (BALF). Results are mean±s.e.m. (n=3-4 mice per group) (C) Number of peribronchial perivacular eosinophils (x1000) and mucus-producing cells (x400) per high-power field (HPF). Results are mean±s.e.m. (n=2-3 mice per group). (D) Cytokine release from in-vitro house dust mite stimulated peribronchial lymphnode cells and (E) CCL20 levels in lung homogenates. Results are mean s.e.m. (n=4 mice per group).

Supplementary Figure 6.2: PP2Ac and beta-actin levels in lung homogenates of allergic and non-allergic WT and Tnfsf10−/− mice.
Supplementary figure 6.3: (A) Positive strand RV1B RNA and (B) IFN mRNA in the airway wall of naïve mice treated with a scrambled siRNA (Nonsense siRNA) or a Midline-1 targeting siRNA (MID-1 siRNA) 24hrs before RV1B challenge intranasally. (C) Positive strand RV1B RNA and (D) IFN mRNA in the airway wall of allergic mice treated with a scrambled siRNA (Nonsense siRNA) or a Midline-1 targeting siRNA (MID-1 siRNA) 24hrs before RV1B challenge intranasally. Results are mean±s.e.m. (n=3-4 mice per group).
Chapter 7: General Discussion
7.1 Overview of thesis

The research that comprises this thesis was conducted over a 4.5 year period and encompasses four individual research chapters. These focus on the therapeutic modulation of novel inflammatory mechanisms in mouse models of AAD. As stated previously the focus on miRNA and TRAIL signalling pathways was specifically designed to modulate multiple aspects of the complex aberrant immune responses that underpin the allergic disease phenotype.

The importance of miRNA for modulating cellular pathways in vivo has only been recently identified and their potential role in the pathogenesis of AAD had not been explored in vivo at the initiation of these studies. The characterisation of a number of miRNA that were significantly dysregulated in the airways of allergic mice confirmed my hypothesis that this recently described regulatory mechanism may play a role in the development of allergic inflammation. Subsequent proof of concept studies demonstrated that the up-regulation of specific miRNA could be corrected within the allergic airways by targeting them with so-called antagonirs. Of particular interest was that although several of these target miRNA were identified, the specific inhibition of only a few altered allergic inflammation and disease. This suggests that many miRNAs are upregulated in AAD, but most may not have a significant role in the regulation of airways hyperreactivity, inflammation, and Th2 responses. They are likely to be involved in other functions relating to the inflammatory environment such as epithelial repair induced by exposure to HDM.
7.2 Antagonism of microRNA-126 suppresses the effector function of Th2 cells and the development of allergic airways disease

For the first time it was demonstrated that a select set of miRNAs are rapidly upregulated within the airways following repeated HDM challenge. Further, in the HDM induced AAD model this mechanism was dependent on TLR4/MyD88 signalling. This data highlighted a potential role for miRNA regulation of the initial innate immune response to HDM that underpins the subsequent adaptive immune response. Moreover, our follow-up experiments proved the concept that miRNA can be synthetically modulated within the allergic airways by direct administration of modified complementary RNA sequences termed antagonirs. Importantly, antagonirs targeted against miR-126 abolished AHR, attenuated mucus hypersecretion, airways eosinophilia and Th2 cytokine production; specifically IL-5 and IL-13. Interestingly antagonirs directed against miR-126 also reduced the number of CD4+ T cells and myeloid DCs recruited into the airway upon HDM challenge. The increase in expression of the miR-126 target BOB.1 following antagonir treatment with the corresponding increase in the pro-inflammatory transcription factor PU.1 and decrease in the Th2 lineage-defining marker GATA 3, demonstrated the potential for miR-126 to regulate the activation of effector T cells within the allergic environment. It may well be that miR-126 acts on other targets to promote Th2 driven inflammation in addition to the BOB.1 pathway identified. However, current target prediction models are prone to both type 1 and type 2 errors and whether the miRNA silencing is always detectable at the mRNA level remains controversial. I was therefore unable to find any other clear targets using a transcriptome wide microarray approach on the Illumina platform (unpublished data). However, the data
presented confirmed the hypothesis that miRNA are upregulated as part of the initial innate response to allergen and some are critical for mounting the Th2 inflammatory response and thus have the potential to influence allergic disease.

