# UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Academic Unit of Clinical and Experimental Sciences

**Role of MicroRNAs in the innate immune response to rhinovirus infection in asthma**

by

**Dr Hitasha Rupani**
BSc BM MRCP

Thesis for the degree of Doctor of Philosophy

March 2015

**UNIVERSITY OF SOUTHAMPTON**

# ABSTRACT

FACULTY OF MEDICINE

Academic Unit of Clinical and Experimental Sciences

Thesis for the degree of Doctor of Philosophy

## ROLE OF MICRORNAS IN THE INNATE IMMUNE RESPONSE TO RHINOVIRUS IN ASTHMA

Dr Hitasha Rupani

Asthma is one of the commonest chronic diseases worldwide and severe asthma sufferers experience recurrent exacerbations. Exacerbations are predominantly virus-induced and have been linked to defective interferon responses by airway immune and structural cells. Ascertaining the molecular mechanisms underlying this deficiency is a major research goal in order to identify new therapeutic targets.

We hypothesised that impaired interferon responses in severe asthma are caused by aberrations in microRNA expression in alveolar macrophages. MicroRNAs are non-coding RNA molecules that down-regulate gene expression. We identified and focused on 3 microRNAs predicted to target Toll-like receptor 7 (TLR7). We hypothesised that reduced expression of TLR7 would lead to reduced innate immune responses to the virus and that manipulating the expression of these microRNAs could restore the defective interferon response in asthmatic alveolar macrophages.

Alveolar macrophages were isolated from bronchoalveolar lavage from healthy and severe asthma subjects. Expression of microRNAs miR-150, -152 and -375 was increased and expression of TLR7 reduced in alveolar macrophages from severe asthma subjects compared to healthy subjects. Using a TLR7-luciferase reporter construct we showed that the 3 microRNAs directly targeted the 3'UTR of TLR7 and operated synergistically to reduce its expression. Reduced TLR7 expression was association with impaired interferon responses to rhinovirus and imiquimod, a specific TLR7 agonist, and correlated inversely with number of disease exacerbations. *Ex vivo* knock-down of these microRNAs restored TLR7 expression with concomitant augmentation of virus-induced interferon production.

In conclusion, the results presented here show that increased expression of miR-150, miR-152 and miR-375 in severe asthma leads to reduced expression of TLR7 in alveolar macrophages and contributes to the impaired innate immune response to rhinovirus. This would certainly predispose the individual to more frequent and prolonged exacerbations due to reduced viral clearance by airway cells. Importantly we show that knock-down of these microRNAs in alveolar macrophages rescues the expression of interferon, providing a novel therapeutic target for the prevention and treatment of asthma exacerbations.

# Table of Contents

# List of Figures

# List of Tables

# DECLARATION OF AUTHORSHIP

I, Hitasha Rupani

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

*ROLE OF MICRORNAS IN THE INNATE IMMUNE RESPONSE TO RHINOVIRUS INFECTION IN ASTHMA*

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed:.....

Date:.....

# Acknowledgements

I would like to start by thanking my PhD supervisors, Dr Tilman Sanchez-Elsner and Professor Peter Howarth. They provided invaluable guidance, assistance and expertise that I needed especially at the start of my PhD. Then, when I felt ready to venture into research on my own, they gave me the freedom to explore different ideas, at the same time continuing to contribute valuable feedback, advice and encouragement. Tilman, thank you for having faith in me and letting a 'clinician' into your lab, I sincerely hope that you are proud of the molecular biologist you have created. Peter, I am truly thankful for the steadfast integrity and immense knowledge you shared with me which motivated me throughout my PhD. I wold also like to express my special appreciation to my mentor- Professor Chauhan who has believed in me and supported me throughout my career and inspired me to pursue my goals and aspirations.

It would not have been possible to complete this thesis without the help and support of all the people I work with. Laurie, thank you for the laughs and all your words of encouragement after each of the bronchoscopies. I was really pleased to finally hear you tell someone that I was a 'good bronchoscopist'. To everyone on Level E- thank you for all the 'science' discussions, the chats in the corridors, the laughs and of course, for asking to see my children's pictures on a regular basis. And I couldn't have asked for a better group to be part of than the JunkRNA group. I've learnt a lot from all of you- from the many memorable conversations on the intricacies of cell biology with Victor to the quest of finding western blot supplies with Marcus. And Rocio and Ana- you have become true friends and I could not have made it through this 'journey' with you. Rocio, your enthusiasm for research is contagious, thank you for always having an answer for every random question I had in the lab. Ana, we started this together and I hope to share more happy times with you in the future.

I would like to thank my parents who have always encouraged me, to do more and do it better. My mum would always say "Aim for the moon….that way you may make it to the top of the coconut tree" (!). Has that helped me get to where I am now? Probably. My final words of acknowledgement are to my children and husband. I started my PhD before both my children were born, so

this has been their journey as much as mine.  But most importantly, my husband helped me laugh, a lot, all the way through this PhD journey.

So, although this may seem like the end, Tilman I think you are right, it is just the beginning.

# List of Abbreviations

| | |
|---|---|
| 3'UTR | 3'untranslated region |
| AAM | alternatively activated macrophages |
| ACQ | asthma control questionnaire |
| AM | alveolar macrophage |
| AP-1 | activator protein 1 |
| BAL | bronchoalveolar lavage |
| BHR | bronchial hyperreactivity |
| BDP | beclometasone |
| BTS | British Thoracic Society |
| cDNA | complimentary DNA |
| CF | cystic fibrosis |
| COPD | chronic obstructive pulmonary disease |
| DNA | deoxyribonucleic acid |
| EGF | epidermal growth factor |
| EMTU | epithelial mesenchymal trophic unit |
| FBS | foetal bovine serum |
| $FEV_1$ | forced expiratory volume in one second |
| FGF | fibroblast growth factor |
| FVC | forced vital capacity |
| GM-CSF | granulocyte monocyte colony stimulating factor |
| HC | healthy control subject |
| HRV | human rhinovirus |
| ICAM-1 | intercellular adhesion molecule 1 |
| ICS | inhaled corticosteroid |
| IFN | interferon |
| IFNα | interferon-alpha |
| IFNAR | interferon α/β receptor |
| IFNβ | interferon-beta |
| IFNγ | interferon-gamma |
| IFNλ | interferon-lambda |
| IgE | immunoglobulin E |
| IL | interleukin |
| IRF | interferon regulatory factor |
| ISG | interferon stimulated gene |
| LABA | long-acting β 2-agonist |
| LDH | lactate dehydrogenase |
| LPS | lipopolysaccharide |
| LRT | lower respiratory tract |
| MA | mild asthma subject |
| MDA-5 | melanoma differentiation associated protein 5 |
| MDM | monocyte derived macrophages |
| miRNA | microRNA |
| MO | moderate asthma subject |
| MOI | multiplicity of infection |
| mRNA | messenger RNA |
| MxA | myxovirus resistance protein A |
| NF-κB | nuclear factor-kappaB |
| NKT | natural killer T cell |
| OAS | oxaloadenylate synthetase |
| PAMP | pathogen-associated molecular patterns |

| | |
|---|---|
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEF | peak expiratory flow |
| PKR | protein kinase R |
| polyI:C | polyinosinic-polycytidylic acid |
| PRR | pattern recognition receptor |
| qPCR | quantitative (or real time) polymerase chain reaction |
| RIG-1 | retinoic-acid inducible gene 1 |
| RISC | RNA-induced silencing complex |
| RNA | ribonucleic acid |
| RT | reverse transcription |
| RV | rhinovirus |
| SA | severe asthma |
| SABA | short-acting β 2-agonist |
| SARP | Severe Asthma Research Program |
| siRNA | small interfering RNA |
| TGFβ | transforming growth factor β |
| Th1 | T helper type 1 lymphocyte |
| Th2 | T helper type 2 lymphocyte |
| TLR | toll-like receptor |
| TNFα | tumour necrosis factor α |
| Treg | T-regulatory cell |
| URT | upper respiratory tract |

# Chapter 1 Introduction

## 1.1 Asthma

Asthma is a global health problem affecting about 300 million individuals worldwide across all ages and ethnic backgrounds (Bousquet et al. 2010). The global prevalence of the disease has markedly increased over the last 50 years as has the burden of this disease to governments, health care systems, families and patients. The World Health Organisation has estimated that 15 million disability-adjusted life years are lost annually due to asthma, representing 1% of the total global disease burden (Masoli et al. 2004). With a mean prevalence of 16.1%, the UK has one of the highest prevalence rates for asthma in the world (GINA 2011).

### 1.1.1 Pathophysiology

The term 'asthma' was derived from the Greek word for shortness of breath and as a condition it has been recognised from early civilisation and been well described by physicians over the centuries. William Osler, in the first issue of his textbook *Principles and Practice of Medicine*, first published in 1860, commented that asthma was caused by spasm of the bronchial muscles, swelling of the bronchial mucous membrane and the presence of mucus in the lumen of the airways, laying the foundation for our current understanding of the nature of asthma (Girard 1981).

Asthma is a common chronic inflammatory disease of the airways that is characterised by variable and usually reversible airflow obstruction in association with airway hyperresponsiveness. This is accompanied by increased sensory irritability of the airway mucosa and mucus hypersecretion. Clinically this manifests as symptoms of chest tightness, wheeze and cough. The differing clinical expressions of asthma are due to varying environmental factors that interact with the airways to cause acute and chronic inflammation, and the varying contributions of smooth muscle contraction, oedema and remodelling of the airways (Holgate 2008). Ultimately these interactions lead to airway narrowing- the final common pathway leading to the symptoms and physiological changes in asthma.

Asthma is a good example of gene-environment interaction, however no single gene or environmental factor has been found that fully accounts for the disease. However it is accepted that a number of host and environmental factors interact to influence asthma susceptibility and the development and expression of asthma (Holgate 1999; Ober 2005).

| Factors influencing the development and expression of asthma | |
|---|---|
| Host Factors | Environmental Factors |
| Genetic e.g.:<br>1. genes pre-disposing to atopy<br>2. genes pre-disposing to airway hyperresponsiveness<br>Obesity<br>Sex | Allergens<br>1. indoor: domestic mites, pets (dogs, cats, mice), cockroach allergen, fungi, moulds, yeasts<br>2. outdoor: pollens, fungi, moulds<br>Infections (predominantly viral)<br>Tobacco smoke (active and passive smoking)<br>Outdoor/indoor air pollution<br>Diet |

**Table 1-1 Host and environmental factors influencing the development and expression of asthma**
(Adapted from GINA 2011)

Most asthma is characterised by a Th2-type inflammatory response with up-regulation of a cluster of cytokines including IL-4, IL-5, IL-9 and IL-13. Bronchial biopsies from patients with asthma are characterised by accumulation of eosinophils, mast cells and CD4+ Tcells producing IL-4 and IL-5 in the epithelium and lamina propria (Bousquet et al. 1990; Choy et al. 2011; Robinson et al. 1992). Animal models, in which the Th2 cytokines are individually knocked out also provide evidence that the Th2 axis can drive bronchial hyperreactivity and eosinophilic inflammation (Lloyd et al. 2010). However, it is now apparent that this Th2 biased response is predominant and detectable in only about 50% of individuals with asthma (Woodruff et al. 2009). This is reflected in clinical trials of biological agents aimed at blocking specific Th2 cytokines failing to show broad efficacy (Flood-Page et al. 2007; Gauvreau et al. 2011; Leckie et al. 2000), except in cases where patient selection was based on careful stratification based on inflammatory sub phenotypes (Corren et al. 2011). Other inflammatory cells and cytokines have also been shown to be involved including invariant NKT cells, Th17 cells and their associated cytokines IL-17A and IL-17F, IL-25, IL-33 and thymic stromal lymphopoietin

(TSLP), all capable of driving either an eosinophilic, neutrophilic, or combined inflammatory response (Holgate et al. 2009). However our understanding of non-Th2 asthma remains poor and incomplete. What is becoming clear is that asthma is not a single disease, but instead consists of multiple phenotypes with distinct, although sometimes overlapping, underlying biology.

The epithelium in asthma is thought to be structurally and functionally defective and responds abnormally to the inhaled environment (Holgate 2007). Inhaled environmental insults include allergens, environmental tobacco smoke, viruses, pollutants and other chemicals. This leads to enhanced signalling between the airway epithelium and the underlying structural (the epithelial-mesenchymal trophic unit- EMTU) and immune cells. This is thought to promote a microenvironment that facilitates allergic sensitisation, supports inflammation and predisposes to exacerbations. Activation of the EMTU is also thought to drive airway wall remodelling, reducing lung function and leading to refractoriness to treatment in asthma.

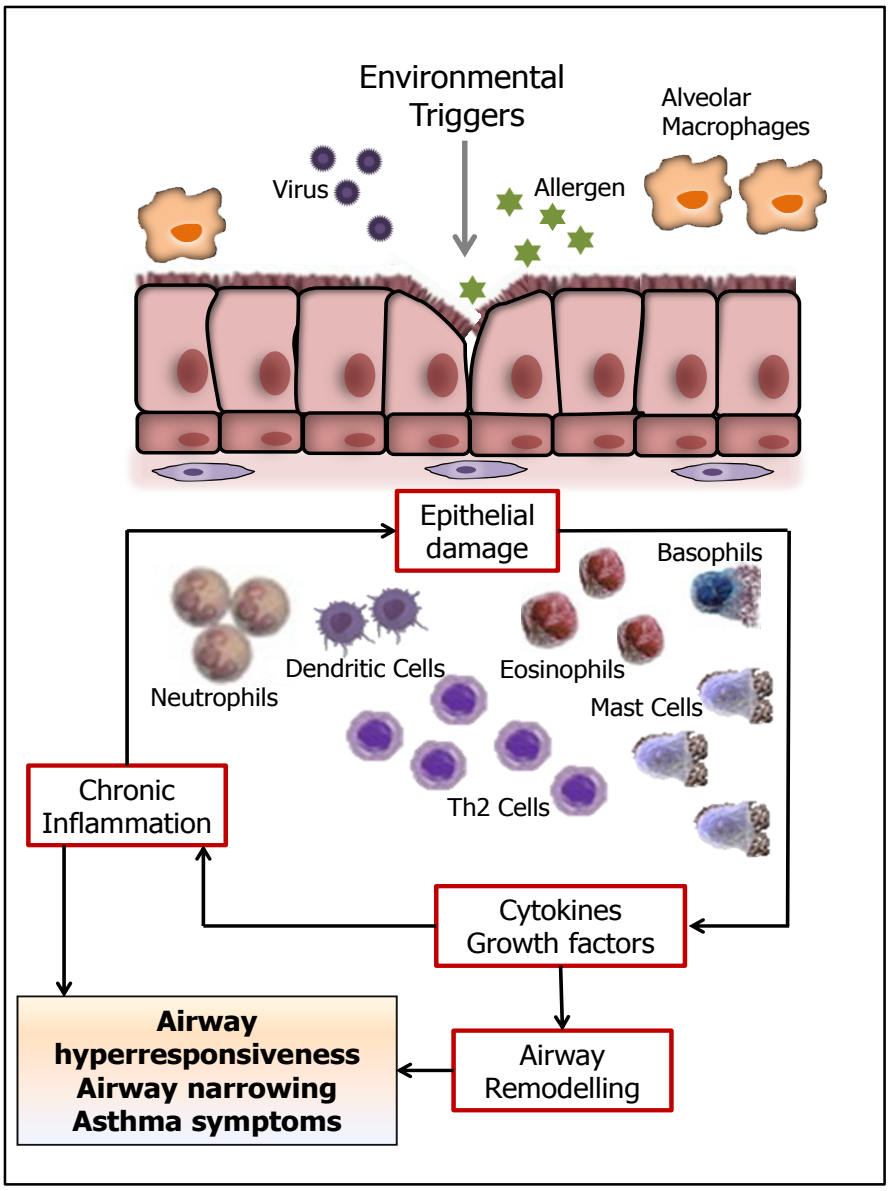


**Figure 1-1 Schematic representation of how epithelial injury and aberrant repair interact leading to the inflammatory changes in chronic asthma.**

The barrier function of airways epithelium in asthma has been shown to be impaired, rendering it more permeable to environmental insults including allergens, pollutants and microorganisms (Chung 2006; Hackett et al. 2007; Roche et al. 1993). Under normal circumstances, the airway epithelium functions as a physical barrier, impeding the access of allergens to underlying structural and immune cells. The integrity of the barrier depends on apical tight junctions and adherents junctions between the bronchial epithelial cells (Lambrecht et al. 2012). Epithelial tight junction formation has been found to be severely disrupted in asthmatic biopsies and epithelial cells brushed from

asthmatic airways and differentiated at an air-liquid interface *in vitro*, are unable to form effective tight junctions (Xiao et al. 2011). In asthma there is also an increase in the number of goblet cells in the epithelium, termed goblet cell metaplasia (GCM). A number of factors lead to GCM including epithelial disruption, exposure to the Th2 cytokines IL-4 or IL-13 and reactive oxygen species. This results in the secretion of abnormally large amounts of highly tenacious and viscous mucus, predisposing the individual to airway obstruction.

The asthmatic airway is also functionally abnormal. The normal airway epithelium is well equipped with anti-oxidant enzymes that play a vital role in maintaining integrity and resisting injury when faced with the numerous chemical, particulate and biological agents that enter the airways and generate reactive oxygen species. In asthma, the antioxidant pathways such as superoxide dismutase catalase and glutathione peroxidase are also defective (Bucchieri et al. 2002; Comhair et al. 2001) resulting in increased epithelial injury. The asthmatic airway is also functionally abnormal because it fails to generate interferons (IFN), key anti-viral cytokines, in response to viral infections, both deficiencies resulting in failure to clear virus, premature cell death and clinically manifests as asthma exacerbations (Contoli et al. 2006; Wark et al. 2005). This defect is discussed in detail later.

Therefore, the scenario that arises is asthma is one in which there is chronic epithelial injury followed by aberrant repair. This leads to an epithelium that is 'chronically wounded' and heals by secondary intention, involving the laying down of matrix and other features of remodelling (Holgate 2011). Damaged epithelium is also a potent source of profibrotic growth factors (TGFβ , EGFs, FGFs), providing a strong stimulus for the deposition of extracellular matrix components and collagen in the basement membrane and airway remodelling (Davies 2009).

Repeated cycles of epithelial injury, due to increased susceptibility, followed by aberrant repair creates a microenvironment enriched in cytokines and chemokines that promote a chronic inflammatory response and drive the generation of growth factors leading to airway remodelling (Figure 1-1) (Holgate 2007). Over time, airway wall thickening can lead to irreversible airflow obstruction and an accelerated decline in lung function, features

characteristic of more severe asthma (Bourdin et al. 2007; ten Brinke 2008). HRCT and endobronchial ultrasound have confirmed the presence of airway wall thickening in more severe asthma (de Blic et al. 2007; Gupta et al. 2009; Shaw et al. 2004). Unfortunately, although inhaled corticosteroid (ICS) therapy, the mainstay of treatment for asthma, can exert some modifying effects on inflammation that is linked to matrix deposition (Dijkstra et al. 2006), chronic aspects of asthma arise and progress despite continuous use of ICS. It is now appreciated that repeated cycles of bronchoconstriction itself induces epithelial stress and initiates a tissue response that promotes airway wall remodelling (Grainge et al. 2011).

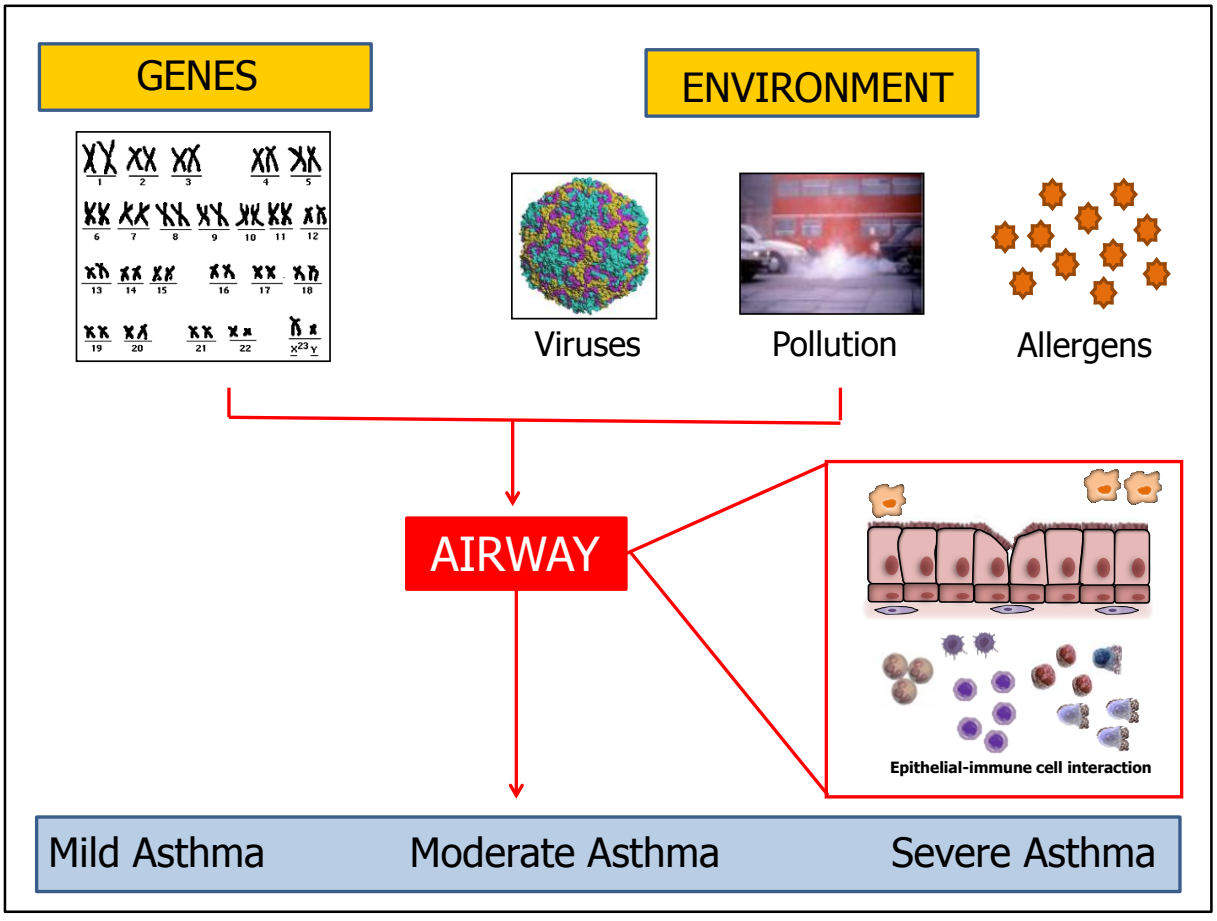


**Figure 1-2 Schematic representation of the asthmatic airway interacting with the external environment to determine asthma phenotypes.**

## 1.1.2 Classification and treatment of asthma

Clinically, asthma is frequently classified according to the treatment being received. The goal of asthma treatment is control of the disease. The British

Thoracic Society (BTS, https://www.brit-thoracic.org.uk/) has defined complete asthma control as:

- no daytime symptoms
- no night-time awakenings due to asthma
- no need for rescue medication
- no exacerbations
- no limitations on activity including exercise
- minimal side effects from medication

Treatment is adjusted to achieve control and patients can be moved up treatment steps in order to improve control and once control is achieved, move down treatment steps in order to find and maintain the lowest controlling step. Frequently, and especially for trial purposes, patients are also classified as having mild, moderate or severe asthma. These can be roughly correlated with the BTS defined Steps of Asthma Treatment:

| **BTS Step** | **Treatment being received** | |
|---|---|---|
| STEP 1 | Inhaled short acting $\beta_2$ agonist (SABA) as required | MILD |
| STEP 2 | Inhaled corticosteroid (ICS) added to SABA | MODERATE |
| STEP 3 | Long-acting $\beta_2$ agonist (LABA) added to ICS and SABA (if no response to LABA leukotriene receptor antagonist (LTRA) or theophylline added) | |
| STEP 4 | Fourth drug e.g. LTRA or theophylline added to ICS+LABA+SABA and ICS dose increased | SEVERE |
| STEP 5 | Daily oral steroids | |

**Table 1-2 The step-wise maanagement of asthma in adults as defined by the BTS and the correlation between these steps and asthma severity.**

As mentioned previously, asthma is a heterogeneous disease and in recent years, attempts have been made to classify it based on underlying pathobiology and natural history in order to provide more personalised care to patients.

### 1.1.3 Severe asthma

Currently available treatments for asthma are largely effective at dealing with the major components of asthma- bronchial obstruction and airway inflammation. They are safe, easy to use and generally well-tolerated. However there remains a group of patients with asthma who despite being on appropriate treatment remain symptomatic and experience recurrent exacerbations. This group of patients represent 10% of the asthma population (Wenzel 2005) but show the greatest morbidity and need for health care utilisation and contribute disproportionately to the overall costs of asthma (Antonicelli et al. 2004). A recent assessment of asthma in Europe (Brussels Declaration) identified that these 10% of patients with severe disease account for 50% of health care costs (Holgate et al. 2008). Not only does this indicate a huge unmet clinical need but also highlights the need for further studies to better understand this condition.