7.3 Inhibition of house dust mite–induced allergic airways disease by antagonism of microRNA-145 is comparable to glucocorticoid treatment

While the inhibition of miR-126 with antagonomir provided -for the first time-proof of concept that modulation of increased miRNA expression could ameliorate hallmark features of AAD, this did not address the relative efficacy of such an approach as compared to the gold standard anti-inflammatory treatment with glucocorticoids. In Chapter 4, I designed experiments to specifically compare miRNA inhibition with dexamethasone. I also examined the potential of targeting other miRNA expressed during allergic inflammation for the treatment of disease. With all of the phenotypic markers of allergic inflammation examined, specific inhibition of miR-145 was able to at least equal the efficacy of systemic dexamethasone treatment for the suppression of disease. In particular, a single administration of antagonomir was able to inhibit the development of AHR in an established allergic environment while a single dose of dexamethasone was not effective. These studies also address for the first time the specificity of antagonomir therapies, as the inhibition of miR-21 and let-7b had no effect on inflammation despite these miRNA -like miR-126 and miR-145-being highly upregulated in AAD. This data highlights the potential of specific inhibition of miRNA as a future therapeutic approach. However, for this approach to be relevant in a clinical setting it would be desirable that the molecular mechanism of miR-145 action be identified. At this time our
understanding of miRNA biology is still a limiting factor. Within these experiments I conducted transcriptome-wide microarrays in an attempt to clarify the target mRNA sequences silenced by miRNA and presumably up-regulated with antagonir treatment. Unfortunately, these experiments did not identify any significant differences at the mRNA level. One potential reason for this is that while translation of protein from miRNA-targeted mRNAs is blocked, these mRNAs may still be detected by array probes because they are not targeted for degradation by the specific miRNA. However, the Bartel group at the Whitehead Institute, Cambridge, Massachusetts recently published a paper arguing that all miRNA targets can be detected at the mRNA level\textsuperscript{332}. It has to be considered, however, that this study only demonstrated the effect for a limited number of miRNA and a subset of their potential targets. It is still not clear whether this will be true for all types of miRNA silencing as other groups have demonstrated instances in which mRNA was not detected at altered levels even though miRNA regulated translational repression was clearly seen at the protein level \textsuperscript{333}. In this instance it may be that the effect in my experiments was also masked by multi-testing correction, or the dilution of the specific cell type in which the miRNA elicit their effect with the influx of other cell types. This could explain the discrepancies between the \textit{in-vitro} findings of the Bartel group in comparison to our \textit{in-vivo} findings.

The value of identifying a single miRNA target may be subject to criticism, as a possibility or even probability remains that the miRNA in question is capable of modulating additional targets in concert. The physiological as well as other roles of the targeted miRNA need to be further clarified to properly understand the implications of any miRNA based therapies. In the case of both miR-145 and
miR-126 an important biological function of these miRNA is to act as tumour suppressors and impairment of these pathways has been implicated in the development of lung cancers\textsuperscript{334 335 336}. Evidently, a better understanding of miRNA regulation and silencing is required to allow some certainty that the specific inhibition of miRNAs employing antagonirs will not lead to detrimental side effects. Importantly, it is anticipated that my proof-of-principle studies will promote further research into the value of miRNA modulation as a novel therapeutic strategy for AAD.

7.4 Targeting miR-126 as a potential therapeutic target for chronic asthma

Asthma is a chronic disease with ongoing airway inflammation and concurrent structural alterations commonly known as airways remodelling. To examine the role of miRNA in chronic disease, I collaborated with Cristan Herbert in Professor Rakesh Kumars’ laboratory at the University of New South Wales (who had developed a mouse model of chronic allergic airways disease (cAAD) based on the extended administration of aerosolised ovalbumin). The miRNA microarray of the airways of mice from this model again demonstrated both miR-126 and miR-145 to be amongst the most upregulated miRNA in the airways after 2 weeks of exposure, although only miR-126 was still significantly upregulated at the conclusion of the 6 weeks of ovalbumin exposure. Considering previous studies this may suggest that some miRNAs play a more significant role in the initiation and exacerbation of allergic airway disease rather than its perpetuation. Inhibition of miR-126 with a single weekly dose of antagonir was able to upregulate the known miR-126 target TOM1, thus indirectly demonstrating treatment efficacy. Once weekly treatment with an antagonir
directed against miR-126 led to the reduction of intraepithelial eosinophils in the conducting airways but not the other chronic inflammatory response nor airway remodelling. This study may suggest that targeting a single miRNA fails to prevent all aspects of allergic asthma to occur, namely chronic structural changes of the lung. Alternatively the importance of a specific miRNA is affected by the allergic stimulus, e.g. HDM versus OVA. It is also possible that more frequent treatments with antagomirs would have lead to higher efficiency. This would explain why AHR was abolished in the HDM model by the more regular treatment with antagomir each 48hrs but not in the chronic OVA model by antagomir treatment once weekly. However, unpublished dose response experiments indicated miRNA knockdown was effective for up to 9 days post antagomir administration. Therefore, the mechanisms that underpin AHR in the chronic model are more complex and abolishment of this important hallmark feature of asthma may require further targeting of specific miRNA at different points of disease progression or inhibiting multiple miRNA that act in concert. Indeed AHR is regulated by IFN-γ in the chronic model and not IL-13. More work is required to investigate these alternatives. It is also important to consider that as more miRNA are targeted the number of off target effects increases dramatically as each miRNA regulates multiple targets. However, as remodelling can also not be inhibited by steroid treatment, new treatments are required.

7.5 The therapeutic potential of targeting TRAIL induced signaling pathways

The elucidation of the pro-inflammatory anti-apoptotic TRAIL signalling pathway identified two novel targets crucial for the development of AAD. The significance of the TRAIL pathway in viral exacerbation models is of particular
clinical significance with up to 80% of asthmatic exacerbations requiring hospitalisation resulting from a viral infection \(^{306}\). Specifically targeting molecules upstream of significant pro-inflammatory transcription factors, such as p38 and NF-κB is a novel approach that capable of exerting an influence over a broad range of effector cytokines and chemokines. This has the advantage of modulating the immune milieu as a whole, rather than isolated pro-inflammatory molecules.

7.6 The E3 ubiquitin ligase Midline-1 links allergen and rhinovirus exposure to asthma via targeting PP2A

In chapter 6, I explored signalling mechanisms downstream of TRAIL activation with the view to therapeutically intervene in the development of AAD. Initial microarray studies identified the ubiquitin ligase Mid1 as a TRAIL-regulated gene during AAD because its expression was upregulated in the airways of allergic WT mice but not altered in allergic TNFSF10 deficient (-/-) mice. MID1 is a microtubule-associated E3 ubiquitin ligase located on the X chromosome at locus Xp22. Mutations in MID1 have been associated with the X-linked Opitz BBB/G syndrome, an inherited midline malformation syndrome characterised by cleft lip/palate, hypertelorism (increased distance between pupils), hypospadias (defect in urinary opening in males), laryngotracheoesophageal abnormalities, developmental delay, imperforate anus, and cardiac defects \(^{337}\). The Midline-1 protein, a member of the Ring-finger B-box coiled coil domain (RBCC) protein family \(^{338}\), is characterised by an N-terminal RING finger, a C-terminus containing fibronectin Type III domain, a coiled-coil domain, two B-boxes \(^{339}\), a COS domain \(^{340}\) and a B30.2 domain \(^{341}\). Mid-1 interacts with the regulatory subunit of
protein phosphatase 2a (PP2a) through the first B-box domain to polyubiquitinate and thereby regulate, the ubiquitin-specific modification and proteasomal degradation of the microtubule-associated catalytic subunit of PP2a (PP2ac). Further investigations revealed that PP2A is an ubiquitination target of Mid1 in-vivo with higher levels of PP2A activity observed after siRNA-mediated Mid1 inhibition. Immunoprecipitation studies demonstrated binding of Mid1 to the catalytic PP2A subunit (PP2Ac). This was the first time that this pathway had been identified to play a role in allergic disease.