One of the most comprehensive attempts at defining severe asthma has been recently published by the European Respiratory Society (ERS) and American Thoracic Society (ATS) (Chung et al. 2014). This report defines severe asthma as asthma that requires treatment with high dose ICS plus a second controller and/or systemic corticosteroids for ≥50% of the year to prevent it from becoming "uncontrolled" or that remains "uncontrolled" despite this therapy. Table 1-3 details the definition of uncontrolled asthma:

| **Uncontrolled asthma is defined as at least one of the following:** |
|---|
| 1. Poor symptom control: ACQ consistently >1.5<br>2. Frequent severe exacerbations: two or more bursts of systemic corticosteroids (>3 days each) in the previous year<br>3. Serious exacerbations: at least one hospitalisation, intensive care stay or mechanical ventilation in the previous year<br>4. Airflow limitation: after appropriate bronchodilator withhold $FEV_1$ <80% predicted |

**Table 1-3 Definition of uncontrolled asthma as per ERS/ATS guidelines on severe asthma**
(Chung et al. 2014)

Our knowledge about the development of severe asthma is limited. It is unclear whether severe asthma develops slowly over time because of certain genetic and environmental interactions, or whether an acute event occurs near the onset of the disease causing irreversible alterations to the lung to promote severe asthma. Additionally, it is uncertain if all patients with asthma are at risk of developing severe asthma or only an undefined subset. However, it is recognised that severe asthma is not a single disease process but comprised of several clinical and inflammatory phenotypes and evidence is starting to emerge that these phenotypes are related to certain genetic factors, disease duration, exacerbations, sinus disease and inflammatory characteristics (Chung et al. 2014).

Currently, longitudinal studies have focused mainly on measurements of lung function, specifically $FEV_1$. Two large longitudinal studies from Australia and New Zealand have suggested that children with reduced lung function in early life are likely to have reduced lung function in adulthood (Oswald et al. 1997; Sears et al. 2003). Data from two European studies suggest that late/adult-onset asthma is associated with a more rapid decline in lung function (ten Brinke et al. 2001; Ulrik et al. 1994). However, although decline in lung function would contribute to more severe disease other factors including worsening levels of inflammation and frequency of exacerbations are likely to play important roles in disease pathogenesis and progression.

### 1.1.4 Asthma Exacerbations

The ATS/ERS guidelines define asthma exacerbations as events clinically identified to be outside the patient's usual range of day-to-day asthma variation, which are troublesome for patients and require a change in treatment, such as at least 3 days of oral corticosteroids, an increase from a maintenance dose of systemic corticosteroids, or hospitalisations/emergency department visits (Reddel et al. 2009). Acute exacerbations of asthma are the main cause of the morbidity and mortality associated with the disease (Johnston et al. 2006). They are also associated with enormous health costs in terms of time off school and work, primary care consultations, hospital admissions and medication costs. Data from the United States show that in 2007 asthma-related hospitalisation constituted about one third of the total $14.7 billion in US annual asthma-related health care expenditures

(http://www.lung.org/). The European Lung Foundations has estimated that the cost of asthma in the European Union is €17.7 billion, of which €9.8 billion is related to lost productivity (http://www.european-lung-foundation.org/), a large proportion of which is due to disease exacerbation. Data from the BTS Severe Asthma Registry shows that in the UK, patients requiring two or more rescue courses of steroids a year had 31% higher costs compared to patients with less frequent exacerbations, illustrating the importance and impact of recurrent exacerbations (O'Neill et al. 2014). In the UK, 20 million working days are lost to asthma each year (Holgate et al. 2010). The Asthma Insights and Reality in Europe survey carried out in 1999 across 7 European countries reported that 36% of children and 28% of adults required an unscheduled urgent care visit in the past 12 months (Rabe et al. 2000). 18% of children and 11% of adults had one or more emergency department visits due to their asthma in the last 12 months and 7% of all patients required overnight admission. These data suggests that asthma exacerbations are common and frequently result in utilisation of unscheduled medical care.

Patients with severe asthma are at risk of frequent severe exacerbations and a high rate of exacerbations has been incorporated in most definitions of severe asthma (Bousquet et al. 2010; ENFUMOSA 2003). The Severe Asthma Research Program (SARP) has shown that 54% of patients with severe asthma have 3 or more exacerbations per year (compared to 5% in the mild asthma group) (Moore et al. 2007). Recent exacerbations are an important predictor of future exacerbations in these patients (Haselkorn et al. 2009; Miller et al. 2007) and an exacerbation in the prior year increases the risk for exacerbation almost 8-fold (Schatz et al. 2014). Table 1-4 lists some of the factors found to be associated with exacerbations in severe asthma.

| Disease exacerbation in severe/difficult-to-treat asthma | References |
|---|---|
| **Factors associated with frequent exacerbations**<br>1. Recurrent respiratory infections<br>2. Psychological dysfunction<br>3. Gastro-oesophageal reflux disease<br>4. Nasal and sinus disease<br>5. Atopy<br>6. Obstructive sleep apnoea<br>7. Raised exhaled nitric oxide (>45ppb)<br>8. Reduced lung function<br>9. Non-steroidal anti-inflammatory drug intolerance | (Koga et al. 2006; Kupczyk et al. 2014; ten Brinke et al. 2005) |
| **Result of frequent exacerbations**<br>1. More rapid decline in lung function<br>2. Excess decline in $FEV_1$<br>3. Worse asthma control (as evaluated by ACQ)<br>4. Lower asthma quality of life (as evaluated by AQLQ) | (Bai et al. 2007; Kupczyk et al. 2014; O'Byrne et al. 2009) |

**Table 1-4 List of factors associated with frequent exacerbations in patients with severe asthma and impact of exacerbations on these patients**

The relationship between exacerbation and accelerated loss of lung function is important because it has been shown that even one severe exacerbation per year leads to more severe airflow limitation after 11 years (Bai et al. 2007). As reduced $FEV_1$ itself in an important risk factor for acute exacerbations, asthma exacerbation become self-perpetuating with one exacerbation causing deterioration in lung function that promote a subsequent exacerbation. Therefore, understanding the pathogenesis of asthma exacerbations is of utmost importance and will aid in the future development of new targeted therapies for this group of patients.

### 1.1.4.1 Origins of asthma exacerbations

#### 1.1.4.1.1 Viruses

The link between viral respiratory tract infections and acute exacerbations of asthma was identified over 40 years ago (Lambert et al. 1972; Minor et al. 1974). Clinical studies performed using PCR suggest respiratory viruses are the cause of the majority of asthma exacerbations in both children (80-85%)

(Chauhan et al. 2003; Johnston et al. 1995) and adults (75-80%) (Grissell et al. 2005; Wark et al. 2002). Of the respiratory tract viruses identified, rhinoviruses (RV) are most commonly found and are detected in approximately 65% of cases (Johnston et al. 1995; Nicholson et al. 1993). Other viruses that have been detected during asthma exacerbations include respiratory syncytial virus, influenza viruses, coronaviruses, human metapneumoviruses, parainfluenza viruses and adenoviruses. However virus detection rates vary depending on study design and the method used to detect virus. For example, a study which used standard serology and culture on nasopharyngeal aspirates had a virus detection rate of only 29% (Teichtahl et al. 1997).

A study by Wark *et al* provides evidence for a causal relationship between virus infection and asthma exacerbation (Wark et al. 2002). Patients admitted to hospital with an acute exacerbation of asthma underwent sputum induction and respiratory viruses were detected in sputum in 76% of admissions. The sputum was also analysed for inflammatory cell numbers and levels of lactate dehydrogenase (LDH) as a marker of virus-induced lower airway cell damage. In acute asthma with viral infection there was intense neutrophil influx and degranulation compared to the non-infective cases which were characterised by increased IL-5 and eosinophil activation but no strong neutrophil response. Acute asthma with viral infection also had increased sputum LDH, implying increased cell lysis. Sputum LDH levels were also found to be the single strongest predictor of severity of the exacerbation, measured in terms of length of hospital stay. This study clearly showed that virus infection is a major cause of acute asthma exacerbations and also severity of infection is strongly related to the clinical severity of the exacerbation.

Finally, observational studies have shown that asthma mortality in adults over 45 years of age peaks in winter months. This suggests that respiratory virus infections, which are more frequent in winter months, may also precipitate asthma deaths in this age group (Campbell et al. 1997; Nichols et al. 1999).

Contrary to common belief, asthmatic individuals are not at greater risk of RV infection than healthy individuals. A study done on co-habiting couples, where one partner had asthma, showed that the risk of RV infection was similar in both groups, as was the severity and duration of upper respiratory tract (URT) symptoms. However, individuals with asthma had a lower respiratory tract

(LRT) clinical illness twice as frequently, more severe and long-lasting LRT symptoms, and a greater fall in PEF when infected with RV than did healthy individuals (Corne et al. 2002). This observation was replicated in a study of experimental RV infection in mild asthmatic and healthy volunteers (Message et al. 2008). In this study, volunteers underwent experimental RV infection. RV infection induced significantly greater LRT symptoms, lung function impairment and increases in bronchial hyperreactivity (BHR) in asthmatic compared with normal subjects. In the asthmatic subjects, viral load was also significantly related to LRT symptoms and BHR, implicating severity of infection as the main determinant of exacerbations severity. These studies clearly demonstrate that respiratory tract viral infections cause a greater degree of morbidity in asthmatic subjects than in the healthy population.

#### 1.1.4.1.2 Allergen

Allergen exposure, in sensitised individuals, leads to eosinophilic infiltration of the airways together with lymphocytes expressing a Th2-like phenotype, secreting high levels of IL-4 and IL-13. Allergen exposure also leads to more severe asthma exacerbations as evaluated by hospital admissions and unscheduled medical visits (Rosenstreich et al. 1997; Sporik et al. 1993). Individuals sensitised to and exposed to indoor allergens tend to have lower $FEV_1$ and increased PEFR variability and BHR (Custovic et al. 1996; Langley et al. 2003). The impact of allergic responses on asthma exacerbations is highlighted by the positive effects of omalizumab, a monoclonal antibody that targets IgE to interrupt the allergic cascade, in reducing number of exacerbations and hospital admissions (Thomson 2014). However, patients are often exposed to both viruses and allergens simultaneously, and it is likely that, rather than being mutually exclusive, these interact to increase the risk of an exacerbation.

#### 1.1.4.1.3 Virus-allergen interactions

Studies done in both adults and children demonstrate that the combination of virus infection, sensitisation and exposure to high levels of sensitised allergen increases the risk of hospital admission due to acute asthma (Green et al. 2002; Murray et al. 2006). Mechanisms that may explain this phenomenon include:

- Viral infection can damage the epithelium, enhancing the absorption of allergens and inflammation (Sakamoto et al. 1984)
- Allergic inflammation can inhibit innate immune IFN responses (Tversky et al. 2008).
- RV infection can stimulate TLR3 dependent secretion of TSLP, a cytokine that can enhance allergic inflammation (Kato et al. 2007).
- RV infection prior to allergen exposure leads to greater histamine release and eosinophil recruitment within the airways and these effects persist even once the acute infection has resolved (Calhoun et al. 1994).
- *In vitro*, IgE crosslinking has been shown to reduce virus induced IFN production by dendritic cells (Gill et al. 2010).

#### 1.1.4.1.4 Bacteria

Numerous studies have investigated a possible association between bacteria (in particular the atypical organisms *M pneumonia* and *C pneumonia*) and asthma exacerbations. However the methods used to detect these organisms are not standardised and many are insensitive and nonspecific leading to a huge variation in detection rates across studies. A recent review on the subject found that 9 of the 12 studies reviewed demonstrated an associated between infection with either *M pneumonia* or *C pneumonia* and exacerbations (Johnston et al. 2005). In the largest of these studies infection with *M pneumonia* was found to be significantly associated with hospitalisation for acute asthma and it was detected in 18% of patients (Lieberman et al. 2003). There is also data to suggest that *C pneumonia* might act as a cofactor increasing the severity of virus-induced exacerbations (Wark et al. 2002).

#### 1.1.4.1.5 Other factors

A number of air pollutants contribute to the symptoms and increase the severity of asthma exacerbations. These include pollutants produced from the combustion of natural gas and motor fuel, such as nitrogen dioxide. In children with asthma, exposure to nitrogen dioxide is associated with increased respiratory symptoms and increased severity of virus-induced exacerbations (Chauhan et al. 2003; Mann et al. 2010). Cigarette smoking also leads to increased emergency department visits and hospital admissions in patients with asthma (Thomson et al. 2004). Finally, exacerbations occur in approximately 13-52% of pregnant women with asthma, depending on the

initial severity classification of the mother (Schatz et al. 2003) and there appears to be a clustering around the late second trimester. Pregnant women with asthma have increased susceptibility to viral infections compared to pregnant non-asthmatics (Minerbi-Codish et al. 1998) and there is some evidence to suggest that this phenomenon is due to an impaired innate immune response to RV during pregnancy (Forbes et al. 2012). However, discontinuation of medication, due to the belief it may harm the foetus is also an important factor.

#### 1.1.4.2 Need for new therapies for asthma exacerbations

ICS are the mainstay of treatment for asthma and are extremely effective at treating many aspects of the disease. Their use is associated with reduced risk of exacerbations. Two observational studies have compared children admitted to hospital with an exacerbation of their asthma with a control group of age-matched children with asthma of similar severity who were not admitted to hospital. Both studies found that the children admitted to hospital were far less likely to have been prescribed ICS compared to the control group (Johnston et al. 2005; Murray et al. 2006). However, even high dose budesonide treatment is only able to reduce asthma exacerbations by 50% in adults with asthma (Pauwels et al. 1997). In school-age children, prophylactic ICS were ineffective at reducing exacerbations frequency, duration or severity (Doull et al. 1997). Another agent used for the treatment of asthma is LABAs. Adding a LABA to high dose budesonide has been shown to reduce the estimated rate of exacerbations by 60%, which still leaves a large group of patients at risk of exacerbations (O'Byrne et al. 2001; Pauwels et al. 1997).

The modes of action of ICS in preventing virus-induced exacerbations are poorly understood. Budesonide and formoteral (a LABA) used in combination has been shown to inhibit the *in vitro* release of RV-induced pro-inflammatory and remodelling-associated mediators in primary bronchial epithelial cells (Skevaki et al. 2009). Virus-induced chemokine production has been associated with impaired lung function in asthma exacerbations, and so this inhibitory effect of ICS may be viewed as beneficial. However, several studies have also shown that ICS can impair the innate immune response to virus. The combination of budesonide and formoterol has also been shown to inhibit the production of type I IFN induced genes (MxA and OAS) from peripheral blood

mononuclear cells (PBMCs) treated with RV *in vitro* (Davies et al. 2011). MxA and OAS are important anti-viral molecules and play vital roles in viral clearance. Therefore the results of this study raises concern that the inhibition of an already impaired type I IFN response in patients with asthma might lead to delayed viral clearance and worsen the pathophysiological effects of the exacerbation.

Eosinophilic inflammation is a risk factor for asthma exacerbations and more severe viral illnesses. In that respect, mepolizumab, a monoclonal antibody against IL-5 which reduces eosinophil levels in the lungs and circulation, significantly reduces asthma exacerbations (Ortega et al. 2014; Pavord et al. 2012). As mentioned above, omalizumab, an anti-IgE antibody also reduces the frequency of asthma exacerbations and interestingly also inhibits seasonal peaks in asthma exacerbations in children, suggesting it may also have a role in preventing virus-induced exacerbations (Busse et al. 2011). However, despite these recent developments in personalised therapeutics in asthma, patients continue to have exacerbations and the associated morbidity, mortality and economic burden highlight the need for novel treatments for the prevention and treatment of disease exacerbations.

Once an exacerbation has developed options for the treatment of these acute asthma exacerbations are currently of limited efficacy. In adults who regularly take an ICS, two separate studies have shown that doubling the dose of ICS at the start of an exacerbation has no effect on the course of the exacerbation (FitzGerald et al. 2004; Harrison et al. 2004). It is thought that during virus-induced asthma exacerbations a state of relative steroid resistance may develop in the lungs due to virus induced activation of transcription factors (such as AP-1 and NF-κB) (Contoli et al. 2005).

## 1.2 Rhinovirus

Rhinoviruses are members of the Picornaviridae family ('pico' = small, 'rna' = ribonucleic acid genome), with more than 100 serotypes. They are the most common cause of the common cold in both adults and children. They are also an important cause of bronchiolitis, pneumonia and otitis in infants and school-aged children. RV infections typically case URT symptoms including rhinorrhoea, sore throat, nasal congestion, sneezing, cough and headache. In addition, as mentioned previously, RV is the pathogen most commonly recovered in acute exacerbations of asthma.

RVs are distributed worldwide with no predictable pattern of infection by serotype. In temperate climates, the incidence of RV infections peaks in the autumn and spring, but infections occur all year-round. These fall and spring peaks correspond closely to peaks in asthma hospitalisations, adding strength to a causal relationship. The 100 serotypes of RV have been classified into HRV-A and HRV-B based on genetic sequence similarity. Recently, PCR-based studies have identified a third group- HRV-C which contributes substantially to the burden of RV-related illness. A number of studies suggest that member of the HRV-C group might be intrinsically more virulent and have a greater propensity to cause asthma exacerbations (Miller et al. 2009).

RVs have an icosahedral capsid which encases a single stranded (ss) RNA genome. The genome is of positive polarity and so can also function as a template for translation. Independent from the classification mentioned above, RVs are also divided into two groups reflecting the receptor they use to enter cells. Approximately 90% of RV serotypes use ICAM-1 for cell entry. They are termed the major group RVs and include RV-16. ICAM-1 is an adhesion molecule and is involved in inflammatory cell recruitment to sites of inflammation. RV infection of airway epithelial cells up regulates expression of ICAM-1 both *in vivo* and *in vitro* (Grunberg et al. 2000; Papi et al. 1999). By increasing the expression of its own receptor, RV can potentially facilitate increased epithelial infection and leukocyte infiltration, contributing to the inflammatory cascade in asthma exacerbations. However, corticosteroids can inhibit induction of ICAM-1 by RV (Suzuki et al. 2000). The minor group RVs (e.g. RV1B) use members of the low-density lipoprotein receptor family for cell

entry. The receptor or receptors for the recently identified group C virus viruses are yet to be identified.

There is some evidence to suggest that allergic inflammation can enhance ICAM-1 expression on airway epithelial cells. *In vitro*, cytokines such as IL-4, IL-5 and IL-13 induce increased ICAM-1 expression on bronchial epithelial cells (Bentley et al. 1993). As the airway mucosa in atopic asthma is predominantly infiltrated by Th2 lymphocytes, these results could partially explain why asthmatic patients have an increased susceptibility to RV infection.

Attachment of the virus to the cellular receptor triggers internalisation of the virus-receptor complex and entry into early endosomes (Fuchs et al. 2010). It is likely that uncoating of the virus is facilitated by both binding to ICAM-1 and the low endosomal pH (Giranda et al. 1992; Hoover-Litty et al. 1993). In the cell cytoplasm, the viral ssRNA is translated using host protein synthesis machinery to provide proteins that will be essential for genome replication and production of new virus particles. The ssRNA is also used to produce a complementary negative (-) strand, forming a double stranded (ds) intermediate. The (-) strand is subsequently used as a template to synthesise copies of the viral genome which are initially used for translation of more viral proteins or, later in the infection cycle, assembled into new virus particles in the cytoplasm. Both ssRNA and the dsRNA intermediate are likely to be stimuli for the host innate immune response.

RV was previously thought to only infect the upper airway epithelium where the mucosal temperatures are relatively cool because optimal replication occurs between 33˚C and 35˚C (Gern et al. 2002). However it is now appreciated that the temperatures found throughout the lower airway lumen also allow RV to replicate effectively (Papadopoulos et al. 1999). Following experimental infection of the upper airways, the presence of RV has been demonstrated in lower airway fluids and cells in most volunteers (Gern et al. 1997; Papadopoulos et al. 2000). When upper airway secretions are compared to sputum (representative of lower airway sampling), increased amounts of RV are detected in the latter compared to the former (Horn et al. 1979; Lahti et al. 2009; Mosser et al. 2005).

Infection of airway cells by RV leads to the production of a variety of pro-inflammatory cytokines and chemokines thereby recruiting inflammatory cells into the airways. In patients with pre-existing airway inflammation, such as patients with asthma, the influx of additional inflammatory cells caused by RV infection would lead to addition or synergistic effects and an exacerbation of airways disease.

## 1.2.1 Host immune response to RV

The innate immune system is highly conserved and serves as the first line of defence for protecting the host from invading microbial pathogens. In response to virus, host cells produce a number of cytokines and chemokines, the most important of which are the type I IFNs. Type I IFNs include IFNα and IFNβ and are produced by all nucleated cells in response to virus infection. Recently, type III IFNs (IFNλ 1, λ 2, λ 3) have been identified as having properties similar to the type I IFNs (Kotenko et al. 2003). The anti-viral IFNs have pleiotropic functions, the most important being inducing apoptosis of virus-infected cells and cellular resistance to viral infection (Samuel 2001). In addition, they also induce the activation of the adaptive immune system (Le Bon et al. 2002). IFNs exert their actions through cognate cell surface receptors that tend to be species specific.

### 1.2.1.1 Type I IFNs

IFNβ is induced first, it then induces IFNα . They then bind to and activate the IFNAR (IFN α/β receptor) resulting in the rapid induction of transcription and translation of more than 300 interferon stimulated genes (ISGs) (Der et al. 1998; Platanias 2005). IFNARs are ubiquitously expressed on all cell lineages to ensure that all cell types can induce an IFN response to virus when infected. The main IFN-induced proteins implicated in the antiviral activity of IFNs are protein kinase R (PKR), 2'5'-oligoadenylate synthetase (OAS), RNase L, myxovirus resistance protein A (MxA) and viperin. PKR binds to dsRNA and ssRNA and by phosphorylating a protein synthesis initiation factor, inhibits translation (Roberts et al. 1976) to limits viral replication. PKR also induces apoptosis thereby triggering the death of virus-infected cells (Balachandran et al. 1998). OAS activates RNaseL and together these two proteins cause degradation of viral RNA (Malathi et al. 2005; Rebouillat et al. 1999). RNA degraded by RNaseL is able to activate cytoplasmic RIG-1 and MDA5 (discussed

below), resulting in further IFN gene induction (Malathi et al. 2007). MxA targets viral ribonucleoprotein structures, inhibiting transcription and viral replication (Haller et al. 2002). Viperin localises on the endoplasmic reticulum and inhibits cell membrane and lipid raft integrity, thereby inhibiting viral budding (Wang et al. 2007). Type I IFNs can also induce apoptosis via activation of the tumour suppressor gene p53 (Takaoka et al. 2003). This would limit virus replication as the infected apoptotic cells would be removed by phagocytosis, thereby limiting the magnitude of the inflammatory responses to infection.

An interesting property of ISG-mediated antiviral activity is that the effector proteins have a gradient of antiviral activity (Schoggins et al. 2011). This is thought to be beneficial to the host as a diverse range of multiple weak effectors would be preferable to a small group of extremely potent genes, as the latter could result in a toxic cellular environment.

#### 1.2.1.2 Type III IFNs

Discovered in 2003 the type III IFNS are referred to as IFNλ 1, λ 2 and λ 3 or IL-29, IL28A and IL-28B respectively (Kotenko et al. 2003; Sheppard et al. 2003). The type III IFNS signal vial distinct receptor complexes compared to the type I IFNs, but they activate the same intracellular signalling pathways. They also have many of the same biological and antiviral activities. However, unlike the receptor for type I IFNs which is expressed on most cell types, IFNλ receptors are largely restricted to cells of epithelial origin (Donnelly et al. 2010).

#### 1.2.1.3 Pattern recognition receptors

A vital feature of the innate immune response is its ability to survey the environment and recognise foreign organisms. It must also be able to discriminate between self and non-self, especially in virus infection. It is now know that this discrimination relies mainly on pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs). PPRs recognise conserved microbial moieties termed pathogen-associated molecular patterns (PAMPs). As dsRNA, which is generated during viral replication, and virus DNA rich in CpG motifs are not normally found in our body, it is relatively easy for our innate immune system

to recognise them as 'foreign'. However, ssRNA is abundant in human cells and at present it is not fully understood how PPRs provide this discrimination. It is thought that they must recognise some additional factor such as methylation state or certain sequences or restrict specific TLRs to endosomal expression, where host nucleic acids have limited access (Ishii et al. 2005; Kariko et al. 2005). The list of viral components identified as ligands for PRRs is shown in Table 1-5.

| PRR | Viral PAMP | PRR location |
|---|---|---|
| TLR2, TLR4 | Viral proteins | Cell surface |
| TLR3 | dsRNA | Endosome |
| TLR7/8 | ssRNA | Endosome |
| TLR9 | Unmethylated DNA | Endosome |
| MDA-5 | Cytoplasmic dsRNA | Cytosol |
| RIG-1 | Cytoplasmic dsRNA, specifically the 5'-triphosphate end | Cytosol |

**Table 1-5 Host PRRs and their interaction with respiratory viruses**

The RLRs are found in the cytoplasm and include RIG-1 and MDA5 (melanoma differentiation associated gene 5). RIG-1 and MDA-5 play a major role in the recognition of RNA viruses in dendritic cells, macrophages and fibroblasts. RIG-1 binds 5'triphosphorylated ssRNA and short dsRNA to stimulate the production of type I IFNs (Pichlmair et al. 2006; Takahasi et al. 2008). In contrast, MDA-5 recognises long dsRNA, including synthetic polyI:C (Kato et al. 2006).