PP2a itself is a phosphoserine-threonine specific enzyme ubiquitously expressed in eukaryotic cells. In its native form PP2a is a heterodimer consisting of a, b and c subunits with the a and c subunits being highly conserved scaffolding and catalytic portions of the enzyme complex, respectively. The b subunit plays a regulatory role defining the substrate specificity. Over 20 distinct classes of b subunit have been identified to date. The catalytic activity and subcellular localization of PP2a holoenzymes are intricately regulated with the c subunit requiring specific post-translational modification and the PP2ac heterotrimer requiring methylation to allow the formation of active complexes with the b subunit. The active complex itself can be targeted by Mid1, initially with the displacement of the c subunit then subsequent polyubiquitination of the PP2ac heterotrimer through interaction with the α4 subunit with subsequent degradation targeting and degradation within the proteasome.

The ability of Mid1 to regulate PP2a is of particular interest to the pro-inflammatory TRAIL signalling pathway as active PP2a has been demonstrated to dephosphorylate and thus deactivate MAP kinase (M KK) 3/6 and IκB kinase...
(IKK) which are critically involved in the activation of p38 and NFκB respectively.

Activation of MAPK and NFκB induces proinflammatory cytokine cascades that play a crucial role in the initiation and progression of AAD.

Modulation of this pathway through either direct inhibition of Mid1 with specific siRNA treatment or synthetic activation of PP2A with the small molecule AAL(s) exerted a significant inhibitory effect on the development of AAD. These data were in accordance to the protection seen in TRAIL deficient mice as described in our previous publication (Weckman, Collison et al. Nat Med 2007) and has lead to the hypothesised pathway shown in figure 7.1. Further data demonstrated that these interventions were also capable of mediating RV induced exacerbations of AAD which is of great clinical significance considering that RV is the major cause of asthmatic exacerbation and current treatments are of limited efficacy. The common role of this pathway in both allergy and viral response means that it is a more versatile target than either miR-126 or miR-145 with preliminary data showing both of these miRNA to be downregulated 24hrs after rhinovirus infection (unpublished data). Therefore, further inhibition of these miRNA is unlikely to yield any therapeutic value and may even exacerbate the viral infection. Subsequent translational studies in both transformed and primary human airway epithelial cells have confirmed that the TRAIL, Mid1, PP2a pathways are relevant in human cells. This study reveals novel targets for the future treatment of allergic asthma as well as RV-induced exacerbation able to address both the underlying AAD and in addition RV infection and associated exacerbation of disease.
Figure 7. 1: The TRAIL dependent upregulation of Mid-1 in the airway epithelium leads to a downregulation of PP2a activity decreasing the inhibition of p38 leading to increased pro-inflammatory signalling. This pathway is hypothesised to drive key pathological features of allergic asthma including eosinophilia, neutrophilia, mucus hypersecretion, collagen deposition and smooth muscle hypertrophy.

7.7 Summary

Together these studies identify the crucial role of several novel factors in the pathogenesis of AAD, namely miR-126, miR-145, Mid1 and PP2a. Synthetic modulation of these pathways using modified small RNA such as the antagonimirs against miR-126 and miR-145, siRNA to silence Mid1, and small molecules such as AAL(s) to reactivate PP2a were effective in correcting the dysregulation of their target. Within the models studied these mechanisms of action were able to significantly abrogate the hallmark features of AAD, thus highlighting the potential for these approaches as novel therapeutic strategies.
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