Toll was initially identified in insects as a receptor essential for dorsoventral polarity during embryogenesis (Akira 2003). Subsequently it was found to play an essential role in the innate immune response against fungal infection in insects (Lemaitre et al. 1996). In humans, TLRs are well expressed in DCs and macrophages, but can be found in nearly all human cells. Unlike RLRs, which are present within the cytoplasm, TLRs are present on the cell surface and within the endosomal compartment in the cytosol. They are transmembrane

receptors comprising an extracellular leucine-rich repeat and a cytoplasmic Toll/Interleukin-1 (TIR) receptor domain. These are connected through a transmembrane domain. The TIR domain shares structural homology with the IL-1 receptor. TLRs recognise and respond to a range of PAMPs. In general, once activated TLRs recruit an adaptor protein to the TIR domain, leading to the engagement of IL-1-receptor-associated kinase (IRAK), additional adaptor proteins and tumour-necrosis-factor-receptor-associated factor 6 (TRAF6). This results in the activation of transcription factors including activator protein 1 (AP-1), nuclear factor κB (NF-κB), interferon regulatory factor 3 (IRF3) and others, depending on the signalling pathway activated (Thompson et al. 2007). The translocation of these transcription factors to the nucleus leads to transcription and subsequent secretion of proinflammatory cytokines (including TNFα, IL-1, and IL-6) and the induction of type I IFNs. This is followed by the expression of ISGs that induce an anti-viral state within cells and limit viral replication. The cytokine profile produced by the activation of each TLR is dependent on the PAMP it recognises, the cell type it is expressed in and the signal transduction cascade activated by that specific TLR.

The cell surface TLRs which recognise virus include TLR2 and TLR4. TLR2 has been shown to recognise RV capsid proteins and components of measles virus and herpes simplex virus (Bieback et al. 2002; Kurt-Jones et al. 2004). Given its ability to recognise RV capsid proteins, TLR2 can also trigger an inflammatory response when exposed to UV-inactivated virus particles in experimental systems (Triantafilou et al. 2011). TLR4 is best known for its ability to recognise bacterial endotoxin or LPS. However, it also responds to RSV and retrovirus (Kurt-Jones et al. 2000; Rassa et al. 2002). The recognition of viral proteins by these TLRs triggers mainly pro-inflammatory responses, but whether they lead to protective or pathological immune responses is thought to depend on the type of virus, route of infection and other host factors (Finberg et al. 2007).

The endosomal TLRs, TLR3, TLR7, TLR8 and TLR9, are ideally placed to respond to viruses because most viruses utilise host endocytic pathways at the cell entry phase or at the point of budding. These 4 TLRs share the property of being activated by nucleic acids and their expression can be increased by type I IFNs. Again, the precise processes that normally prevent self-ligands from

reaching intracellular TLRs remain poorly define. It is also unclear if these TLRs occupy the same sub-compartments or have distinct intracellular localisations. However, it is likely that there is dynamic movement between the sub-compartments (Barton et al. 2009).

TLR3 is expressed in and can mediate activation of a number of immunologically relevant cell types including macrophages, dendritic cells (DCs), B cells and CD8+ T cells (See et al. 2008). TLR3 responds to the presence of dsRNA which is formed as a product of replication for the majority of RNA viruses (including RV) and some viruses with DNA genomes (Weber et al. 2006). It also responds to the artificial dsRNA mimic polyI:C when it is used in experimental systems. Given that TLR3 is expressed on various immune and non-immune cells and the observation that its activation leads to the production of Type I IFNs, it has been surprising that studies have been unable to demonstrate impaired immunity to viral challenge in TLR3 deficient mice. Recent work has suggested that TLR3 is involved in recognising viral dsRNA in the context of phagocytosed apoptotic cells (Schulz et al. 2005). It has also been suggested that TLR3 has evolved principally to detect RNA viruses within infected cells undergoing apoptosis of necrosis (Barton 2007).

Unlike the other endosomal TLRs, TLR3 is also constitutively expressed on the surface of bronchial epithelial cells (Hewson et al. 2005). Using a human bronchial epithelial cell line, BEAS-2B, it has been demonstrated that RV infection and replication increases TLR3 mRNA and protein expression on the cell surface. Inhibition of TLR3, using a blocking antibody, led to a greater than two-fold increase in virus released from the cell, indicating greater viral replication and release. These results suggest that surface-expressed TLR3, which recognises extracellular dsRNA, plays an important role in the innate immune response to RV.

TLR9 mediates recognition of unmethylated DNA with a CpG motif is activated by the DNA viruses herpes simplex virus (HSV)-1, HSV-2 and cytomegalovirus (CMV). CpG motifs are CG pairings in which the cytosine is unmethylated and are substantially more prevalent in pathogenic DNA than mammalian DNA (Krieg 2002).

TLR7 and TLR8 recognise ssRNA and induce innate immune responses to ssRNA viruses (Heil et al. 2004). However, precisely which aspects of ssRNA are required for TLR7/8 binding and activation remains unclear. Infection of TLR7-/- mice *in vivo* or of immune cells *in vitro* with influenza virus has been shown to result in abrogated IFN and cytokine production (Diebold et al. 2004; Lund et al. 2004). A family based association study has shown that polymorphisms in the TLR7 (and TLR8) gene on the X-chromosome are associated with asthma and related atopic disorders including rhinitis and eczema (Moller-Larsen et al. 2008).

In summary, it is likely TLR2 on the cell surface recognises RV viral proteins. Once the virus enters the cells the single stranded viral genome is detected by endosomal TLR7 and TLR8. Once the virus begins replication and dsRNA molecules are formed, it is likely that TLR3, RIG-1 and MDA5 are activated and continue the pro-inflammatory antiviral response already initiated, thereby limiting viral replication.

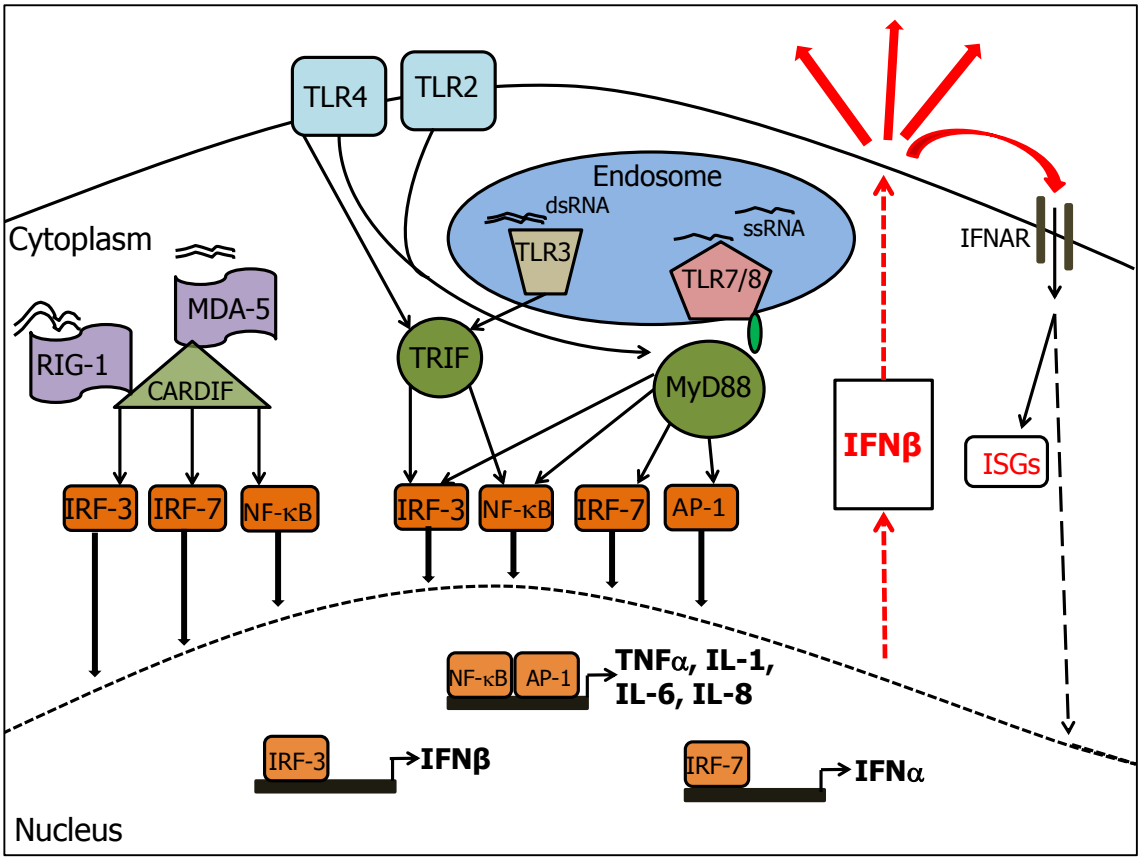


**Figure 1-3 Schematic representation of PRRs involved in recognition of virus and responses elicited.**
Retinoic-acid-inducible protein (RIG)-1 and melanoma-differentiation-association gene (MDA)-5 detect viral replication in the cytosol and signal through CARD adaptor inducing IFNβ (CARDIF) to phosphorylate interferon response factor (IRF)-3 and IRF-7 inducing a type I IFN response. CARDIF also activates NF-κB leading to pro-inflammatory cytokine production. TLR3 is expressed within the endosome and signals via TIR-domain-containing adaptor protein-inducing IFNβ (TRIF) leading to the phosphorylation of IRF-3, resulting in the expression of IFNβ. Signalling can also occur that leads to the translocation of NF-κB to the nucleus. TLR7 is also expressed within the endosome and signals through a MyD88-dependent pathway, leading to the translocation of IRF-7, AP-1 and NF-κB. TLR4 is expressed on the surface of cells and is able to signal via both MyD88-dependent and -independent pathways and is able to activate a response via IRF-3, NF-κB and AP-1. Following ligand stimulation TLR2 signals via a MyD88-dependent pathway. Release of type I IFNs is recognised by infected and neighbouring cells through the IFN α/β receptor (IFNAR) leading to the transcription of type I IFNs and interferon stimulated genes (ISGs).

#### 1.2.1.3.1 Expression of TLRs in asthma

A number of studies have looked at the expression of TLRs on peripheral blood cells in asthma. Using flow cytometry, Lun *et al* have shown that asthmatic

monocytes had significantly decreased expression of TLR2 and TLR4, increased expression of TLR3, while the expression of TLR7 was not shown to be significantly different between asthmatic patients and control subjects (Lun et al. 2009). Stimulation of TLR2 is thought to lead to IL-10 secretion which is an anti-inflammatory cytokine that can dampen the production of Th2 cytokines (Fiorentino et al. 1991; Re et al. 2004). Therefore decreased expression of TLR2 in asthmatic patients may lead to decreased IL-10 production and diminished regulation of Th2 cytokines. 87% of the patients in this study were on ICS treatment, suggesting that as a group they included patients with mild-to-moderate disease. Another flow cytometry based study evaluated the expression of TLR1, 2, 3, 4, 6 and 9 on PBMCs (Chun et al. 2010). Expression of TLR1, TLR2, and TLR9 was found to be significantly higher in the asthmatic group, while TLR6 showed reduced expression in the asthmatic group compared to healthy subjects. This study also included a group of patients with more severe disease and this group showed markedly increased expression of TNF$\alpha$ after the cells were stimulated with a TLR4 ligand, LPS. This suggests that severe asthmatics have an intrinsic tendency to over-produce TNF$\alpha$ in response to TLR stimulation (through bacterial infection), which can promote persistent airway inflammation. However it is important to remember that alveolar macrophages (AM) can have a distinct receptor profile compared to peripheral blood monocytes. This has been elegantly demonstrated in a study which looked at the expression of TLR2, TLR4 and TLR9 on AM and autologous monocytes and found that surface expression of TLR2 was lower on AM than blood monocytes, expression of TLR4 comparable and surface expression of TLR9 higher on AM than monocytes (Juarez et al. 2010). Therefore it appears that TLR expression is tissue specific and enables the compartmentalisation of immune responses within the lung. This study also highlights the need to study tissue specific cells when trying to understand disease processes i.e. study AM when investigating the asthma and not to always generalise based on results from peripheral blood cells. AM from subjects with well-controlled asthma (ICS naïve and on low dose ICS treatment i.e. on BTS treatment steps 1 and 2) have been found to have comparable TLR3, TLR7 and TLR8 mRNA to healthy AM (Sykes et al. 2012) but the expression of these receptors in airway cells from subjects with severe asthma has not been studied.

### 1.2.2 Defect in innate immune response to RV in asthma

The observation that asthmatic patients have more severe and prolonged virus-induced LRT symptoms compared to non-asthmatic subjects was made over a decade ago. This suggested for the first time that there might be some inherent differences in the way asthmatic subjects respond to viral respiratory tract infections. Since then evidence has accumulated showing that this phenomenon may be due to a defect in IFN production. This defect in IFN production has been demonstrated to occur in asthmatic bronchial epithelial cells (BECs), asthmatic AM and asthmatic PBMCs (Contoli et al. 2006; Iikura et al. 2011; Sykes et al. 2012; Wark et al. 2005).

The first study was performed on primary (cultured) BECs infected with RV-16 *ex vivo* (Wark et al. 2005). Asthmatic BECs showed profound impairment of virus-induced IFNβ release. The deficiency of IFNβ was associated with increased viral replication and cytolysis with increased viral release and infection of neighbouring cells in the asthmatic BECs. In non-asthmatic cells, infection of BECs was limited by the induction of apoptosis in infected cells, while impairment of apoptotic responses was observed in the asthmatic cultures. An interesting finding of this study was that exogenous IFNβ , given to infected asthmatic cells, was able to restore apoptotic responses and limit virus replication to levels observed in normal cells.

As mentioned previously, the recently discovered type III IFNs (IFNλ 1 and IFNλ 2/3) are also important in the innate immune response against viruses. It has also been shown that asthmatic BECs and bronchoalveolar lavage (BAL) cells (which are predominantly AM) have a deficient induction of IFNλ s by RV *in vitro* (Contoli et al. 2006). When the same subjects underwent experimental RV infection, this deficiency was found to be highly correlated with the severity of the exacerbation and viral load.

Extending on from BECs and AM, it has also been found that PBMCs from young patients with asthma (age 7-19 years) had a deficient IFNα response to RV infection *ex vivo* (Iikura et al. 2011). This defect was not present in adults (age >20 years) with asthma, or young children (age 2-6 years). The results of this study suggest that perhaps the innate immune defect is not just localised to the lung, but also present, to some extent, in circulating immune cells. To

further support this, PBMC from atopic asthmatic adolescents have been shown to have an impaired IFN response to TLR7 stimulation (Roponen et al. 2010).

In order to investigate the mechanism of defective RV-induced IFN production, the expression of TLRs, RLRs and key signalling molecules implicated in IFN induction has been measured in asthmatic patients and healthy subjects (Sykes et al. 2012). BAL cells (which in this study was >95% AM) from patients with atopic mild-to-moderate asthma and healthy volunteers were stimulated with RV16. The induction of IFN$\alpha$ and IFN$\beta$ was significantly deficient in BAL cells from asthmatic patients. However, expression of PPRs (TLR3, TLR7, TLR8, RIG-1 and MDA-5), their adaptor proteins (TRIF, MyD88 and CARDIF) and signalling kinases and transcription factors downstream of these PRRs (IKK1, IKKB, IRAK4, IRF3, and IRF7) was not different in asthmatic patients and healthy subjects. Additionally (and confirming previous observations), in this group of asthmatics who had a mean age of 33 years, no deficiency in RV-induced type I IFN production was observed in PBMCs. Interestingly, the study did find that IFN deficiency in asthmatic patients correlated with degree of airway hyperresponsiveness and degree of atopy (as measured by number of positive skin prick test responses), suggesting that defective IFN production might be related to greater disease severity and/or allergic status.

There has also been some suggestion in the literature that impaired IFN production by asthmatic BECs is related to asthma severity and/or treatment. While Wark *et al* demonstrated profound reduction of virus-induced IFN production by asthmatic BECs (Wark et al. 2005), a recent study by Sykes et al has not been able to reproduce those results (Sykes et al. 2014). On closer examination, patients in the latter study all had well controlled disease with over a third not requiring any ICS therapy and with a mean $FEV_1$ % predicted of 89%. In contrast, impaired RV-induced IFN production has been detected in BECs from asthmatic subjects that could be considered as having more severe disease as they had a mean $FEV_1$ % predicted of 65% and mean ICS dose of 2300 mcg (BDP equivalent) (Parsons et al. 2014).

As highlighted by these studies, further work to investigate mechanisms of defective virus-induced IFN production in asthmatic patients is needed. These studies also highlight the importance of careful patient selection in terms of age, disease severity and atopic status. However what is clear is that the

deficiency in type I and III IFNs in asthmatic individuals creates a local environment within the lung in which the RV is able to survive and replicate , leading to worsening inflammatory changes within the asthmatic airways and recurrent asthma exacerbations (Figure 1-4)

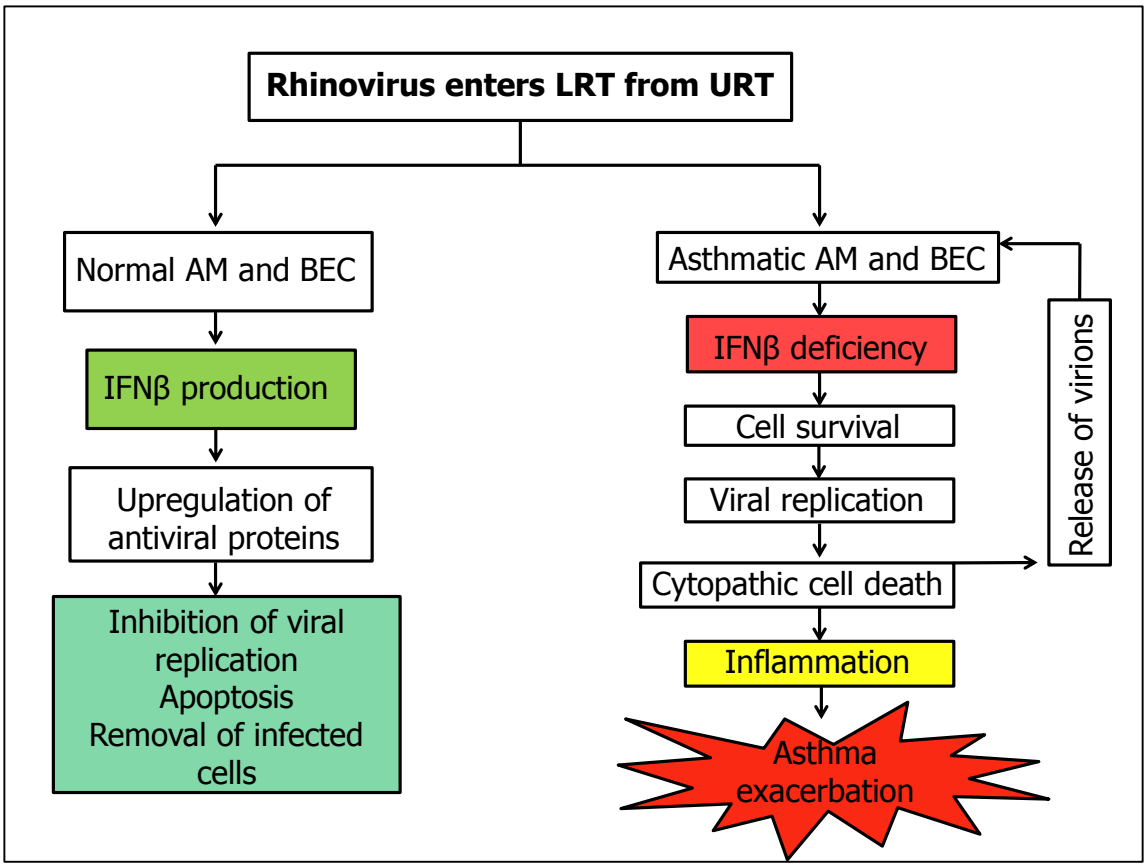


**Figure 1-4 Comparison between healthy and asthma airway cells in their response to rhinovirus**

Interestingly, it appears that defective epithelial production of type I and III IFNs in response to RV infection is not restricted to asthmatic BECs but is also seen in cystic fibrosis (CF) (Vareille et al. 2012) and chronic obstructive pulmonary disease (COPD) (Mallia et al. 2011). BECs from patients with CF were shown to produce significantly less IFNβ and IFNλ when exposed to RV *in vitro*. In CF, this defect was also present in nasal epithelial cells. Deficiency of IFNs was also associated with a deficiency in the production of the ISGs MxA, OAS and viperin. Again, as was demonstrated in asthmatic BECs, application of exogenous IFN to CF cells restored control of virus replication and levels of ISGs produced. BAL cells from subjects with COPD produced significantly less

IFNβ compared to control subjects when incubated with RV *in vitro*. The subjects then underwent experimental RV infection, and virus load in nasal lavage, sputum and BAL was significantly higher in subjects with COPD, reflecting the IFN deficiency. Therefore, it is obvious that IFN deficiency does not only occur in asthma and from this observation it can be inferred that the impaired IFN response is not caused by some genetic/epigenetic change or environmental interaction unique to asthma. As it is also seen in other chronic inflammatory lung diseases, it may be that the defective IFN response to RV is caused by a chronic pro-inflammatory state within the lower airways and/or bacterial infection within the lung.

## 1.3 Alveolar macrophages

Within the airways, the AM is the predominant immune effector cell and plays a key role in host defence against pathogens. AMs are distributed from the upper airways to the alveoli and are quickly recruited during inflammatory processes of the lung (Gordon et al. 2002). All macrophages originate from precursor cells in haemopoeitic organs and enter the respiratory tract via blood and lymph. They can be derived from peripheral blood monocytes that leave the circulation and develop into extravascular mature tissue macrophages or from a stable self-sustaining population of resident lung macrophages (Yang et al. 2012). The major resident macrophages responsible for lung defence are AMs and DCs, other lung macrophages include pleural, interstitial and intravascular macrophages (Gordon et al. 2002). AM are strategically situated at the air-tissue interface in the alveoli and are the first cells to encounter inhaled antigens in the lower respiratory tract. They are involved in the elimination of inhaled foreign materials and express high levels of immunoglobulin receptors, complement receptors and mannose receptors to facilitate phagocytosis of opsonised and non-opsonised particles. Once activated they release a number of cytokines including IL-12 (activates NK cells), LTB4 (recruitment of neutrophils), T-cell stimulatory and pro-inflammatory cytokines (TNF$\alpha$) and antibacterial products such as lactoferrin and lysozyme. They also play a pivotal role in antigen presentation and produce inflammatory cytokines to recruit cells of the adaptive immune system (See et al. 2008).

Macrophages are frequently classified as classical or alternative. Classically activated macrophages, also designated as M1 macrophages, modulate host defence against intracellular pathogens, tumour cells and tissue debris (Mantovani et al. 2004; Martinez et al. 2006). The classical pathway for macrophage activation is driven by IFN$\gamma$ and its purpose is to enhance macrophage dependent phagocytosis and killing of microbes and antigen presentation to activate the adaptive immune response and ensure full clearance and long term protection against pathogen (Tyner et al. 2005). M1 macrophages are associated with Th1 immune responses and marked by production of IL-1$\beta$, IL-6, IL-12 and TNF$\alpha$. Exposure to IL-4 and IL-13 triggers the alternative pathway of macrophage activation (Martinez et al. 2009).

Alternatively activated macrophages (AAM) are also designated as M2 macrophages. Other cytokines such as IL-33 and IL-25 can amplify AAM induction (Gordon et al. 2010). M2 macrophages have been shown to have a role in models of allergic inflammation of the airways (Kim et al. 2008) and to promote Th2 reactivity, including parasite elimination, suppression of inflammation and tissue remodelling (Biswas et al. 2010). Chemokines produced by M2 macrophages are also likely to be functionally relevant in asthma- for example CCL24 cooperates with IL-13 in the recruitment of eosinophils (Pope et al. 2005). BAL fluid and airway wall tissue from asthmatic subjects has been shown to have higher number of M2 macrophages compared to healthy subjects (Melgert et al. 2011). They even appear to have a role in asthma exacerbations as it has been shown that during acute exacerbations circulating monocytes demonstrate upregulated expression of genes that are indicative of an alternatively activated phenotype (Subrata et al. 2009). Finally, in severe asthma, higher levels of IL-13 producing macrophages are seen in BAL fluid, suggesting that M2 macrophages may also contribute to reduced lung function in asthma (Kim et al. 2008). In a mouse model of chronic asthma, AAM were shown to replace Th2 cells as the major cellular source of IL-13 (Kim et al. 2008). Contrary to these findings, recent human studies suggest that BAL fluid from severe asthma patients have higher levels of M1 macrophages compared to M2 macrophages, suggesting that M1 macrophages may also play a key role in the development of severe asthma (Goleva et al. 2008). However, despite the demonstration of these two distinct macrophage phenotypes, several studies have shown not only that there is a functional overlap between subtypes but that there is significant plasticity between subtypes and so cells can first have an M1 phenotype and subsequently develop an M2 phenotype (Hume 2008; Murray et al. 2011).

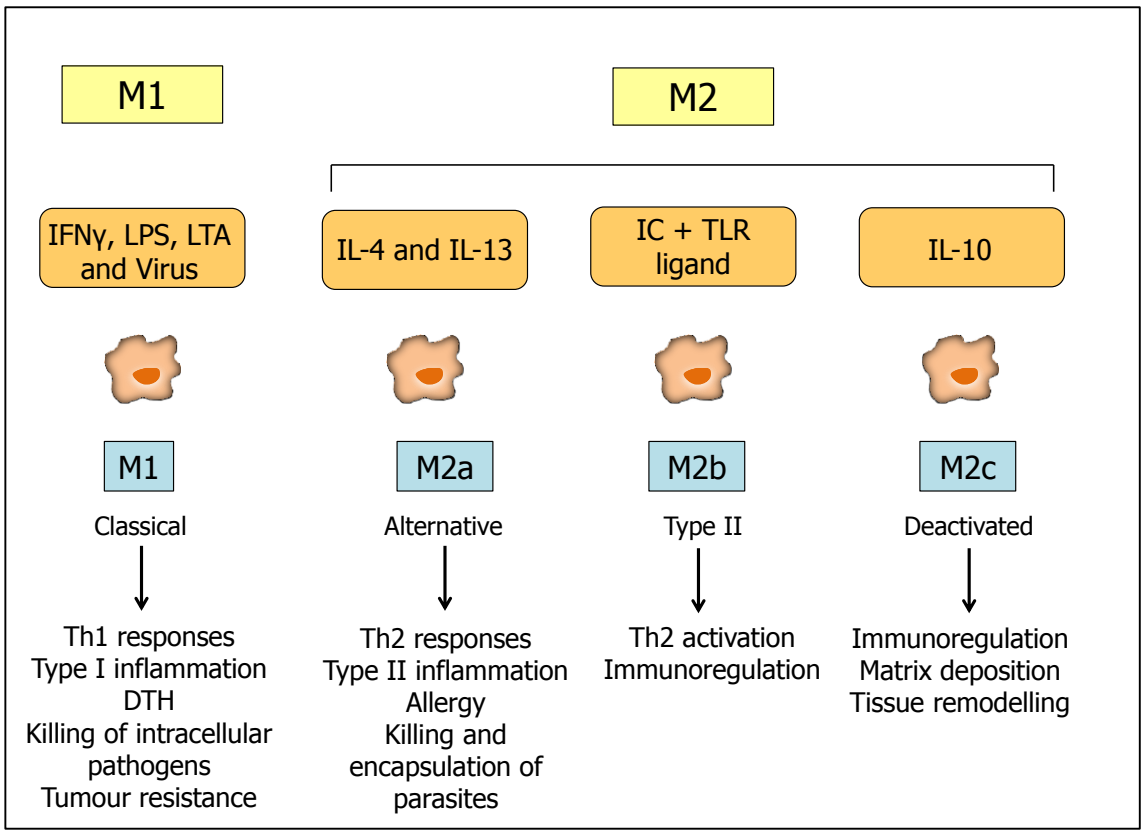


**Figure 1-5 Inducers and selected functional properties of different polarised macrophage populations**
Macrophage exposure to IFNγ , bacterial and viral products drives M1 polarisation with potentiated cytotoxic and antitumoural properties. The immunoregulatory M2 macrophages can be subdivided into M2a (induced by IL-4 and IL-13), M2b (induced by combined exposure to immune complexes and TLR) and M2c (induced by IL-10). M2a and M2b exert immunoregulatory function and drive type II responses while M2c macrophages are more related to suppression of immune responses and tissue remodelling. DTH, delated-type hypersensitivity; IC, immune complexes; IFNγ , interferonγ , LPS, lipopolysaccharide; LTA, lipoteichoic acid; TLR, toll-like receptor.

Although AMs are the most abundant pulmonary innate immune cell and are crucial in modulating chronic inflammation, our understanding of their role in asthma and asthma exacerbations is currently limited. Since AM can produce both Th1 and Th2 cytokines they are implicated in the activation and maintenance of airway inflammation in asthma (Fuller 1989). Interestingly, transfer of AM from allergy-resistant rats to allergy susceptible rats protects the latter against the development of asthma symptoms after allergen exposure (Careau et al. 2004), suggesting an immunomodulatory role for AM in allergic asthma.

AMs express high levels of the RV receptors ICAM-1 and LDL-receptor making them prime targets for RV infection (Laskin et al. 2001). They also express a number of PRRs and are therefore potent releasers of type I IFNs in response to viruses, limiting virus spread by inducing an antiviral state in nearby bronchial epithelial cells. In addition to type I IFNs, the interaction between RV and AM stimulates the secretion of pro-inflammatory cytokines, such as IL-1, IL-8, IFNγ , CCL3 and TNFα. However, an excessive inflammatory response can perturb gas exchange and so AM are "ambidextrous"- capable of both enhancing and suppressing inflammatory responses appropriate to the needs of the moment (Peters-Golden 2004).

Whether the AM supports RV replication is still debated. Gern *et al* used AM recovered from BAL fluid and reported that although RV bound to and entered AM they did not replicate in AM (Gern et al. 1996). Viral replication was assessed by measuring total RNA in AM with or without actinomycin D, which inhibits cellular transcription but has little effect on picornavirus RNA synthesis (Reich et al. 1962). No increase in RNA was noted, suggesting no new viral RNA was being synthesised. However despite not replicating in AM, infection with RV was a potent stimulus for TNFα secretion. In contrast, using monocyte derived macrophages (MDM) as a surrogate for AM Laza-Stanca *et al* showed that RV16 does replicate in MDMs, leading to the release of TNFα through activation of NF-κB (Laza-Stanca et al. 2006). Viral replication was confirmed by showing persistence of viral release and high levels of intracellular viral RNA up to 72 hours after infection and by demonstrating synthesis of new viral proteins. When UV-inactivated RV16 was used, which is unable to replicate, TNFα release was completely abrogated. However replication of RV was significantly less in MDMs compared to BECs and THP-1 cell, a monocytic cell line. The differing results of these two studies are difficult to explain. Part of the reason maybe that the former study used primary human AM while the latter utilised a surrogate for AM- MDM. Additionally, there are differences in the way they assessed viral replication. However, both studies confirm that AM are key responders to RV and produce antiviral and pro-inflammatory cytokines.

# 1.4 Toll-like receptor 7

TLR7 is of particular relevance to asthma and TLR7 gene polymorphisms have been associated with human asthma (Moller-Larsen et al. 2008) implicating this PRR as particularly relevant to the pathogenesis of asthma. It is expressed in bronchial epithelial cells (Cherfils-Vicini et al. 2010), airway smooth muscle (Ekman et al. 2011) and airway immune cells. TLR7 is an endosomal PRR and recognises ssRNA from the viral genome and replication intermediates. It signals through a Toll/IL-1 receptor (TIR) domain containing adapter- myeloid differentiation factor 88 (MyD88). MyD88 then binds to and forms a signalling complex with IRAK-4, IRAK-1, tumour necrosis factor-receptor associated factor 3 (TRAF3), and TRAF6. This results in the phosphorylation of Iκ B kinase β (IKKB) and activation of interferon regulatory factor (IRF)7 and NF-κ B, resulting in the production of type I IFNs, cytokines and inflammatory cell recruitment (see Figure 1-3). Evidence is starting to emerge supporting the relevance of TLR7 in asthma exacerbations, airway inflammation and bronchoconstriction.

## 1.4.1 Viral exacerbations and TLR7

Respiratory viruses, especially ssRNA viruses such as RV are a frequent cause of asthma exacerbation. The detection of respiratory viruses by TLR7 activates Th1 antiviral responses, promoting viral clearance. In rats, TLR7 agonist treatment was able to increase IFN production, thereby reducing parainfluenza virus replication and attenuating virus-induced BHR (Stokes et al. 1998). TLR7 function has been shown to be reduced in adolescents with atopic asthma compared to healthy and atopic non-asthmatic controls (Roponen et al. 2010). It has been proposed that this defect may contribute to defective viral clearance and increased susceptibility to LRT viral infections in patients with asthma. In this study PBMCs were stimulated *ex vivo* with imiquimod or PolyI:C, synthetic agonists of TLR7 and TLR3 respectively, and the production of ISGs was measured. Following TLR7 activation, PBMCs from asthmatic patients had deficient production of OAS and MxA mRNA. In contrast to the results with the TLR7 ligand, the ability of the TLR3 ligand to induce key anti-viral molecules was similar in asthmatic and control groups. The asthmatic patients in this study had mild to moderate disease with 11 out of the 17 asthmatic subjects prescribed an ICS at the time of the study. They also found that there was no difference in the baseline expression of TLR7 in asthmatic

versus healthy controls. In contrast, the expression of TLR7 has been shown to be decreased in the bronchial epithelium from subjects with severe asthma (Shikhagaie et al. 2014). The clinical significance of this is unclear as the main stimulus for epithelial derived IFN is dsRNA, which signals via TLR3, RIG1 and MDA5.

Several prospective birth cohort studies have shown that wheezy LRT infections in infancy are a risk factor for the development of asthma in later childhood (Jackson et al. 2008; Kusel et al. 2007; Sigurs et al. 2000; Stein et al. 1999). A recent interesting publication has suggested that TLR7 could also be involved in the pathogenesis of asthma. Pneumovirus infection in TLR7 knockout mice predisposed toward a bronchiolitis-like disease and promoted Th2 responses to virus alone or bystander allergen (Kaiko et al. 2013). On subsequent infection in later life, these Th2 cells were reactivated and the mice developed an asthma-like pathology and airway hperreactivity. This was associated with defective anti-viral cytokine production and greater viral load leading to epithelial sloughing and necrosis.

### 1.4.2 TLR7 attenuates airway inflammation

As mentioned previously asthma is a complex inflammatory disease, largely characterised by a predominance of Th2-type immune responses. The observation that the prevalence of asthma has increased significantly in Western countries, coupled with the decrease in childhood infections has led to the proposition of the hygiene hypothesis (Strachan 1989). The hypothesis proposes that frequent exposure to a range of microbial products in childhood results in a predominant Th1 phenotype and protects against asthma, while a lack of such interactions due to more hygienic living conditions, the increasing use of antibiotics and sterile food conditions, could promote Th2-driven allergic diseases. In support of this hypothesis, it has been found that countries with low levels of health and hygiene standards have the lowest prevalence of allergic diseases (Yazdanbakhsh et al. 2002). Additionally, the prevalence of atopy was found to be significantly lower in children from anthroposophic families (people who use antibiotics restrictively, have few vaccinations and consume fermented foods) than in children from other families in a developed city (Alm et al. 1999). Therefore a number of studies have focused on the use of TLR agonists in suppressing the development of

Th2-mediated responses by skewing the innate immune system towards induction of Th1 cytokine production. This is supported by animal data showing that mice deficient in MyD88 (resulting in a lack of TLR signalling) have markedly increased Th2 responses and overwhelming IgE production (Schnare et al. 2001).

In a mouse model of allergen-induced airway disease, local administration of a mouse TLR7 ligand, resiquimod, before repeated airway allergen challenge attenuated the development of airway inflammation and *in vivo* airway hyperreactivity (Quarcoo et al. 2004). It also significantly reduced the numbers of eosinophils and lymphocytes in BAL fluid, inhibited mucus gland hyperplasia and reduced Th2-cytokine production. The beneficial effect of resiquimod was not seen in mice lacking the TLR7 receptor, indicating that the above responses were mediated through TLR7. Carrying on from this study, it has also been shown that imiquimod, another TLR7 ligand, was able to inhibit airway remodelling in a mouse model of chronic asthma (Du et al. 2009). Like resiquimod in the previous study, imiquimod was able to decrease eosinophilia, total inflammatory cells and Th2 cytokines in BAL fluid and abolish the number of mucus producing goblet cells. Additionally, it also reduced total serum IgE, suppressed the deposition of sub epithelial collagen, reduced allergen-induced increase in airway smooth muscle and decreased lung expression of the profibrotic cytokine TGF-β 1. Finally it has been shown that TLR7 mediated suppression of airway responses is long-lived (Xirakia et al. 2010). Again, using a mouse model of allergic asthma, intranasal resiquimod was capable of suppressing allergic airway inflammation triggered by inhaled allergen challenge 4 weeks after treatment. This study also showed that signalling through TLR7 on immune cells leading to the induction of type I IFNs was mediating the suppression of allergic airways disease.

The inhibition of TLR7 agonists on IgE production has also been demonstrated with human PBMCs *ex vivo* (Frotscher et al. 2002). PBMCs from patients with atopic dermatitis and seasonal allergic rhinitis were analysed in the presence of resiquimod. Resiquimod was able to inhibit the spontaneous production of IgE by approximately 75%. It was also shown that this effect was partly due to the inhibition of CD23, a low affinity IgE receptor capable of modulating IgE production, expression on B cells and induction of IFNγ .

### 1.4.3 TLR7 reverses airway hyperresponsiveness

Finally, animal studies have also shown that TLR7 agonists are potent and rapid bronchodilators (Ekman et al. 2011; Kaufman et al. 2011). In guinea pigs, imiquimod was able to rapidly (within minutes) inhibit bronchoconstriction *in vivo*, and *in vitro* experiments showed that this was through relaxation of contracted airway smooth muscle (Kaufman et al. 2011). This effect of imiquimod was shown to be TLR7 dependent, through a nitric oxide pathway, and TLR7 independent, through mechanisms involving prostaglandins and calcium-activated potassium channels. As mentioned previously, on recognition of viral ssRNA, TLR7 signalling leads to the production of antiviral cytokines and inflammatory cytokines. Although required to clear the virus, the resulting inflammation also results in airway wall oedema and sloughing of dead cells leading to airway obstruction. Therefore, this additional role attributed to TLR7 might represent a potent mechanism by which airflow can be maintained during virus-induced inflammation, counteracting virus induced airflow obstruction. It is tempting to speculate that reduced function of TLR7 (Roponen et al. 2010) or TLR7 polymorphisms may predispose to asthma exacerbations by eliminating this compensatory bronchodilatation during viral infections, leaving the bronchoconstrictive effects of viral infection unopposed.

Given the beneficial effects of TLR7 agonists in animal models of asthma, it seems surprising that their use has not translated into human studies. Repeated intranasal TLR7 stimulation has been shown to reduce allergen responsiveness in patients with allergic rhinitis (Greiff et al. 2012), supporting its role as a therapeutic target for allergic airway inflammation. However there are justifiable concerns regarding predictable side effects from induction of systemic Th1 cytokines. Dose-dependent development of flu-like symptoms (pyrexia, myalgia and shivering) have been reported with nasal administration of a TLR7 agonist (Greiff et al. 2012) and systemic resiquimod administration (Pockros et al. 2007) for allergic rhinitis and chronic hepatitis C virus infection respectively. However, direct inhalation into the lungs is an attractive route for administration and would enable avoidance of systemic side effects.

# 1.5 MicroRNAs

MicroRNAs (miRNA) are small non-coding RNA molecules (approximately 22 nucleotides in length) that constitute a newly recognised layer of gene regulation. They usually act as endogenous repressors of gene activity by translational repression and mRNA degradation. They have been found to be crucial to several biological processes including cell proliferation, apoptosis and differentiation. Indeed computational analyses suggest that miRNAs may regulate over 60% of the human genome (Friedman et al. 2009; Lewis et al. 2005). First discovered in the nematode *C. elegans* (Lee et al. 1993), the term 'microRNA' was not introduced until 2001 (Lagos-Quintana et al. 2001). Over 1000 human miRNA sequences are now recognised, many of which are highly conserved in other organisms, suggesting that they represent a relatively old and important regulatory pathway (Bartel 2004; Stefani et al. 2008; Williams 2008). Several human diseases have been associated with deregulated miRNA expression, ranging from metabolic and chronic inflammatory diseases to malignancy (Care et al. 2007; Fiore et al. 2008; Krutzfeldt et al. 2006; Lu et al. 2005; Poy et al. 2004). MiRNAs are also pivotal to both adaptive and innate immunity, with regulatory effects on cell differentiation and immunological function (Lu et al. 2009). It is also becoming apparent that miRNAs frequently operate in a coordinated fashion- multiple miRNAs act together to regulate the expression of an mRNA or a group of functionally related mRNAs. Therefore, although the individual targeting for each of these miRNAs and its seed match may be weak, the combination of multiple miRNAs targeting multiple seed matches in the same 3'UTR may have considerable biological significance (Peter 2010).

MiRNAs are distinct from the better known small interfering RNAs (siRNA) that knock down single genes, as a single miRNA may target several hundred different mRNAs. Table 1-6 summarises some of the differences between miRNAs and siRNAs.

| | MicroRNA | Short interfering RNA |
|---|---|---|
| **Structure** | Single stranded<br>19-25 nucleotides long | Double stranded<br>21-22 nucleotides long |
| **Origin** | Endogenous to every cell<br>Transcribed from genomic DNA | Chemically synthesised molecules that are transfected into mammalian cell.<br>Occur naturally in viruses, plants and lower animals |
| **Complementarity to target mRNA** | Not perfect<br>Therefore a sing miRNA may target several hundred mRNAs | 100% perfect complementarity<br>Therefore siRNAs knock down specific genes |
| **Biogenesis** | Expressed by genes whose purpose is to make miRNAs | Regulate the same genes that express them |
| **Action** | Inhibit mRNA translation<br>mRNA destabilisation | Cleave mRNA |
| **Function** | Regulate gene expression | Gene silencing<br>Proto-immune system in some organisms |

**Table 1-6 Differences between miRNAs and siRNAs**
(Mack 2007; Metias et al. 2009)

## 1.5.1 MiRNA biogenesis

MiRNA biogenesis involves a series of complex steps which start in the nucleus and are completed in the cytoplasm (Figure 1-6). MiRNA genes are localised in the non-coding regions or introns of protein-coding genes in genomic DNA. Within the nucleus, primary miRNAs are transcribed and are generally >1kb long. The primary miRNA undergoes a series of endonucleolytic steps including cleavage by a nuclear RNase III enzyme termed “Drosha”. The resulting 70nt pre-miRNA is actively transported into the cytoplasm and processed into a 22nt double stranded miRNA by a cytoplasmic RNase III enzyme termed “Dicer”. One strand of this duplex, representing the mature miRNA is incorporated into the RNA-induced silencing complex (RISC). Once assembled into the RISC the miRNA regulates gene expression by base-pairing to the target mRNA.

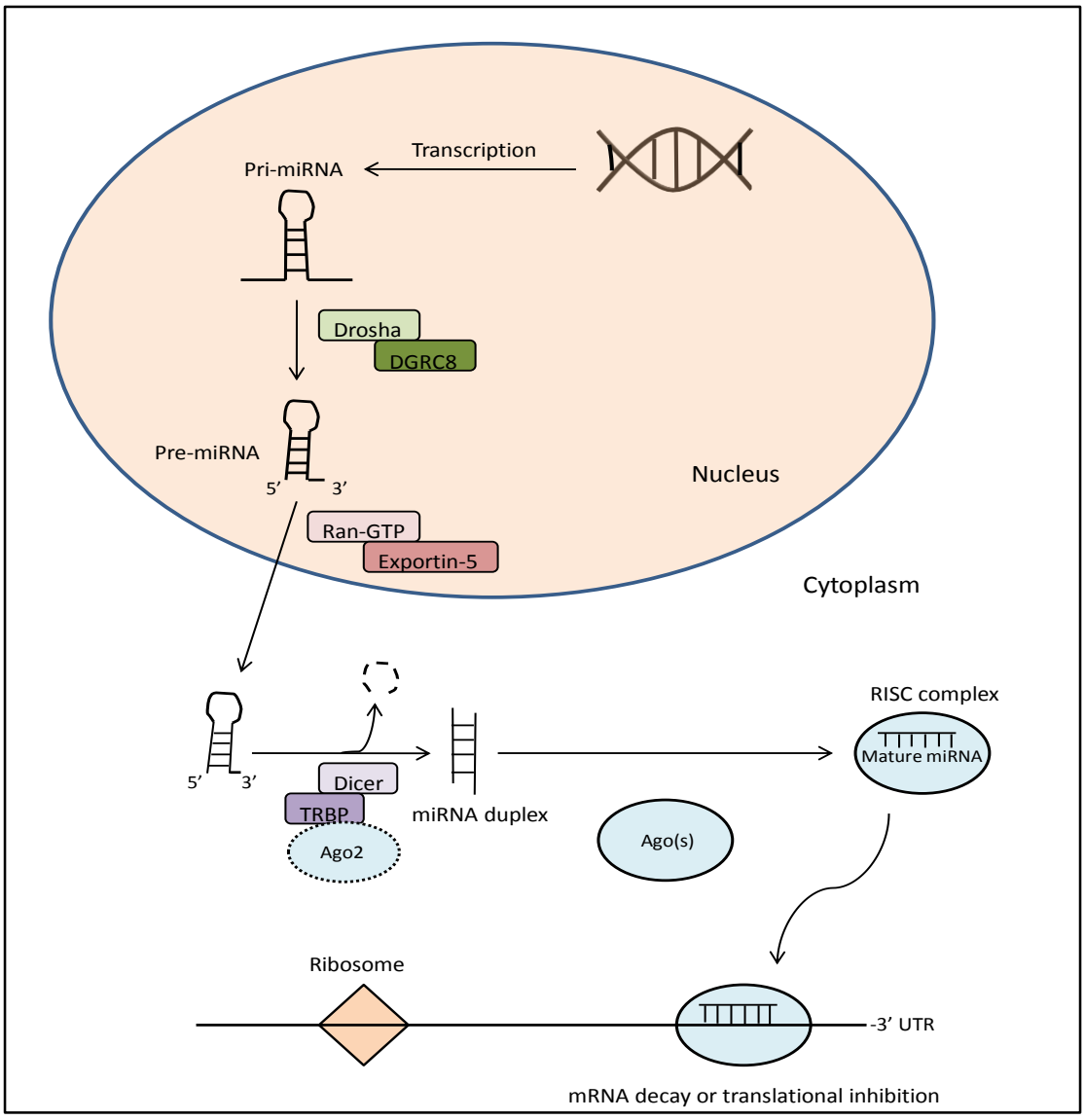


**Figure 1-6 MiRNA biogenesis**
Primary miRNA (Pri-miRNA) is transcribed by RNA polymerase III and then cleaved by the Drosha-DGCR8 microprocessor complex to precursor miRNA (pre-miRNA). After export into the cytoplasm by the nuclear transport Exportin-5 and the co-factor RanGTP. Within the cytoplasm, the pre-miRNA is further processed into a ~22nt double stranded miRNA duplex by Dicer, a cytoplasmic RNase III enzyme. Argonaute 2 (Ago2) can sometimes support Dicer processing by cleaving some pre-miRNAs. One strand of this duplex incorporates into a large protein complex the RNA-induced silencing complex (RISC), to finally become the mature miRNA. Argonaute proteins (Ago) are key factors in the assembly and function of RISCs. The RISC directs the miRNA to binding sites in the target mRNA leading to inhibition of protein translation or degradation of the mRNA.

### 1.5.2 Mechanisms of miRNA action

MiRNAs act at the post-transcriptional level and in most cases cause a reduction in protein output from the target mRNA. This occurs through either translational repression or mRNA degradation via interactions between the miRNA 5' region (the seed sequence) and mRNA 3' untranslated region (3'UTR) (Figure 1-7).

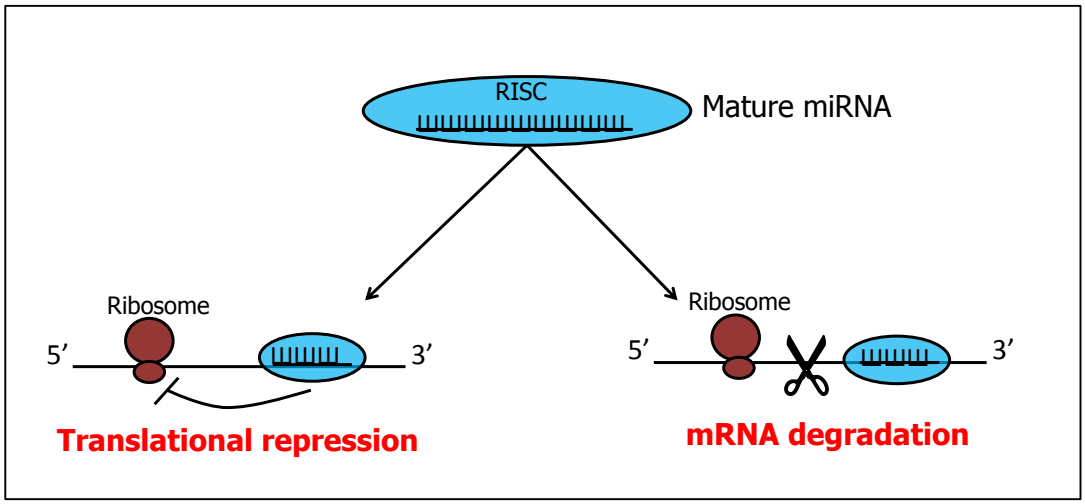


**Figure 1-7 Mechanism of action of miRNAs**
The mature miRNA is incorporated into the large protein complex RNA-induced silencing complex (RISC). This can repress protein production by binding to the target messenger RNA (mRNA) by base-pairing, causing inhibition of protein translation or degradation of the target mRNA.

MiRNAs are thought to employ multiple mechanisms to repress the translation of targeted mRNA:

i) inhibiting the initiation of translation (Humphreys et al. 2005; Kiriakidou et al. 2007; Mathonnet et al. 2007)
ii) inhibiting translation elongation (Olsen et al. 1999; Seggerson et al. 2002)
iii) causing premature termination of translation (Petersen et al. 2006) and
iv) inducing co-translational inhibition of nascent polypeptides (Nottrott et al. 2006)

However, recently it has recently been shown that target mRNA degradation, or destabilisation, may be the predominant mechanism for reduced protein output. In one study, about 84% of target repression mediated by miRNAs was shown to be due to decreased mRNA levels (Guo et al. 2010). Very occasionally, miRNAs can promote translation, thereby increasing protein expression (Orom et al. 2008).

### 1.5.3 Regulation of miRNAs

Most miRNAs are predicted to target many 100s of mRNAs, simultaneously targeting multiple gene pathways to fine tune gene expression. Therefore multiple mechanisms have evolved to stringently regulate miRNA function at multiple levels- during transcription, miRNA processing and target interaction (Breving et al. 2010). For example at the transcription level, the tumour suppressor gene p53 has been shown to bind to the promoter regions of miR-34 family and activate its expression (Chang et al. 2007). The over-expression of miR-34 in vitro has been shown to lead to cell cycle arrest and apoptosis (Raver-Shapira et al. 2007). Polymorphisms within the 3'UTR region of the target mRNA may also compromise miRNA regulation and binding (Tan et al. 2007). For example, a single nucleotide polymorphism in the 3'UTR of HLA-G influences the targeting of three miRNAs to this gene, suggesting that allele-specific targeting of these miRNAs could account for the observation that HLA-G is an asthma susceptibility gene (Nicolae et al. 2005). The immune response itself has the potential to regulate miRNA levels. IL-13, a major cytokine in the asthmatic airway has been shown to down-regulate the expression of miR-133a in bronchial smooth muscle cells, thereby altering the phenotype of these cells (Chiba et al. 2010). MicroRNAs can also be regulated by environmental factors such as cigarette smoke, pollutants and diet (Breving et al. 2010; Kulshreshtha et al. 2007; Schembri et al. 2009).

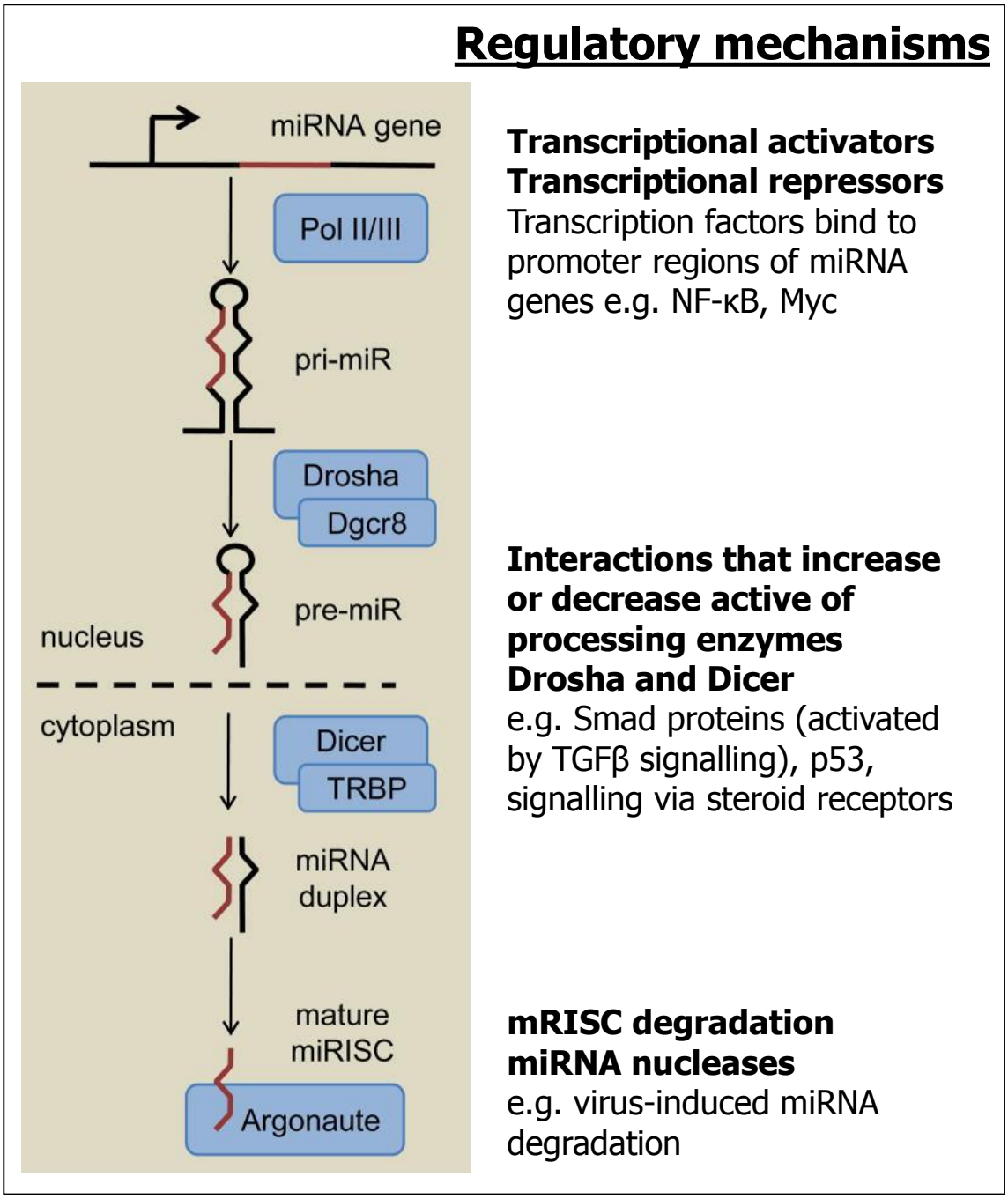


**Figure 1-8 Regulation of miRNA biogenesis and action**
Several transcription factors can bind promoters of miRNA genes to reduce expression of miRNA genes (e.g. Myc) or up-regulate the expression of miRNAs (e.g. NF-κ B, p53). SMAD proteins and p53 can directly interact with Drosha and Dicer to affect nuclear and cytoplasmic processing of miRNA precursors. Several intracellular signalling pathways can affect Argonaute stability, loading and localisation, thereby affecting miRNA activity. Finally miRNA turnover can be accelerated in certain situations (e.g. murine cytomegalovirus encodes factors that cause miRNA degradation). (Adapted from Bronevetsky and Ansel 2013)

### 1.5.4 Prediction of miRNA targets and experimental validation

A number of computational algorithms exist that can predict miRNA targets e.g. miRanda/miRbase (www.microna.org), PicTar (www.pictar.mdc-berlin.de) and TargetScan (www.targetscan.org). Each uses slightly different prediction criteria leading to the occasional lack of overlap in targets between the different methods (Rajewsky 2006). Therefore it is necessary to undertake experimental confirmation of miRNA targets.

Some of the commonly used principles of miRNA target recognition include:

i) The miRNA sequence (especially the 5'end) is complementary to the 3'UTR sequence of potential target mRNAs (Brodersen et al. 2009). Rarely, miRNAs can also base-pair to the 5'UTR and coding regions (Lee et al. 2009).
ii) Assessing the thermodynamic properties of the miRNA:mRNA duplex by calculating the free energy of the putative binding. If the free energy is low, the RNA duplex is in a more stable state thermodynamically, which means that the binding of the miRNA to the mRNA is stronger (Lewis et al. 2005). A stable miRNA:mRNA duplex will remain paired longer, giving the RISC proteins time to carry out their enzymatic activities.
iii) Accessibility of the target binding sites. In order to form a miRNA:mRNA duplex, RISC-associated miRNAs must gain access to the binding sites on the mRNA transcript (Hammell 2010).
iv) Clustering and position of binding sites within UTRs. Many targets contain tandem miRNA binding sites, spaced within 50 nucleotides of each other. Experimental assays have shown that tandem binding sites have a synergistic effect on the expression of the target, even when the binding sites respond to different miRNAs (Hon et al. 2007; Krek et al. 2005). Binding sites positioned at either end of the 3'UTR have also been reported to have increased efficacy (Grimson et al. 2007).
v) Interaction of other RNA-binding proteins can affect miRNA mediated regulation of the target mRNA (Kedde et al. 2007).

Despite the complexities of target prediction, the above mentioned bioinformatic tools frequently produce extremely long lists of targets for any

given miRNA. In validating a target, the first step is to ensure the miRNA and the target is present in the same cell *in vivo*. One commonly used technique for validating miRNA target genes is to either overexpress or inhibit the miRNA in the selected cell, assess if this corresponds to a predictable change in the amount of protein encoded by the target mRNA and demonstrate that this equates to changes in biological function. To complement this and confirm a direct molecular link between the miRNA and mRNA, the 3'UTR of the target mRNA can be cloned immediately downstream of a luciferase reporter gene. The recombinant plasmid, along with the miRNA of interest can then be transfected into a cell and luciferase activity or light emission measured. If the miRNA is able to bind to the target, reporter protein production will be repressed which can be measured and compared to a control (Figure 1-9).

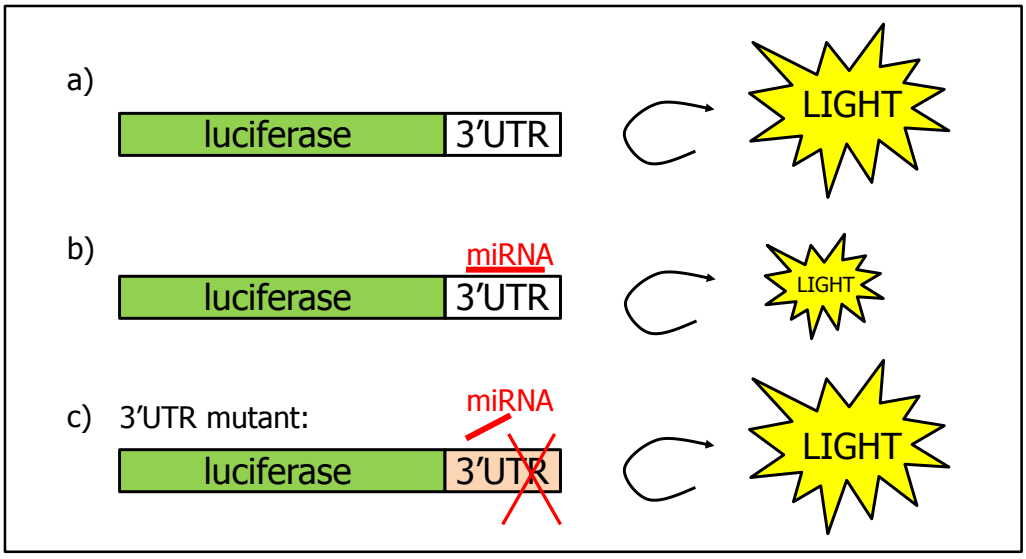


**Figure 1-9 Use of the luciferase assay to validate miRNA/mRNA interactions.**
The 3'UTR of the target gene is cloned into a luciferase reporter and the recombinant plasmid is transfected into a cell and luciferase activity can be assessed by measuring luminescence (a). In (b) the recombinant plasmid is transfected along with the miRNA of interest and the binding of the miRNA to its target within the 3'UTR will repress production of the luciferase reporter protein and less 'light' is produced. To further confirm the results, luciferase reporter constructs harbouring mutant versions of the 3'UTR can be co-transfected with the miRNA (c). Due to lack of sequence homology between the miRNA and the mRNA, the miRNA is unable to exert its inhibitory effects on the luciferase reporter protein and reduction in 'light' production is NOT observed.

### 1.5.5 MicroRNAs and asthma

In the last few years a number of studies have looked at the role of miRNA in asthma pathogenesis. Initial studies were unable to show differential miRNA expression between mild asthma and non-asthmatic controls, although it is likely that this may be due to the use of mixed cell populations in specimens studied (Williams et al. 2009). The study by Williams *et al* did show that the expression of over 200 miRNAs remained unchanged after a period of ICS treatment. Subsequent studies have studied individual airway cells, in particular BECs. Jardim *et al* showed differential expression of 66 miRNAs in patient with mild asthma (Jardim et al. 2012) and Solberg et al demonstrated changes in 217 miRNAs in steroid naïve asthmatics, with ICS treatment normalising only 9 of these (Solberg et al. 2012). This modest effect of steroids on normalising abnormalities in miRNA expression suggests miRNAs may be valuable therapeutic targets.

Airways smooth muscle cells (ASMs) play an important role in airway constriction and contribute to airway thickening and chronic inflammatory responses in asthma. At present no studies have examined asthmatic ASMs, but there are multiple reports on the function of individual miRNAs in isolated ASMs (Table 1-7). This includes a role for miR-140-3p (Jude et al. 2012) and miR-133a in contractility (Chiba et al. 2010), miR-26a in proliferation (Mohamed et al. 2010) and miR-221 in proliferation and the inflammatory response (Perry et al. 2014).

The functional relevance of individual miRNAs have also been assessed in a number of mouse models of asthma and are shown in Table 1-7.

| miRNA | Relevance in asthma | Targets |
|---|---|---|
| miR-126 | Increased expression in human asthmatic BECs (Wu et al. 2014)<br>Silencing reduces allergic inflammation in HDM-induced mouse model of asthma (Collison et al. 2011; Mattes et al. 2009) | Unknown |
| miR-221 | Upregulated in OVA-induced mouse models of asthma, silencing reduces allergic inflammation (Qin et al. 2012)<br>Regulates ASM hyperproliferation and release of IL-6 by ASM (Perry et al. 2014) | Spred-2 |
| miR-145 | Silencing reduces allergic inflammation in HDM-induced mouse model of asthma (Collison et al. 2011) | Unknown |
| miR-106a | Silencing reduces allergic inflammation in HDM-induced mouse model of asthma (Sharma et al. 2012) | IL-10 |
| Let-7a | Overexpression reduces allergic inflammation in OVA-induced mouse model of asthma (Kumar et al. 2011) | IL-13 |
| miR-21 | Increased in asthmatic cultured epithelial cells and also by IL-13 treatment of BECs (Wu et al. 2014)<br>Overexpressed in lung-specific IL-13 expressing transgenic mice (Lu et al. 2009)<br>OVA-induced model of asthma: miR-21 deficient mice show increased Th1 cytokines and reduced eosinophilia (Lu et al. 2011) | IL-12p35 |
| miR-146 | Reduced expression in CD8+ and CD4+ cells in patients receiving oral steroids (Tsitsiou et al. 2012) | Unknown |
| miR-133a | Downregulated by IL-13 in mouse bronchial smooth muscle cells; leads to augmented airway contraction and hyperresponsiveness (Chiba et al. 2010; Chiba et al. 2009) | RhoA |
| miR-1 | Downregulated by VEGF in lung endothelium<br>Administration of miR-1 inhibits inflammatory responses to OVA, HDS and IL-13 (Takyar et al. 2013) | Mpl |
| miR-26a | Upregulated by mechanical stretch; induces smooth muscle proliferation (Mohamed et al. 2010) | Glycogen synthase kinase-3β |
| miR-140-3p | Expression is reduced by TNFα leading to increased CD38 expression in airway smooth muscle cells; CD38 important in smooth muscle contractility (Jude et al. 2012) | CD38 |

**Table 1-7 Function and mechanism of action of some of the miRNAs found to be relevant to asthma**

Together, these studies demonstrate that a complex network of miRNAs can impact the development of asthma and contribute to the related inflammatory

processes. The specific role of miRNAs in disease exacerbations has not yet been explored.

## 1.6 Project rationale and Hypothesis

As discussed above asthma exacerbations are the major cause of the morbidity associated with the disease and patients with severe/difficult to treat asthma suffer recurrent exacerbations, most of which are caused by RV. In addition to having a deleterious effect on quality of life recurrent exacerbations are also associated with declining lung function and aberrant remodelling changes within the airways. It has been proposed that a defect in the innate immune response of airway cells to virus drives the vulnerability of asthmatic subjects to recurrent lower respiratory tract RV infection but the precise mechanisms underlying this defect are unclear and it is unknown if this is relevant in severe asthma.

**We hypothesised that**:

The differential expression of specific miRNAs in patients with severe asthma leads to reduced expression of TLR7 by their alveolar macrophages and a deficient IFN response against viral pathogens.

The project aims are to:

1) Identify miRNAs that are differentially expressed in asthmatic and healthy AMs
2) Confirm that a group of miRNAs that are differentially expressed in asthmatic AM target TLR7
3) Determine whether miRNA deregulation affects
    a. TLR7 expression in severe asthma
    b. TLR7 function in severe asthma
4) Explore whether TLR7 function can be restored, *ex vivo*, in AM from patients with severe asthma

# Chapter 2 Materials and Methods

## 2.1 List of materials used

### 2.1.1 List of reagents

Accutase® (#A6964, Sigma-Aldrich)

All Taqman® qPCR primers were from Applied Biosystems

Anti-Human CD3 FITC (#11-0038 eBioscience)

Anti-Human CD45 PE (#12-9459, eBioscience)

Anti-Human HLA-DR APC (#17-9956, eBioscience)

Anti-miR™ miRNA Inhibitors- hsa-miR-150

Anti-miR™ miRNA Inhibitors- hsa-miR-152

Anti-miR™ miRNA Inhibitors- hsa-miR-375

Anti-miR™ miRNA Inhibitors- Negative Control (#AM17010, Ambion)

Anti-mouse IgG-HRP (#NA931, GE Healthcare Life Sciences)

BCA Protein Assay Kit (#23225, Thermo Fisher Scientific Inc)

Blocking agent (#RPN2125, GE Healthcare Life Sciences)

Bromophenol blue (#114405 Sigma-Aldrich)

BSA (Bovine Serum Albumin) nuclease free (#B9001S, New England Biolabs)

Chemically Competent E. coli (Invitrogen)

Chloroform (#C2432, Sigma-Aldrich)

D-MEM Medium with GlutaMAX™-I (#61965-059, GIBCO, Invitrogen)

Dexamethasone (#D2915 SIGMA-ALDRICH)

Dual-Luciferase Reporter Assay System (#E1910, Promega)

ECL Advance Western Blotting Detection Kit (#RPN2135, GE Healthcare Life Sciences)

FBS (Foetal Bovine Serum) heat inactivated (#10108-165, GIBCO, Invitrogen)

Genopure Plasmid Maxi Kit (#03143422001, Roche Applied Science)

Glycerol (#G/0650/08 C35, Fisher Scientific)

Glycogen 20mg (#10901393001, Roche)

High Capacity cDNA Reverse Transcription Kit (#4374967, Applied Biosystems)

Imiquimod (#tlrl-imp, Source Bioscience, Invivogen)

Immobilon polyvinylidene difluoride membrane (#IPVH00010, Millipore Ltd.)

Isopropanol (#P/7505/17, Fisher Scientific)

LB Agar (#L2897 Sigma- Aldrich)

LB Broth (#L3022 Sigma-Aldrich)

Methacholine chloride 32mg/ml sterile inhalation challenge solution (Stockport Pharmaceuticals)

Methanol (#M/4056/17, Fisher Scientific)

Meso Scale Discovery Human IFN$\alpha$ ultra-sensitive (K151ACC-2)

Meso Scale Discovery Human IFN$\beta$ Tissue Culture (K151ADB-2)

Monoclonal anti-$\beta$-actin-peroxidase antibody (#A3854, Sigma-Aldrich)

Monoclonal mouse anti-TLR7 antibody for western blotting (#ab28048, Abcam)

NP40 Cell Lysis Buffer (#FNN0021, Invitrogen)

NuPAGE MOPS SDS Running buffer 20x (#NP0001, Invitrogen)

NuPAGE Novex 4-12% Bis-Tris Gel 1.0mm, 10 well (#NP0321BOX, Invitrogen)

NuPAGE Transfer buffer 20x (#NP00061, Invitrogen)

PBS (10010-056, Life Technologies)

Pen/Strep 100x (Penicillin-Streptomycin) liquid (#15070-063, GIBCO, Invitrogen)

Phenylmethanesulfonyl fluoride, PMSF (#P7626, Sigma-Aldrich)

Poly:IC (#tlrl-pic, Source Bioscience, Invivogen)

Protease Inhibitor (#P2714, Sigma Aldrich)

QIAquick Gel Extraction Kit (#28704, QIAGEN)

QIAquick Spin Miniprep Kit (#27104, QIAGEN)

Random hexamers (Applied Biosystems)

Recombinant Human IFN-γ (#285-IF-100, R&D Systems)

Recombinant Human IL-4 (#204-IL-010, R&D Systems)

Recombinant Human IL-13 (#213-ILB-005, R&D Systems)

Recombinant Human TNFα (#210-TA-005)

Restriction enzymes used were purchased from New England Biolabs (NEB)

RPMI Medium 1640 with GlutaMAX™-I (# 72400-054, GIBCO, Invitrogen)

Skin Prick Test reagents were from Allegopharma, Allergy Diagnostics Products, Diagenics

SmartLadder (#MW-1700-10, Eurogentec)

Sodium Azide (#S2002-100G, Sigma Aldrich)

Spectra™ Multicolour Broad Range Protein Ladder Lot 34744 (#SM1841, Fermentas)

SuperFect Trasfection Reagent (#301305, QIAGEN)

Taqman® Universal PCR MasterMix, No AmpErase UNG (#4364341, Applied Biosystems)

TRI-Reagent Solution (#AM9738, Applied Biosystems)

Tris Acetate EDTA (TAE) Buffer (#BP1332-1 Fisher Scientific)

Tris Base Ultra Pure (#BPE152-1 C131, Fisher Scientific)

Tris hydrochloride 1M pH7.5 (#BPE1757-500, Fisher Scientific)

Tween20, Molecular Grade (#41116134, Promega)

VeriKine™ Human Interferon Beta ELISA Kit (#41410-2, PBL Interferon Source)

Water, Ultrapure DNAse/RNAse-Free distilled water (#10977035, Invitrogen)

### 2.1.2 List of equipment

7900HT Fast Real-time PCR System (#4329001, Applied Biosystems)

DNA TETRAD™ (#TA001175, Esco Technologies Inc., USA)

Dry Wedge Bellow Spirometer R model (Vitalograph®)

FACSCalibur (BD Biosciences)

Jaeger Masterscreen with APS system and Viasys® Software (Care Fusion)

Luminometer

Nanodrop (Fisher Scientific)

SECTOR™ Imager 2400 (Meso Scale Discovery)

XCell *SureLock*® Mini-Cell and XCell II™ Blot Module (#E10002, Invitrogen)

### 2.1.3 Buffer recipies

FACS blocking solution: 10% FBS in PBS

Lysis Buffer (for western blotting): NP40 lysis buffer supplemented with 1 mM PMSF and protease inhibitor just prior to use

SDS-loading Buffer 4x: 8% SDS, 20% 2-mercaptoethanol, 40% glycerol, 0.008% bromophenol blue, 0.250M Tris HCl, pH 6.8

## 2.2 Patient recruitment and selection

Healthy subjects and subjects with asthma were recruited into the Medical Research Council funded study: Pathophysiology of airway diseases such as asthma and COPD. The study had been approved by the Southampton and South West Hampshire Local Research Ethics Committee REC number 05/Q1702/165. Samples from patients with mild asthma were kindly provided by Dr Christopher Grainge (currently Associate Professor, University of Newcastle Australia). Protocols for his study were approved by the Southampton and South West Hampshire Research Ethics Committee (approval number 08/H0502/6).

All subjects provided written informed consent.

General inclusion criteria included:

- Age 18-75
- For female patients: menopausal >2 years or using efficient contraception and having a negative pregnancy test
- Able to provide informed consent

Exclusion criteria for the study are detailed in Appendix 1.

**Patients with severe asthma**

Patients with severe asthma were recruited from the Difficult Airways Clinic held at University Hospitals Southampton Foundation Trust. All these patients required bronchoscopy and airway sampling in order to provide further information on airway inflammation as part of their clinical work-up. Patients consented to providing samples for research purposes, if tolerated, during the bronchoscopy.

Subjects were included in this arm of the study if they satisfied the following criteria

- Physician diagnosis of severe asthma
- ≥2 exacerbations in the last 12 months
- Step 4/5 of treatment based on the BTS Treatment of asthma
- If history of smoking present, then no evidence of emphysema on CT thorax

**Volunteers with moderate asthma**

Volunteers were recruited using the database of volunteers held by the respiratory department. They were required to have:

- Physician diagnosed asthma (> 1 year duration)
- Step 3 of treatment based on the BTS treatment of asthma
- Good asthma control as assessed by the Asthma Control Questionnaire (score ≤ 1)
- $PC_{20}$ to methacholine of <8mg/ml or
- Positive $PD_{20}$ to methacholine or
- ≥12% improvement in FEV1 following administration of salbutamol (if volunteers had historical evidence of any of these tests within the previous 12 months, they were not repeated)
- No history of smoking

**Healthy volunteers**

Volunteers were recruited by advertisement and using the database of volunteers held by the respiratory department.

Volunteers were required to have:

- No diagnosis of respiratory disease
- No significant response (defined as a drop in $FEV_1$ by ≥ 20%) to methacholine
- No history of smoking

**Volunteers with mild asthma**

Samples from these subjects were kindly provided by Dr Grainge. Volunteers were required to have:

- Physician diagnosed asthma
- Step 1 of treatment based on the BTS treatment of asthma
- Positive skin prick test to HDM
- $PC_{20}$ to methacholine of <8mg/ml

## 2.2.1 Baseline investigations

### 2.2.1.1 Methacholine challenge

Methacholine challenge testing (MCT) is a well-established method of assessing airway responsiveness and is used in asthma diagnostics and research. In subjects with symptomatic asthma, it has been found to have a negative predictive value of 100% (Cockcroft et al. 1992) and so it is also frequently employed when screening for healthy volunteers in asthma studies.

The five-breath standard dosimeter (SDM) protocol was performed for the large majority of subjects in this study as per recommendations of the American Thoracic Society (Crapo et al. 2000). In the SDM protocol incremental concentrations of methacholine are administered to calculate the concentration causing a 20% drop in $FEV_1$ in an individual ($PC_{20}FEV_1$). An alternative method for MCT is available called the aerosol provocation system (APS). In this method, incremental doses of methacholine are administered using a single concentration until there is a 20% drop in FEV1 ($PD_{20}$). It is less time consuming and dilution error can be avoided. The APS is widely used and has been shown to be suitable and reliable when compared to the SDM protocol (Schulze et al. 2009). For these reasons, in the latter part of the study, the APS was employed.

#### 2.2.1.1.1 Five-breath standard dosimeter protocol

Spirometry was measured using a dry wedge spirometer (Vitalograph). The initial step involved 5 breaths of 0.9% saline from an air driven nebuliser. $FEV_1$ was recorded 3 times at 1 and 3 minutes after saline inhalation and the best reading from each time point was recorded and used as the baseline value (for comparison when assessing drop in $FEV_1$). Doubling concentrations of methacholine were then delivered by an air driven nebuliser, starting with 0.03mg/l, to a maximum of 8mg/l. Again, $FEV_1$ was recorded at 1 and 3 minutes following methacholine inhalation. The challenge was stopped when the $FEV_1$ dropped ≥20% compared to the post-saline value and the concentration causing this drop was recorded as the $PC_{20}$.

#### 2.2.1.1.2 Aerosol provocation system

Spirometry was measured using the Jaeger Masterscreen with Viasys® software. The initial step involved inhaling 0.9% saline from the APS nebuliser. In this

system, dosing is breath activated and when the full dose is delivered, nebulisation will automatically stop. $FEV_1$ was recorded 2 times at 1 and 3 minutes after saline inhalation. The best reading from each time point was compared, and the lower of these two reading recorded. This was used as the baseline value. Increasing doses of methacholine were then administered by increasing nebulisation time. The APS nebuliser allows computer controlled production of aerosol and automatically determines the administered dose of methacholine based on nebulisation time. Again, $FEV_1$ was measured at 1 and 3 minutes post methacholine and the lower of the two highest values was recorded. The provocative dose that caused a 20% drop in $FEV_1$ was measured as the $PD_{20}$. In healthy subjects there was ≤20% drop in $FEV_1$ after the last administered dose which was recorded as a 'negative $PD_{20}$ test'.

#### 2.2.1.2 Skin prick testing

Skin prick testing was performed using stock solution of allergen extract from the following: *Aspergillus fumigatus*, *Alternaria tenius*, birch tree pollen, mixed grasses, mixed tree pollen, rape pollen, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, dog fur and cat dander. These were tested along with a positive (histamine) and negative (saline) control. A drop of the solution was placed on the skin of the volar aspect of the forearm and this was applied intradermally by pricking the skin with a sterile lancet (over the bead of the allergen extract). The maximum wheal diameter and the wheal diameter perpendicular to the former were measured in mm 15 minutes later. A positive test was defined as a wheal of 3x3mm greater than the saline negative control and subjects were deemed atopic if they had positive reaction to at least one of the aeroallergens tested.

#### 2.2.1.3 Asthma Control Questionnaire (ACQ) ©

The ACQ has been validated as a tool to assess the adequacy of asthma control (Juniper et al. 1999). It has been shown to have strong discriminative and evaluative properties and is very sensitive to within-patient change in asthma control over time. It has 7 questions and patients are asked to recall how their asthma has been during the previous week. The response to all 7 questions is on a 7-point scale and the ACQ score is the mean of the 7 items. In general, patients with a score ≤1 have adequately controlled asthma and a change or

difference in score of 0.5 is considered clinically important. The ACQ is shown in Appendix 2.

# 2.3 Bronchoscopy and isolation of AM

Bronchoscopy was performed according to BTS guidelines (BTS Bronchoscopy Guideline Committee, 2001) and the local departmental standard operating procedure in the Southampton Centre of Biomedical Research (SCBR).

BAL was performed by instilling 120mls (6 x 20ml aliquots) pre-warmed (37 ˚C) normal saline into a segment of the right upper lobe and recollecting the fluid by suction into a bronchial lavage fluid trap. Some patients with severe asthma could not tolerate the entire 120mls and in these cases, the procedure was stopped after 80mls.

## 2.3.1 BAL processing

The initial processing of the BAL fluid was performed by Dr Laurie Lau (research scientist, Division of Clinical and Experimental Sciences, University of Southampton) so that the procedure could be standardised for all the bronchoscopies done in the department. Briefly, BAL fluid was filtered using a 100µm nylon filter and then centrifuged at 1300G for 10 mins at 4˚C. The supernatant was removed, aliquoted and stored at -80˚C for later analysis. The cells were resuspended in PBS and the first cell count was performed using a Neubauer hemocytometer and the trypan blue exclusion method was used to assess for cell viability. An aliquot of cells was used to prepare cytocentrifuge slides. One slide was used to obtain a differential cell count by staining with rapid Romanowsky stain (this was performed by Dr Jon Ward, research scientist Histochemistry Research Unit). The remaining slides were stored at -80˚C for later analysis.

## 2.3.2 Isolation of AM

30mls of RPMI 1640 with GlutaMAX™ was added to the remaining cells and they were centrifuged at 1500rpm for 10 minutes. The supernatant was discarded and a repeat cell count was performed using a Neubauer haemocytometer. Cells were resuspended in RPMI 1640 with GlutaMAX™ at $1x10^6$ cells/ml and depending on their use, the cell suspension was either placed in 96-well plates using 200µl/well ($2x10^5$ cells/well) or 24-well plates using 1ml/well ($1x10^6$ cells/well). The plates were then placed at 37˚C in a humidified 5% $CO_2$ incubator for 2 hours. AM are known to adhere to plastic

and so after 2 hours, the media was gently removed and adhering AM were carefully washed with PBS.

At this point AM were either:

1. Collected immediately in TRI-Reagent (for RNA isolation) and NP-40 protein lysis buffer (for use in western blotting) in order to assess the baseline expression of cellular miRNAs and genes (eg. TLR7). Samples were stored at -80˚C until used.
2. Cultured at 37˚C in a humidified 5% $CO_2$ incubator for a period of 24-72 hours in RPMI 1640 with GlutaMAX™ supplemented with 10% heat inactivated FBS, 50 IU/ml penicillin and 50 IUmg/ml streptomycin, depending on the experiment.

### 2.3.3 Evaluation of AM purity

The purity of adherent BAL cells was checked by flow cytometry and FlowJo Software. Adherent cells were gently detached using Accutase® which enables cell detachment without affecting cell surface markers. Cells were blocked in FACS Blocking Solution for 15 minutes at 4˚C and then incubated with Anti-human HLA-DR APC, Anti-human CD3 FITC, Anti-human CD45 PE and isotype control antibodies for 40 minutes at 4˚C. After washing cells were analysed by flow cytometry.

# 2.4 RNA extraction

RNA was extracted with TRI-Reagent (Ambion) using 500µl/well for 24 wells plates or 200µl/well for 96 well plates. The procedure was performed according to the manufacturer's guidelines. Briefly, cells were lysed in Tri-Reagent by passing the lysate several times through the pipette tips. Lysates were incubated at room temperature for approximately 5 minutes and then either stored at -80˚C until further use, or processed straight away.

## 2.4.1 Phase separation

Chloroform was added in the measure of 20% compared to the volume of TRI-Reagent used: 100µl chloroform for 500µl TRI-Reagent or 40µl chloroform for 200µl TRI-Reagent. The tube was vortexed vigorously for 15 seconds and incubated at room temperature for 5 minutes. It was then centrifuged at 10,000 rpm in a bench top centrifuge at 4˚C for 20 minutes. This allowed the mixture to separate into a lower red phenol-chloroform phase, an interface and a colourless upper aqueous phase. RNA is contained exclusively in the aqueous phase, while DNA and protein are in the underlying 2 layers. This aqueous phase (which is normally about 50% of the volume of TRI-Reagent originally used) was carefully removed and placed into a fresh eppendorf.

## 2.4.2 RNA precipitation

RNA was precipitated by adding 100% isopropanol in the measure of 50% compared to the aqueous phase. 5-10µg of glycogen was added to help in the visualisation of the precipitate as glycogen co-precipitates with the RNA. The sample was vortexed for 15 seconds, then placed at -20˚C for at least 20 minutes, after which it was centrifuged at maximum seed in a bench top centrifuge at 4˚C for 30 minutes. At this point the RNA is visible as a pellet at the bottom of the tube.

## 2.4.3 RNA wash

After removing the supernatant from the tube, the pellet was washed with 1ml of chilled 75% ethanol, vortexed briefly and then centrifuged again at maximum speed, at 4˚C for 15 minutes. After discarding the supernatant, the pellet was allowed to air-dry for 5-10 minutes and then resuspended in 20-50µl

of RNAse free water depending on whether the sample had been collected from a 96-well or 24-well plate.

RNA samples were quantified using the spectrophotometer Nanodrop 1000. RNA was then stored at -80˚C.

# 2.5 Reverse Transcription (RT) and Real Time PCR (qPCR) analysis

## 2.5.1 mRNA RT

mRNA RT was performed using a High Capacity cDNA Reverse Transcription Kit following manufacturer's instructions. 200ng to 500ng of total RNA was used in the RT reaction (this amount was kept consistent for samples that were compared to each other) and it was performed using Random Hexamer primers. The recipe for the reaction and the RT protocol employed is shown in Table 2-1.

| **Reverse transcription recipe** | | | **Reverse transcription thermal cycling condition** | |
|---|---|---|---|---|
| 10x RT buffer | 1.00µl | | Step 1 | 10 mins at 25˚C |
| dNTPs (100mM) | 0.40µl | | Step 2 | 2h at 37˚C |
| 10x Random hexamers | 1.00µl | | Step 3 | 5 min at 85˚C |
| RNAse inhib (20U/µl) | 0.50µl | | Step 4 | Hold at 15˚C until samples removed |
| Multiscribe (RT enzyme) | 0.50µl | | | |
| RNA (200ng) + water | 6.60µl | | | |
| Total | 10.00µl | | | |

**Table 2-1 mRNA RT recipe and thermal cycling conditions**

After RT, the resulting cDNA was diluted 1:10 and stored at -20˚C until qPCR was performed.

## 2.5.2 MicroRNA RT

For the detection of miRNAs, 5ng of total RNA was used with the High Capacity cDNA Reverse Transcription kit with specific stem loop primers for each of the miRNAs being measured and RNU44 which was used as a housekeeping gene. The recipe for the reaction and the RT protocol employed is shown in Table 2-2.

| Reverse transcription recipe | | | Reverse transcription thermal cycling condition | |
|---|---|---|---|---|
| 10x RT buffer | 0.75µl | | Step 1 | 30 mins at 16˚C |
| dNTPs (100mM) | 0.08µl | | Step 2 | 30 mins at 42˚C |
| 5x specific primer | 1.50µl | | Step 3 | 5 min at 85˚C |
| RNAse inhib (20U/µl) | 0.10µl | | Step 4 | Hold at 15˚C until samples removed |
| Multiscribe (RT enzyme) | 0.50µl | | | |
| Water | 4.08µl | | | |
| RNA (10ng/ul) | 0.50µl | | | |
| Total | 7.50µl | | | |

**Table 2-2 MiRNA RT recipe and thermal cycling conditions**

After RT, cDNA was stored at -20˚C or used immediately for qPCR.

### 2.5.3 Real Time PCR

Real time PCR follows the general principle of PCR and its key feature is that the amplified DNA is detected as the reaction progresses in real time. PCR is based on thermal cycling: repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. By adding primers (both forward and reverse) that are complimentary to the sequence of interest in the DNA (or cDNA constructed in the RT reaction), a target gene can be amplified by the DNA polymerase enzyme. Initially the temperature is raised, denaturing the dsDNA into individual strands, following which the reaction is cooled to allow primer annealing. Each strand is used as a template for DNA synthesis by the DNA polymerase. This cycle of temperature change is repeated 40-50 times giving an exponential amplification of the DNA of interest.

In qPCR, the amount of DNA product produced is measured in real time by the use of a fluorescent probe. A number of different fluorescent probes exist, in this project Taqman® probes were employed (Figure 2-1).

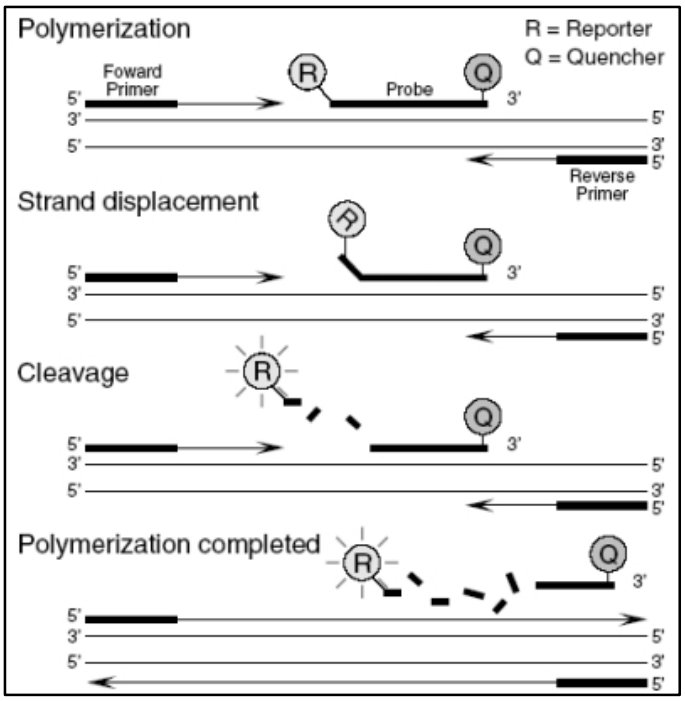


**Figure 2-1 Taqman® assay chemistry:**
The Taqman® probe bears a fluorescent reporter dye (R) at the 5'end and a nonfluorescent quencher (NFQ- Q) at the 3'end, rendering the molecule non-fluorescent. During amplification the probe binds specifically to a complementary sequence on the template between the forward and reverse primer sites. The DNA polymerase enzyme also has 3'exonuclease activity. It only cleaves probes that are hybridised to the target resulting in the separation of the reporter dye from the NFQ. This renders it free to fluoresce.

Reactions were performed following manufacturer's instructions and using Taqman® Universal PCR Master Mix, No AmpErase UNG on the Real-time PCR machine. Standard Taqman® thermal cycling conditions were used as shown in Table 2-3.

| | Temperature (°C) | Time | Function |
|---|---|---|---|
| | 95 | 10 min | Denaturing and enzyme activation |
| 40 cycles | 95 | 15 sec | Denaturing |
| | 60 | 1 min | Annealing/ extension/ data collection |

**Table 2-3 Taqman® thermal cycling conditions**

Fold differences in gene expression were calculated using the comparative $C_T$ method. The Ct value is the cycle number at which the exponential amplification curve (of fluorescence) crosses the threshold mark. A difference

between two Ct values of 1 unit (=1 PCR cycle) represents a 2 fold difference in the expression of the gene and of 2 units, a four-fold difference in gene expression. The ΔCT value is the result of the gene of interest Ct value minus the Ct value of the housekeeping gene (eg. GAPDH). The housekeeping gene is normally stably expressed in each cell. The ΔΔCT is the ΔCT of the sample of interest relative to the baseline/control sample. Relative gene expression or fold induction can then be calculated by exponential transformation of the ΔΔCT value: $2^{-(\Delta\Delta CT)}$. For miRNA expression evaluation the housekeeping gene used was RNU44 while for cellular gene expression it was GAPDH. These choices were based on experience within our research group and previous work showing stable expression in macrophages. The expression of RNU44 was also compared to other potential housekeeping genes e.g. RNU42 and found to be superior in terms of stable expression in AM.

The recipes for qPCR reactions are shown in Table 1-4:

| | **mRNA qPCR** | **miRNA qPCR** |
|---|---|---|
| qPCR mastermix (2x) | 2.50µl | 2.50µl |
| Taqman® primer | 0.25µl | 0.25µl |
| Water | 1.00µl | 1.90µl |
| cDNA | 1.25µl | 0.35µl |
| Total | 5.00µl | 5.00µl |

**Table 2-4 Recipes for mRNA and miRNA qPCR.**
Note in the qPCR for mRNA the cDNA is diluted 1:10, while in the miRNA qPCR undiluted cDNA is used.

# 2.6 MicroRNA microarrays

The Applied Biosystems Taqman® Low Density Array system was used following the manufacturer's instructions. A set of two microfluidic cards (Array A and Array B) were used which enabled quantification of up to 380 miRNAs and controls each. Briefly, 400-600ng of total cellular RNA (extracted as described above), was used to generate cDNA using the Megaplex™ RT Primers (Human Pool A and Pool B). These pools contain up to 380 RT-primers each and enable the simultaneous synthesis of cDNA that can be loaded onto the array card for PCR amplification and real-time analysis. If the total amount of available RNA was less that 400ng, a preamplification step was carried out prior to the qPCR as per the manufacturer's instructions. The components of the RT reaction and the thermal cycling parameters were:

| **RT reaction mix components** | |
|---|---|
| Megaplex RT primers (10x) | 0.80µl |
| dNTPs (100mM) | 0.20µl |
| MultiScribe Reverse Transcriptase (50U/µl) | 1.50µl |
| 10x RT Buffer | 0.80µl |
| MgCl2 (25 mM) | 0.90µl |
| RNase Inhibitor (20U/µl) | 0.10µl |
| RNase free water | 0.20µl |
| RNA (total 400-600ng) | 3.00µl |
| Total | 7.50µl |

| **Thermal Cycling conditions** | | |
|---|---|---|
| Stage | Temp | Time |
| 40 cycles | 16˚C | 2 min |
| | 42˚C | 1 min |
| | 50˚C | 1 sec |
| Hold | 85˚C | 5 min |
| Hold | 4˚C | ∞ |

**Table 2-5 RT reaction for microarrays**

If preamplification was required, the components of the mix and the thermal cycling conditions are shown is Table 2-6. The preamplification product was diluted by adding 75µl of 0.1X TE buffer pH 8.0. Real-time PCR was then carried out on the RT product/preamplified product and the components of that reaction are shown in Table 2-7. The array was run using the 384-well Taqman® Low Densitiy Array default thermal-cycling conditions.

| PreAmp reaction mix components | |
|---|---|
| Taqman® PreAmp Mastermix (2x) | 12.5µl |
| Megaplex PreAmp Primers (10x) | 2.5µl |
| RNase free water | 7.5µl |
| RT product | 2.5µl |

| Thermal cycling parameters: | | |
|---|---|---|
| Stage | Temp | Time |
| Hold | 95˚C | 10 min |
| Hold | 55˚C | 2 min |
| Hold | 72˚C | 2 min |
| 12 cylces | 95˚C | 15 sec |
| | 60˚C | 4 min |
| Hold | 99.9˚C | 10 min |
| Hold | 4˚c | ∞ |

**Table 2-6 Preaplification step for microarrays**

| Component | Volume |
|---|---|
| Taqman® Universal PCR Master Mix, No AmpErase® UNG, 2x | 450µl |
| Diluted PreAmp Product or RT product | 9µl or 6µl |
| RNase free water | 441µl or 444µl |
| Total | 900µl |

**Table 2-7 Microarray qPCR reaction components**

Arrays were carried out on 4 patients with mild asthma and 4 healthy subjects. Analysis was carried out using Excel. For the analysis, all the miRNAs that had a Ct value higher than 35 in 2 or more of the 4 samples (from a either mild asthma or healthy subjects) were discarded. For each of the remaining miRNAs, the mean Ct value, of the 4 samples, was calculated. These mean Ct values were compared between the 2 clinical states and miRNAs that had a cycle difference of ≤1 unit were discarded.

## 2.6.1 Bioinformatic analysis

The prediction programme Targetscan 4.2 (http://www.targetscan.org/vert_42/) was used with default options. Each miRNA highlighted by the arrays was searched and a list of predicted targets generated. These lists were carefully assessed manually, in order to highlight PRRs involved in the innate immune response to RV in human cells (TLR3, 7 and 8, RIG-1 and MDA5). Particular interest was focused on proteins predicted to be targeted by more than 1 miRNA highlighted by the arrays. We then cross-referenced miRNAs and targets of interest in another online prediction tool- miRanda (http://www.microrna.org/microrna/getMirnaForm.do) to ensure target predictions were present in more than one database.

# 2.7 Protein Detection

## 2.7.1 Western Blotting

Cells were lysed in NP-40 lysis buffer (supplemented at the time of use with PMSF and protease inhibitor cocktail) on ice and then frozen immediately at -80˚C until use. On the day of use, lysates were centrifuged at 1300 rpm in a bench top centrifuge at 4˚C. Supernatants were collected and quantified using the BCA assay following manufacturer's instructions. 40-50µg of cell lysates were mixed with 4x SDS loading buffer (containing beta-mecaptoethanol), placed in boiling water for 5 minutes and then immediately on ice prior to use.

For electrophoresis of the samples, precast gels were used: NuPage Novex 4-12% Bis-Tris Gel 1.0mm, 10well, in the manufacturer's electrophoresis system XCell *SureLock*™ Mini-cell. The samples were run using NuPage MOPS SDS Running Buffer at 120V until stacked, after which the voltage was increased to 150V until completion. Protein samples were then transferred onto an Immobilon polyvinylidene difluoride membrane during 90min at 30V using the Xcell II™ Blot Module within the XCell *SureLock*™ Mini-Cell using : NuPage Transfer Buffer made up using 20% methanol. The membrane was then incubated in a blocking solution composed of 2% (w/v) ECL Advance Blocking Agent in PBS-0.1% Tween for 1 hour at room temperature. Following this they were incubated overnight at 4˚C with the primary antibody in the blocking solution.

The membrane was washed 3 times in PBS-0.1% Tween and then incubated for 1 hour at room temperature with the horseradish peroxidase-conjugated secondary antibody in blocking solution. After 3 successive washes with PBS-0.1% Tween, protein detection was performed using an ECL Advance Western Blotting Detection Kit. The membrane was then washed again in PBS-0.1% Tween after which it was incubated for 1 hour at room temperature with an anti-β-actin-horseradish peroxidase conjugated antibody. Again, 3 washes were performed and protein detection performed as previously described. The membrane was not stripped prior to visualisation of actin because the protein of interest (TLR7) is much bigger in size compared to actin and so would not interfere with the detection of the house-keeping protein.

### 2.7.2 Enzyme-linked immunoabsorbant assay (ELISA)

Verikine™ Human IFNβ ELISA kit (assay range 25-2000 pg/ml) was used which employs a sandwich immunoassay. This was done using manufacturer's instructions and standards, samples and blanks were run in duplicate. Wash buffer, antibody solution, HRP solution TMB substrate and Stop solution were supplied in the kit. Briefly, 100µl of interferon standards, samples and blanks was added onto the pre-coated microtiter plate and incubated for 1 hour. The wells were then washed 3 times after which 100µl of antibody solution was added to the wells and the plate covered. After 1 hour the contents of the plate were emptied, wells washed 3 times in the wash buffer and then incubated for 1 hour with 100µl of HRP solution. Following 3 washes, 100µl of TMB substrate was added to each well and the plate was incubated in the dark for 15 minutes. Finally, 100µl of Stop solution was added to each well and the plate read using a microplate reader set at an absorbance of 450nm. By plotting the optical densities using a 4-parameter fit for the standard curve, IFNβ concentrations in the samples were determined. To convert from units/ml to pg/nl a conversion factor of about 10-25 pg/units is applicable.

### 2.7.3 Meso Scale Discovery (MSD®) Cytokine Assay

The MSD Assay is an electrochemiluminescence immunoassay and was used to measure IFNβ and IFNα levels in cell supernatants. It was used in preference to standard ELISA because much smaller sample volumes are required. Like a standard ELISA it employs a sandwich immunoassay format. The cytokine in the sample binds to capture antibody immobilised at the bottom of the plate. The labelled detection antibody binds to the cytokine to complete the sandwich and addition of the read buffer emits electrochemiluminescence signals.

Briefly, the supplied plate was washed with PBS and 50µl of sample added. The plate was incubated for 2 hour with vigorous shaking (1000 rpm) after which it was washed and the detection antibody solution added. Again the plate was incubated for 2 hours with vigorous shaking, followed by 3 washes and addition of the read buffer. The plates were read immediately on a SECTOR Imager. For the IFNβ assay, an additional first step was required and involved incubating the plate for 1 hour with the capture antibody. Standards, samples and blanks were run in duplicate. The lower limit of detection was 0.7pg/ml and 34pg/ml for IFNα and IFNβ respectively.

# 2.8 Infection and Transfection of AM

## 2.8.1 Virus Culture

RV16 was a gift from Dr Jens Madsen (Faculty of Medicine, University of Southampton) and was grown for use by our research group by Dr Victor Bondanese (Research Fellow, Faculty of Medicine, University of Southampton). RV16 was the only strain used in this thesis and all further mention of RV in our experimental work refers to RV16.

## 2.8.2 Infection

After AM were washed with PBS and culture media added, they were treated with imiquimod, a synthetic TLR7 agonist, RV and poly:IC, synthetic dsRNA that stimulates TLR3, RIG-1 and MDA5. The concentrations used were as follows:

| | Concentration |
|---|---|
| Imiquimod | 5 µg/ml |
| RV | MOI 0.6 |
| PolyI:C | 10 µg/ml |

After 24 hours the supernatant was carefully removed and the adherent cells lysed with TRI-Reagent for RNA extraction. Both the supernatant and lysed cells were stored at -80˚C until use.

## 2.8.3 Transfection + Infection

AM were transfected with a combination of 3 anti-miRs- individual concentration 50nM, cumulative concentration 150nM, for 48 hours. A scrambled control was also used at a concentration of 150nM. No transfection reagent was necessary as these cells are transfected by natural phagocytosis or passive diffusion. After 48 hours, cells were treated with imiquimod and RV, and if sufficient cells were present, polyI:C at the concentration described above. After 24 hours the supernatant was collected and Tri-Reagent added to the adhering AM and samples frozen at -80˚C until use. Transfection efficiency was checked by assessing expression of the specific miRNAs in transfected vs. mock-transfected cells.

### 2.8.4 Transfection with pre-miRs

To evaluate miRNA induced changes in TLR7 protein expression healthy AM were transfected with pre-miR-150, pre-miR-152 and pre-miR-375 (500nM each) or scrambled control. Pre-miRs are chemically modified RNA molecules designed to mimic endogenous mature miRNAs. As before, no transfection reagent was required. At 48 hours AM were lysed in NP-40 protein lysis buffer (supplemented with PMSF and protease inhibitor cocktail) and the lysate stored at -80˚C until use. Western Blotting was performed as previously described.

# 2.9 Investigating the role of the asthmatic environment on miRNA and TLR7 expression

## 2.9.1 Investigating the effect of the asthmatic microenvironment

Healthy AM were exposed to BAL fluid from 10 subjects with SA which had been previously obtained and stored at -80˚C. Briefly, BAL recovered at bronchoscopy was washed, spun and the cell pellet used for recovery of AM while the supernatant fluid stored (in aliquots). Each aliquot was only thawed once and 100ul applied onto adherent healthy AM along with 100µl RPMI+4%FCS (with antibiotics, P/S) to give a final concentration of 2% FCS. This was done to avoid induction of intracellular pathways by components of FCS. After 24 hours cells were collected in Tri-Reagent for RNA extraction.

In addition, healthy AM were also exposed to IL-4, IL-13, TNF$\alpha$ and IFN$\gamma$ (all 10ng/ml) in RPMI+2%FCS (+P/S) for 24 hours and then collected in Tri-Reagent.

AM from 6 healthy subjects were used for these experiments.

## 2.9.2 Investigating the effect of steroids

ICS are the mainstay of treatment in asthma. Patients with severe asthma are also frequently on oral steroids, either continuously or recurrent courses. In order to investigate the effect of steroids on miRNAs and TLR7 2 methods were employed:

1. A group of well-controlled moderate asthmatics were recruited (as described above). These patients were on continuous ICS, but unlike patients with severe asthma they had well controlled asthma. This was the *in vivo* comparison group.
2. *In vitro*: AM from healthy subjects were treated with dexamethasone at 3 concentrations ($10^{-6}$, $10^{-7}$ and $10^{-8}$) and incubated at 37˚C with 5% $CO_2$. Cells were collected at 24 hours in TRI-Reagent and stored at -80˚C until analysis. AM from 7 healthy subjects were used for these experiments.

# 2.10 Cloning

## 2.10.1 Generation of vectors

**pCDNA3.1_150**: This vector contains the coding region for miR-150. To clone it, genomic DNA was amplified by PCR and cloned into the pCR2.1-TOPO® vector using the TOPO® TA Cloning kit and called pCR2.1_150 (Figure 2-2). The primers employed were:

Forward: 5' GGA TCC TGG GTA TAA GGC AGG GAC TGG G

Reverse: 3' CTC GAG AGC AGA GAT GGG AGT ACA GGG

These were designed in order to clone the restriction sites BamHI at the 5' end and XhoI at the 3'end (highlighted bases) which will be required later when constructing a vector containing coding regions for all 3 miRNA. pCR2.1_150 was checked by sequencing and then excised at Xba and Hind III sites (pre-existing within the vector). This fragment was subcloned into Xba/Hind III sites of pCDNA3.1 (-) using T4 DNA Ligase overnight at 16 ˚C.

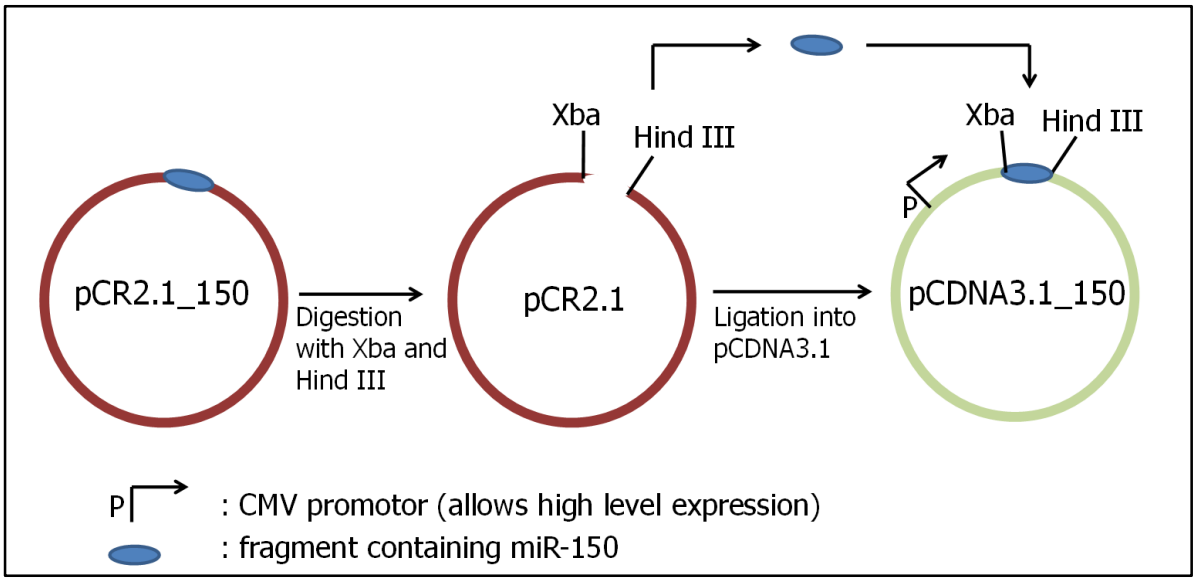


**Figure 2-2 Cloning steps to obtain the mir-150 expression vector construct.**
The genomic region encompassing miR-150 was cloned into pCR2.1-TOPO® vector= pCR2.1_150. This was digested with Xba/Hind III enzymes and the excised fragment was cloned into pCDNA3.1 (-) leading to the contruct pCDNA3.1_150 (vector represented on the right).

**pCDNA3.1_152**: This vector contains the coding region for miR-152. To clone it, genomic DNA was amplified by PCR and cloned into the pCR2.1-TOPO® vector using the TOPO® TA Cloning kit and called pCR2.1_152 (Figure 2-3). The primers employed were:

Forward: 5' CTC GAG CCG GCC AGG GAT CAG CTG G

Reverse: 3' GGT ACC ACG CGT GAG TGG GCG CTG TGC CCG TTG GG

These were designed in order to clone the restriction sites XhoI at the 5' end and KpnI at the 3'end (highlighted bases). pCR2.1_152 was checked by sequencing and then excised at these sites (XhoI and KpnI). This fragment was subcloned into XhoI/KpnI sites of pCDNA3.1 (-) using T4 DNA Ligase overnight at 16 ˚C.

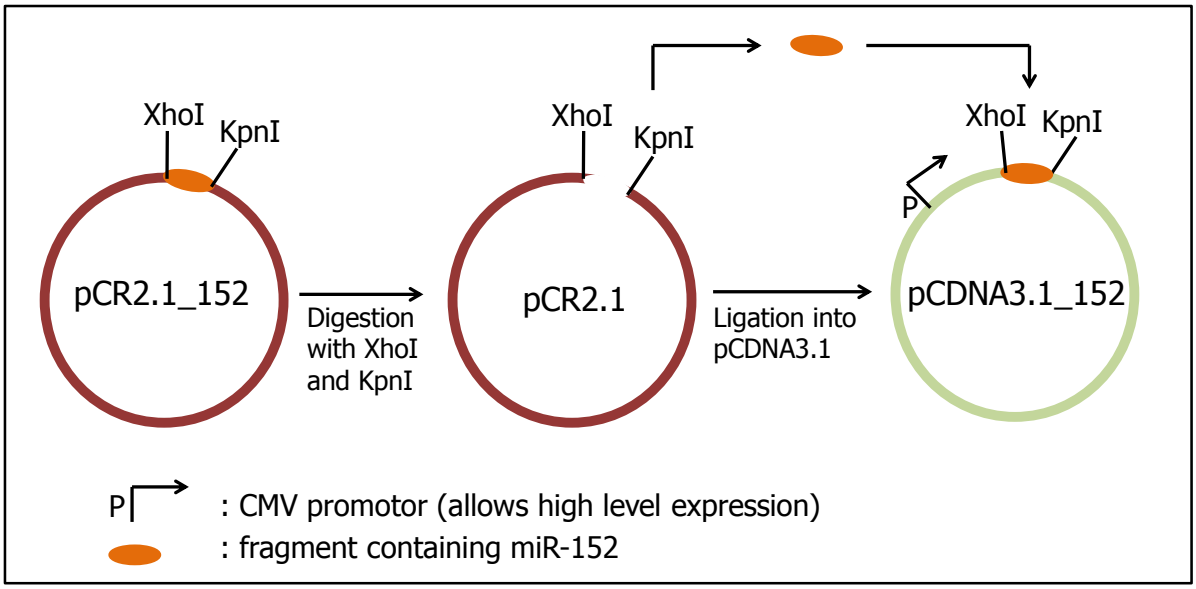


**Figure 2-3 Cloning steps to obtain the mir-152 expression vector construct.**
The genomic region encompassing miR-152 was cloned into pCR2.1-TOPO® vector= pCR2.1_152. This was digested with XhoI/KpnI enzymes and the excised fragment was cloned into pCDNA3.1 (-) leading to the contruct pCDNA3.1_152 (vector represented on the right).

**pCDNA3.1_375**: This vector contains the coding region for miR-375. To clone it, genomic DNA was amplified by PCR and cloned into the pCR2.1-TOPO® vector using the TOPO® TA Cloning kit and called pCR2.1_375 (Figure 1-4). The primers employed were:

Forward: AAG CTT TCT AGA GAC CAG GAG ATC ACC GAG GG

Reverse: GGA TCC GGT GCC TGC GTG GCG ATC AGG C

These were designed in order to clone the restriction sites XbaI at the 5' end and BamHI at the 3'end (highlighted bases). An additional site for the restriction enzyme HindIII was also inserted at the 5' of XbaI. pCR2.1_375 was checked by sequencing and then excised at the sites XbaI and BamHI. This fragment was subcloned into XbaI/BamHI sites of pCDNA3.1 (-) using T4 DNA Ligase overnight at 16 ˚C.

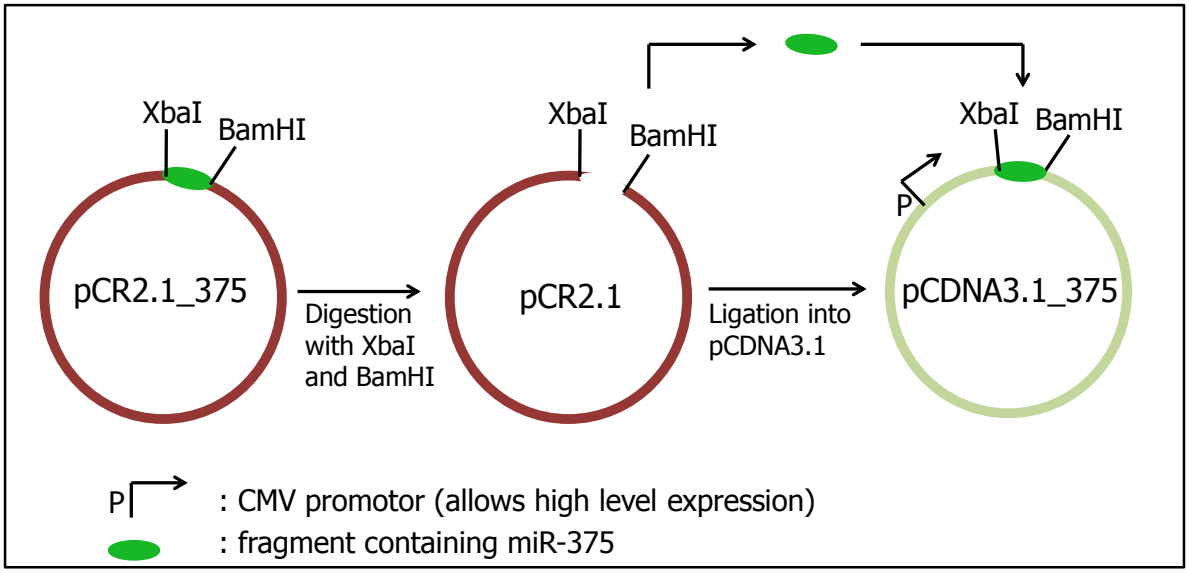


**Figure 2-4 Cloning steps to obtain the mir-375 expression vector construct.**
The genomic region encompassing miR-375 was cloned into pCR2.1-TOPO® vector= pCR2.1_375. This was digested with XbaI/BamHI enzymes and the excised fragment was cloned into pCDNA3.1 (-) leading to the contruct pCDNA3.1_375 (vector represented on the right).

**pCDNA3.1_mix**: This vector contains the coding regions for miR-150, miR-152 and miR-375. In order to construct this vector pCR2.1_375 was digested with XbaI and BamHI, pCR2.1_150 was digested with BamHI and XhoI and pCR2.1_152 was digested with XhoI and KpnI. This led to the release of fragments containing miR-375, miR-150 and miR-152 respectively (Figure 2-5). pCDNA3.1(-) was digested with XbaI and KpnI thereby 'opening' it up and a 'quadruple' ligation was carried out overnight at 16˚C using T4 DNA ligase.

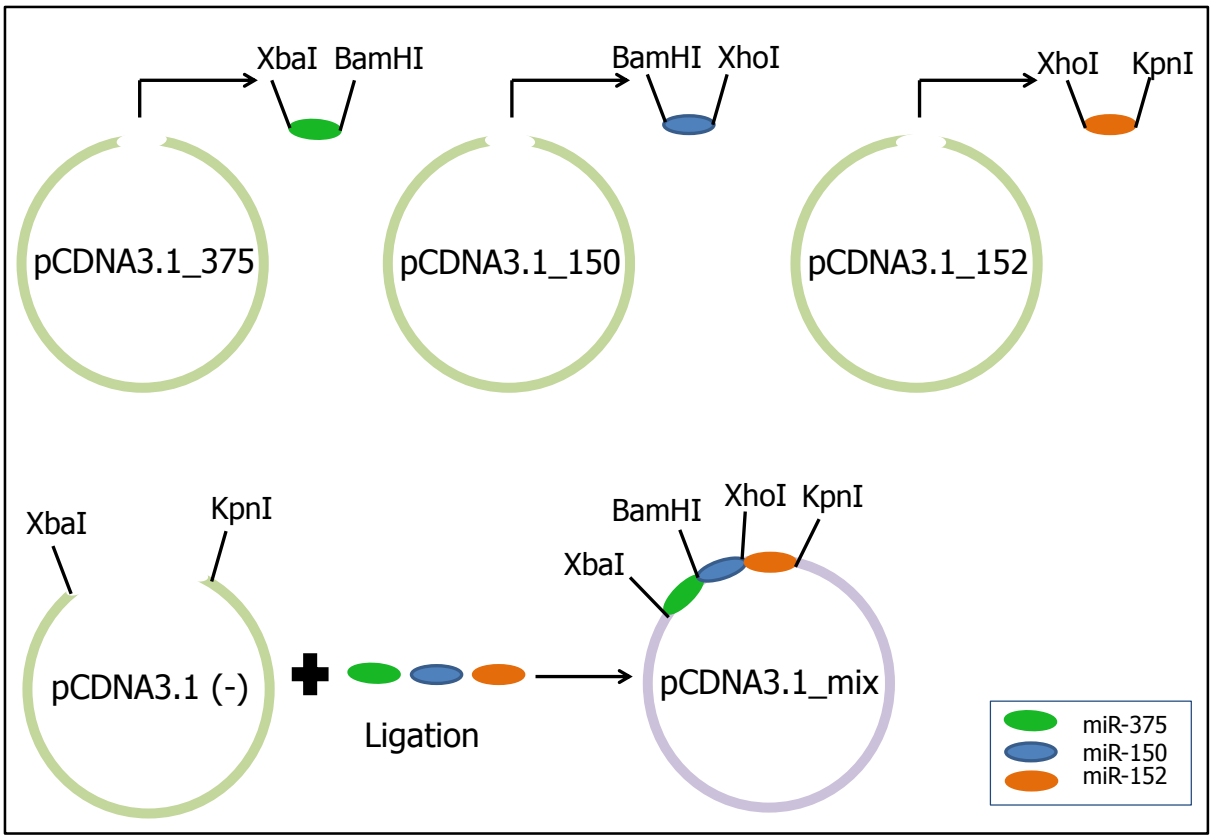


**Figure 2-5 Construction of vector containing all three miRNAs- termed pCDNA3.1_mix**

**pRLTK_3'UTR_TLR7**: This vector contains the 3'UTR of TLR and a cDNA encoding Renilla luciferase which is an enzyme that can function as a genetic reporter following cellular translation. The pRL-TK vector contains the herpes simplex virus thymidine kinase (HSV-TK) promoter which provides low to moderate levels of Renila luciferase expression in co-transfected cell. The 3'UTR of TLR7 was amplified from genomic DNA by PCR using the following primers:

Forward: TCT AGA CCA TAT TTC AGG GGA GCC ACC AA

Reverse: GCG GCC GCG GAA AAT ACG ACA TCG CCA ATC TAA

The primers were designed in order to clone the restriction sites XbaI at the 5' end and NotI at the 3'end (highlighted bases). The amplified region was cloned into the vector pCR2.1-TOPO® using the TOPO®TA Cloning Kit and called pCR2.1_3'UTR_TLR7 (Figure 2-6). Clones were checked by sequencing. pCR2.1_3'UTR_TLR7 was digested by XBA I and Not I restriction enzymes and

the excised fragment was subcloned into the reporter vector pRL-TK. These were ligated overnight with T4 DNA Ligase at 16˚C and the resulting vector was called pRLTK_3'UTR_TLR7.

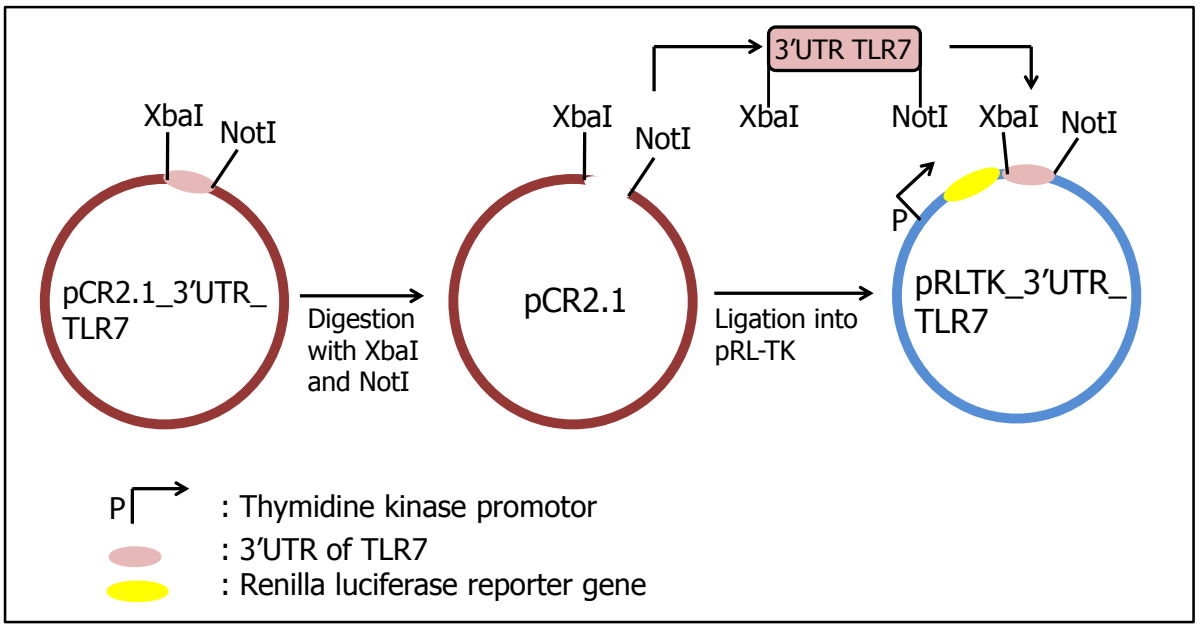


**Figure 2-6 Steps used in the generation of the pRLTK_3'UTR_TLR7 vector.** The 3'UTR was first cloned into pCR2.1 (the vector on the far left). It was then excised using XbaI and NotI enzymes and subsequently ligated into pRL-TK which had been digested with the same enzymes. This led to the formation of pRLTK_3'UTR_TLR7 reporter vector (far right).

## 2.10.2 Generating mutants of the pRLTK_3'UTR_TLR7

Vectors were generated that contained mutations in the binding site(s) for miR-150, miR-152 and miR-375. MiR-150 has 2 putative binding sites in the 3'UTR of TLR7 while the other 2 miRNAs only have one site each. When designing the primers the following guidelines were used, however it was not always possible to follow all of them:

1. Both primers contain the same mutation
2. Primer length should be between 25-45 nucleotides (nt)
3. Primer melting temperature should be between 65 and 85˚C
4. Primer guanosine+cytosine (GC) content should be at least 40%
5. Primers should preferentially terminate in one or more G or C bases

| Primer | Details | | |
|---|---|---|---|
| 150_1 | For: 5' CTT GTA ATC CCA GCA CTC TCG AGG GCC GAG GCA GGT GGA T<br>Rev: 5' ATC CAC CTG CCT CGG CCC TCG AGA GTG CTG GGA TTA GAA G | | |
| | Length 40 nt | GC content 60% | Melting Temperature: 74.2˚C |
| 150_2 | For: 5' CCT GTA ATC CCA GCT ACT TCT AGA GCT GAG GCA GGA GAA TCG C<br>Rev: 5'GCG ATT CTC CTG CCT CAG CTC TAG AAG TAG CTG GGA TTA CAG G | | |
| | Length 43 nt | GC content 54% | Melting Temperature 75.5˚C |
| 152 | For: 5' CCT GCT TAA ATG TTT TTA TCC TCG AGG CAA AGT ACT GTA TCC<br>Rev: 5' GGA TAC AGT ACT TTG CCT CGA GGA TAA AAA CAT TTA AGC AGG | | |
| | Length 42 nt | GC content 41% | Melting Temperature 67.8˚C |
| 375 | For: 5' CAG AGC TAG ACT GTC TCA AAA CTC GAG AAA AAA AAA AAC AC<br>Rev: 5' GTG TTT TTT TTT TTC TCG AGT TTT GAG ACA GTC TAG CTC TG | | |
| | Length 41 nt | GC content 37% | Melting Temperature 65.2˚C |

**Table 2-8 Details of primers designed in order to obtain mutants of pRLTK_3'UTR_TLR7**

The contents of the PCR reaction and the thermal cycling parameters were:

| Contents of PCR reaction | |
|---|---|
| Forward primer (2.5 pmoles/µl) | 0.75µl |
| Reverse primer (2.5 pmoles/µl) | 0.75µl |
| dNTP | 2.00µl |
| DNA fusion high fidelity enzyme | 0.20µl |
| Buffer | 4.00µl |
| DNA (pRLTK_3'UTR_TLR7, 4ng/µl) | 1.00µl |
| DMSO | 0.60µl |
| RNase free water | 10.70µl |

| Thermal cycling program | | |
|---|---|---|
| Hold | 95˚C | 5 mins |
| 18 cycles* | 95˚C | 50 s |
| | variable** | 50 s |
| Hold | 68˚C | 7 mins |

**Table 2-9 PCR reaction and thermal cycling parameters used to obtain mutants of pRLTK_3'UTR_TLR7.**
*18 cycles were performed when primers for 152 and 375 were used. For primers for 150_1 and 150_2, 5 cycles were performed with the forward primer only to help increase the template number. After this, the reverse primer was added and 18 cycles were performed. ** This temperature varied depending on the primer pair used: 150_1: 58˚C; 150_2: 60˚C; 152: 55˚C; 375: 55˚C

The PCR product then underwent a DpnI digestion by the addition of 0.25µl DpnI to the reaction followed by incubation for 1 hr at 37˚C. This enables any

methylated DNA to be destroyed, leaving mutants (all pRL-TK vectors are unmethylated) only. This was transformed into competent cells to yield the 4 vectors:

- pRLTK_MUT_3'UTR_TLR7_150_1
- pRLTK_MUT_3'UTR_TLR7_150_2
- pRLTK_MUT_3'UTR_TLR7_152
- pRLTK_MUT_3'UTR_TLR7_375

### 2.10.3 Dual-Luciferase® Reporter Assay System (DLR)

The DLR system is an in vitro tool used to study gene expression. It allows for the simultaneous expression and measurement of two individual reporter enzymes within a single system: the 'experimental' reporter and the co-transfected 'control' reporter. The 2 reporter genese are Firefly and Renilla luciferase and each have different enzyme structures and substrate requirements, allowing discrimination between their respective bioluminescent reactions. Both do not require post-translational modification and can be used as a genetic reported immediately following translation. To assay post-transcriptional regulation, as would be exerted by miRNAs, sequences are cloned at the 3'end of the reporter (as shown in Figure 1-9). Figure 2-7 explains the DLR assay:

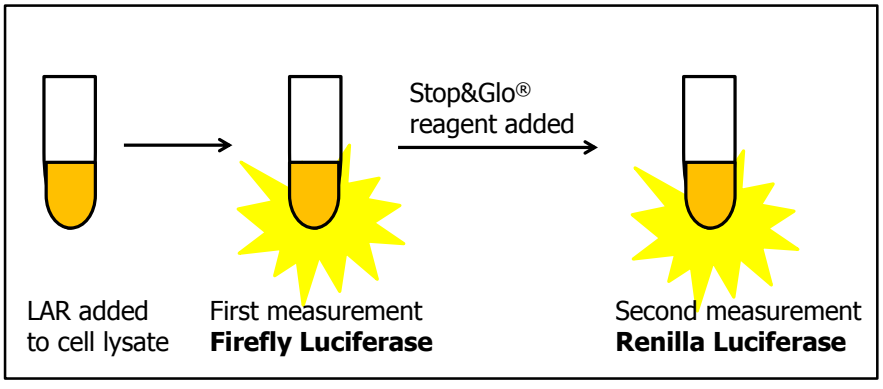


**Figure 2-7 Schematic description of the DLR Assay.**
The activity of the firefly luciferase reporter is measured first by adding Luciferase Assay Reagent (LAR) to an eppendorf containing the cell lysate. This generates a luminescent signal. The reaction is then quenched and the Renilla luciferase reaction simultaneously initiated by adding Stop&Glo® to the same tube. Light emission is captured by a luminometer and the ratio between the 2 readouts represents translation activity.

This system was used to determine if miR-150, miR-152 and miR-375 directly targeted the 3'UTR of TLR7. As previously described the 3'UTR of TLR7 was cloned into pRL-TK at the 3'end of the reporter Renilla luciferase. Renilla luciferase was used as the reporter gene for assaying the effects of the miRNAs and Firefly luciferase (encoded in pGL3) was used as the normaliser.

### 2.10.3.1 Plasmid transfections

HeLa cells were plated in 24-well plates at a concentration of 40,000 cells per well. The following day they were co-transfected with pRLTK_3'UTR_TLR7 plasmid (100ng/well) and 800ng of pCDNA3.1_150 or pCDNA3.1_152 or pCDNA3.1_375 or pCDNA3.1 (-). In parallel, pRLTK_3'UTR_TLR7 (100ng/well) mutants were also co-transfected into HeLa cells along with their corresponding miRNA vector:

i) pRLTK_MUT_3'UTR_TLR7_150_1 was co transfected with pCDNA3.1_150
ii) pRLTK_MUT_3'UTR_TLR7_150_2 was co transfected with pCDNA3.1_150
iii) pRLTK_MUT_3'UTR_TLR7_152 was co-transfected with pCDNA3.1_152
iv) pRLTK_MUT_3'UTR_TLR7_375 was co-transfected with pCDNA3.1._375

pGL3 was also co-transfected into cells at a concentration of 25ng/well for normalisation. Transfections were carried out using Superfect and following the manufacturer's instructions. After 3 hours the cells were washed with PBS and left overnight in D-MEM Medium with GlutaMAX™. Experiments were performed three times in duplicate. The following day, measurements were made using the Dual-Luciferase® Reporter Assay System.

The cumulative effect of 3 miRNAs acting on the 3'UTR of TLR7 was shown by co-transfecting the pRLTK_3'UTR_TLR7 plasmid (100ng) into HeLa cells with pCDNA3.1_mix or pCDNA3.1 (-) (at a concentration of 800ng/well). Superfect was used according to manufacturer's instructions as the transfection agent and normalisation was done by co-transfecting pGL3 (25ng/well) into all cells. Experiments were performed three times in duplicate, and measurements were

made using the Dual-Luciferase® Reporter Assay System. The Luciferase System is explained schematically in Figure 2-8.

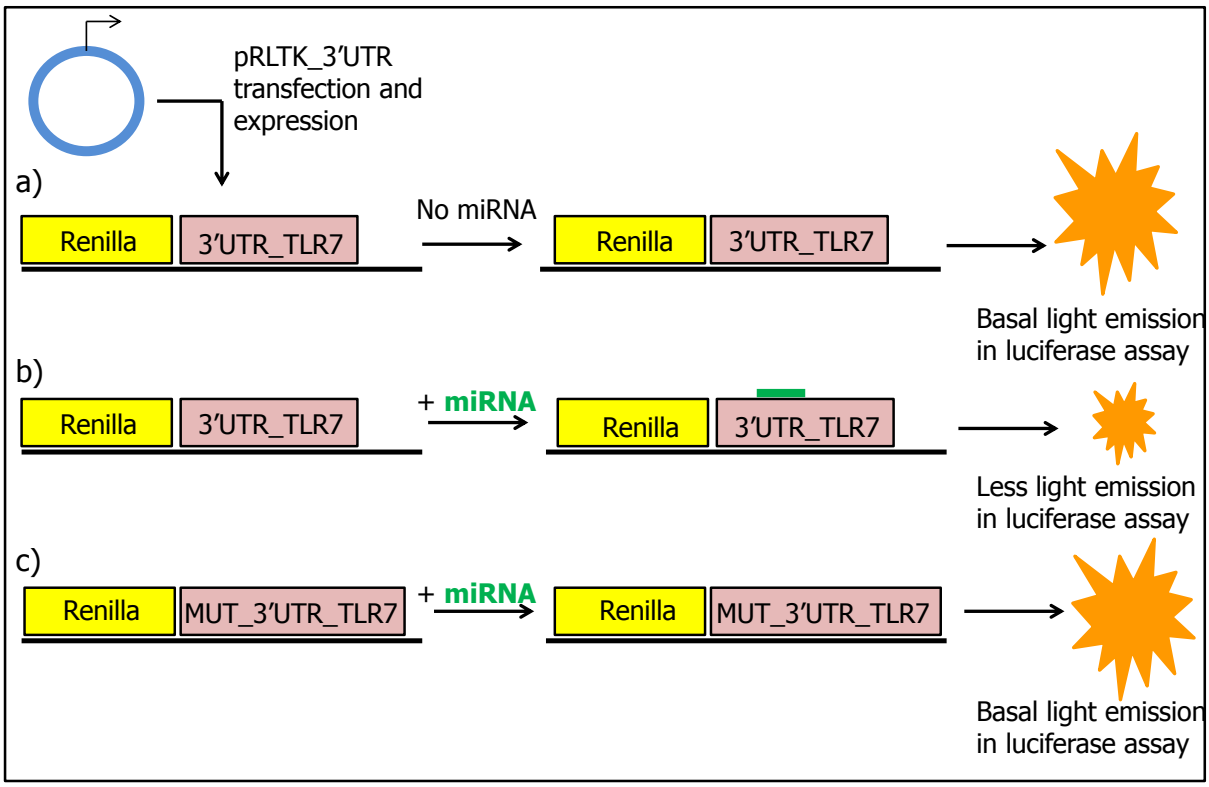


**Figure 2-8 Schematic representation of the Luciferase system**
(a)pRLTK_3'UTR_TLR7 is co-transfected with pCDNA3.1(-) i.e. no miRNA. This leads to basal expression of the construct with the concomitant light emission in the luciferase enzymatic assay. In (b) pCDNA3.1_150 or pCDNA3.1_152 or pCDNA3.1_375 is co-transfected into the cell. The miRNA binds to the 3'UTR of TLR7 and inhibits the expression of the Renilla construct leading to decreased light emission in the luciferase enzymatic assay. If the 3'UTR of TLR7 is mutated (c), the miRNA is unable to bind. This leads to basal expression of the construct with concomitant light emission.

## 2.11 Statistical Analysis

Data was analysed using GraphPad Prism. The specific tests used are indicated in figure legends and depended on sample number, normality of data and if data were paired. $p<0.05$ was considered significant. In general, as our data was from biological samples, for sample numbers >10 non-parametric tests were used. A cut-off of 10 was used because with small samples non-parametric tests have little or no power to find a significance difference. This 'conservative' approach was adopted because if a parametric test had been used and the data actually normally distributed, then the p-value obtained would not be very meaningful. If in fact the data was normally distributed, using a non-parametric may lead to a p-value that is too large but it would not lead us to make incorrect conclusions from out data.

# Chapter 3 Characterisation of study model

## 3.1 Patient characteristics

| | Healthy | Mild Asthma | Moderate Asthma | Severe Asthma | p value |
|---|---|---|---|---|---|
| **Number** | 51 | 19 | 8 | 35 | |
| **Age** | 27.5 (18-54) | 21.3 (18-30) | 39.8 (23-54) | 45.0 (21-68) | <0.0001 |
| **Sex (M/F)** | 28/23 | 6/13 | 3/5 | 11/24 | 0.11 |
| **$FEV_1$** | 4.1 (0.1) | 3.8 (0.3) | 2.9 (0.2) | 2.0 (0.1) | <0.0001 |
| **%predicted $FEV_1$** | 105.3 (1.9) | 101.0 (3.5) | 86.8 (4.6) | 69.8 (4.0) | <0.0001 |
| **FVC** | 4.9 (0.2) | 4.7 (0.3) | 4.3 (0.2) | 3.0 (0.2) | <0.0001 |
| **%predicted FVC** | 106.7 (2.5) | 108.8 (2.8) | 106.5 (3.9) | 85.8 (3.4) | <0.0001 |
| **BMI** | 24.8 (0.5) | 22.2 (0.6) | 28.7 (1.4) | 31.9 (1.3) | <0.0001 |
| **Atopy (%)** | 0 | 19 (100) | 5 (63) | 23 (68)* | <0.0001 |
| **ACQ score** | N/A | Not done | 0.7 (0.3-1) | 3.0 (0.8-4.9) | <0.0001 |
| **ICS dose (BDP equivalent, mcg)** | N/A | N/A | 725 (400-1000) | 2379 (1000-4000) | <0.0001 |
| **Number on daily oral steroids** | N/A | N/A | N/A | 10 | - |
| **Number of exacerbations in previous year** | N/A | 0 | 0 | 5.9 (1-12) | - |
| **BAL cell differential** | | | | | |
| **% macrophages** | 87.0 (73-97) | 85.8 (69-93) | 81.2 (57-98) | 68.5 (3-97) | 0.03 |
| **% neutrophils** | 3.3 (0-14) | 2.8 (0.5-9) | 7.8 (0-30) | 15.2 (0.5-95) | 0.008 |
| **% eosinophils** | 0.4 (0-2.3) | 2.1 (0-7) | 0.8 (0-2.5) | 2.0 (0-30) | 0.028 |

**Table 3-1 Volunteer baseline characteristics.**
Values are means with SEM in parenthesis except for age, ACQ score, ICS dose, number of exacerbations and BAL cell differential for which range is shown and atopy for which % is shown. ACQ, asthma control questionnaire; BAL, bronchoalveolar lavage; BDP, beclometasone; BMI, body mass index; $FEV_1$, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroid; N/A, not applicable; *data unavailable for one subject. p values calculated using Kruskal Wallis test except for comparison of sex and atopy for which Chi-square test was used and comparison of ACQ and ICS dose for which Mann Whitney test was used.

Study subjects were recruited as described in Chapter 2. The clinical characteristics of all study subjects are shown in Table 3-1 and baseline characteristics of individual subjects are shown in Appendix 3.

Severe asthma subjects were asked about their exacerbation history in the previous 12 months and we did not attempt to confirm this from the GP. In line with ATS/ERS recommendations (Reddel et al. 2009), exacerbations were defined as events characterised by a change from the patient's baseline status and requiring urgent action on the part of the patient and physician including a change in treatment (at least 3 days' use of OCS or an increase from a maintenance dose of OCS).

Healthy volunteers, mild asthma and MO subjects were all non-smokers with no previous history of smoking. However, within the severe asthma group, there were 3 current smokers (8%), 8 ex-smokers (23%) and 24 never-smokers (69%).

### 3.1.1 Demographics of study subjects

As a group the severe asthma patients were significantly older than healthy and mild asthma subjects. They also had a higher BMI than healthy and mild asthma subjects. There was no difference in the sex distribution between groups. Atopic status of individuals was assessed by skin prick testing to the allergens listed in Chapter 2. Individuals were deemed to be atopic if they tested positive (defined as a wheal of 3x3 mm greater than the saline negative control) to one or more of the allergens tested, in addition to the positive control used (histamine).

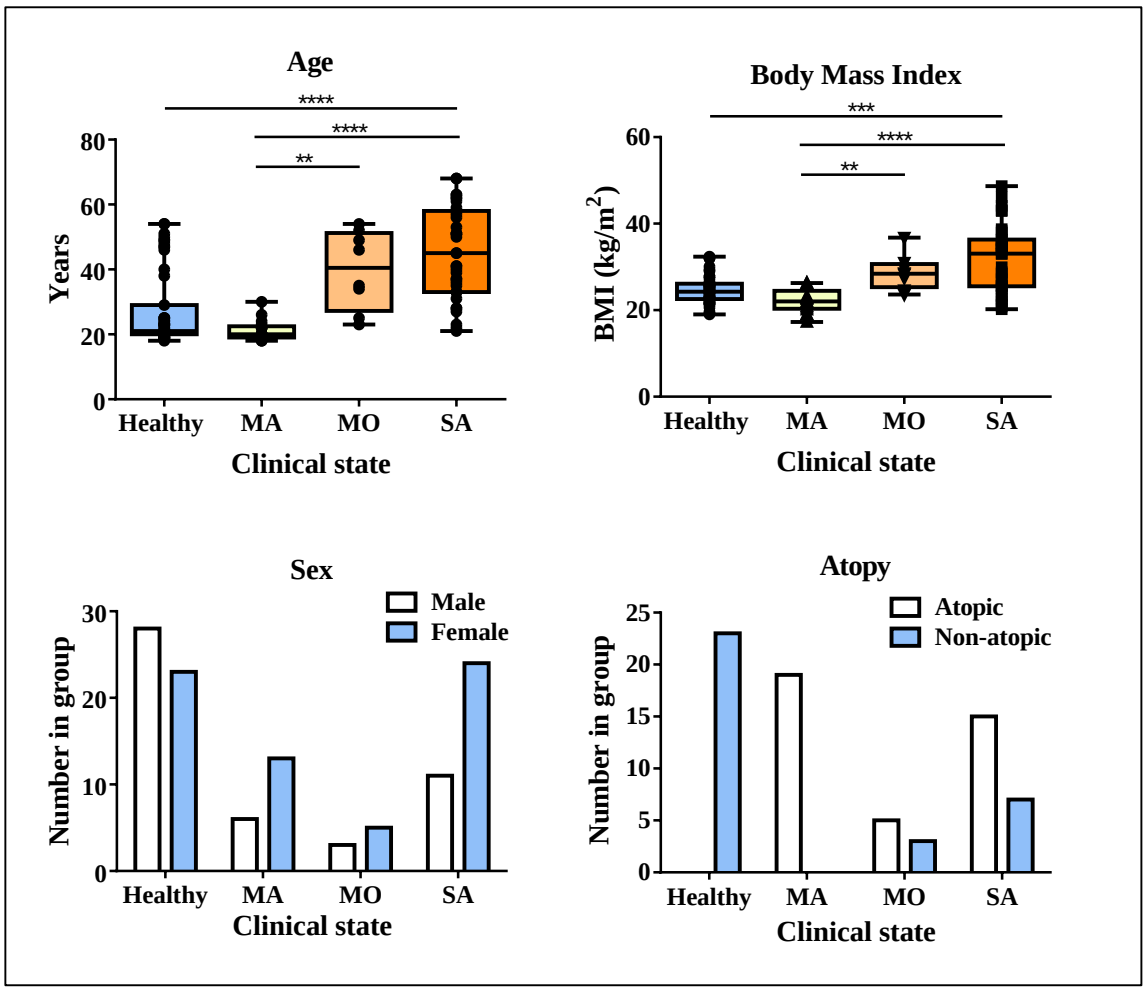


**Figure 3-1 Age, Body mass index, sex and atopic status of subjects used in study according to clinical state.**
Box and whisker plots show values for each individual, the median and interquartile range. MA=mild asthma, MO=moderate asthma, SA=severe asthma. **p<0.01, ***p<0.001, ****p<0.0001 using Kruskal-Wallis test with Dunn's multiple comparisons test.

## 3.1.2 Lung function

All subjects had baseline lung function tests performed using either the Dry Wedge Bellow Spirometer or the Jaeger Masterscreen with Viasis® software. Both $FEV_1$ and FVC decreased with asthma severity with severe asthma subjects having significant lower measures compared to healthy and mild asthma subjects. For both $FEV_1$ and FVC, mild asthma subjects were not significantly different from healthy.

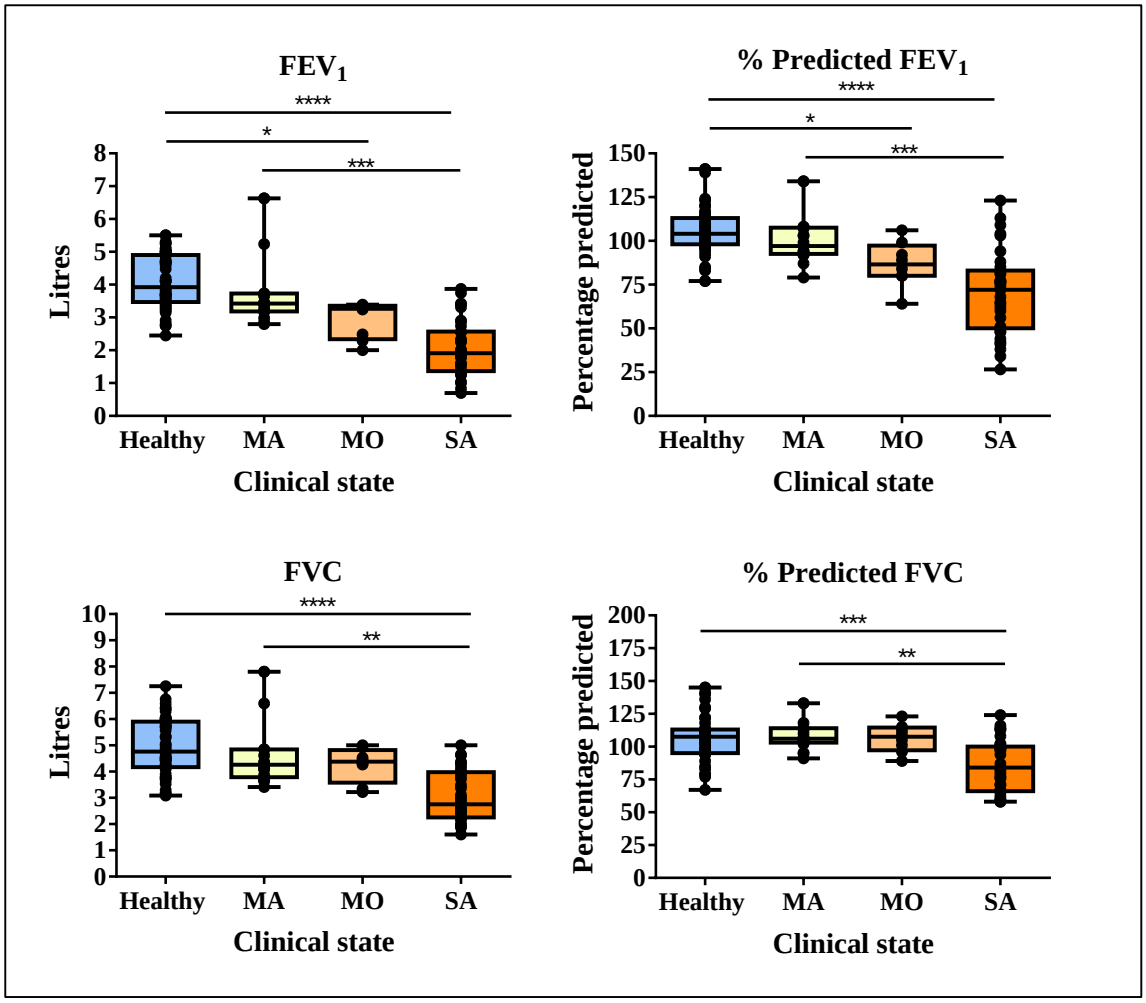


**Figure 3-2 Baseline lung function of study subjects according to clinical state.**
Baseline $FEV_1$ (forced expiratory volume in one second), % predicted $FEV_1$, FVC (forced vital capacity) and % predicted FVC. Box and whisker plots show values for each individual, the median and interquartile range. MA=mild asthma, MO=moderate asthma, SA=severe asthma. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 using Kruskal-Wallis test with Dunn's multiple comparisons test.

### 3.1.3 Asthma control and treatment

Asthma control was evaluated using the Asthma Control Questionnaire© which is shown in Appendix 2. The ACQ has been developed and validated to measure asthma control as defined by international guidelines (Juniper et al. 1999). It has 7 questions, five of which concern symptoms (night time and day time) and activity limitation, one about the use of rescue bronchodilator therapy and the final one about the subjects $FEV_1$ % predicted. The items are equally weighted and the ACQ score is the mean of the 7 items and therefore between 0 (well controlled) and 6 (extremely poorly controlled). An ACQ score

of ≤1 is considered to represent good control. Unfortunately the ACQ data for volunteers with mild asthma was not available.

To compare the dose of ICS between patients, the equivalent dose of beclometasone is frequently used because a number of different formulations of ICS exist. However, this is not always accurate as the deposition of the drug into the lung, especially the smaller airways, can vary quite widely between formulations.

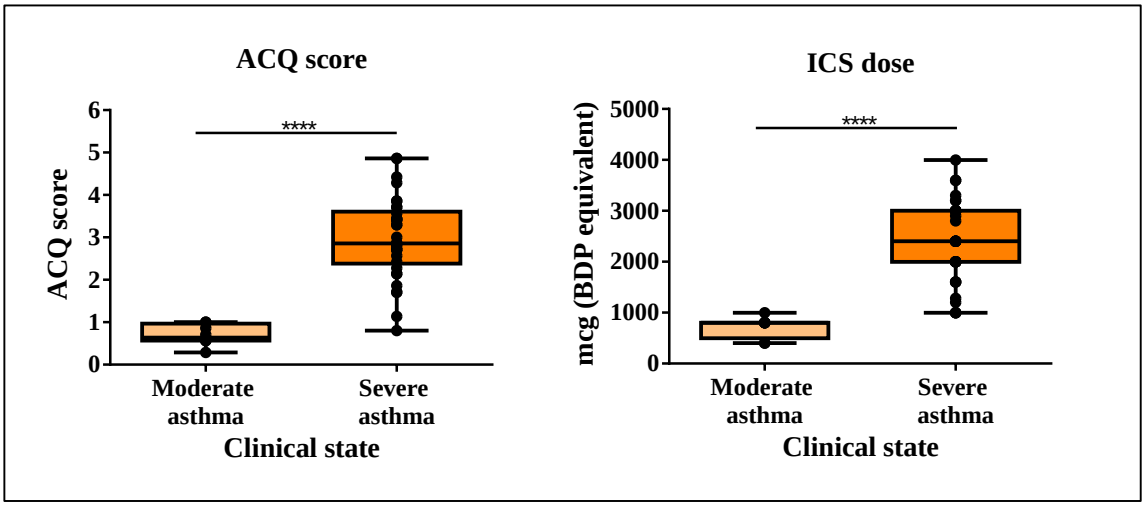


**Figure 3-3 Asthma control questionnaire score and dose of ICS therapy for moderate and severe asthma subjects**
Asthma control questionnaire (ACQ) score and dose of inhaled corticosteroid (ICS) therapy (BDP equivalent) is shown. Box and whisker plots show values from each individual, the median and interquartile range. ****p<0.0001 using Mann Whitney test.

As can be seen from Figure 3-3, subjects with severe asthma had a significantly higher ACQ score than MO subjects, indicating that their disease control was much poorer. This was despite them receiving higher doses of ICS treatment.

### 3.1.4 Discussion

Samples from 113 subjects have been used in the study- 51 healthy subjects, 19 mild asthmatics, 8 moderate asthmatics and 35 severe asthmatics. As mentioned previously the samples from subjects with mild asthma were provided by Dr Grainge. As the focus of this project had always been to study severe asthma, we did not attempt to recruit more subjects with mild asthma in order to equalise subject numbers across the clinical states. A small group of subjects with moderate, well-controlled asthma were recruited later in the

study in order to provide a 'control' group for steroid therapy and to investigate if the changes observed in miRNA and TLR7 expression were disease-related or the result of treatment (ICS). Patients with moderate asthma were all on Step 3 of the BTS Treatment algorithm i.e. they were all taking an ICS along with a LABA inhaler. All patients with severe asthma were also on these 2 treatments, plus other therapy, but unlike the patients with moderate asthma, their symptoms were un-controlled and they had recurrent frequent exacerbations.

There was a significant difference in the ages of subjects between clinical states. Healthy subjects tended to be younger (mean age 27.5 years) because most were recruited through advertising in the local university and medical school. Patients with severe asthma tend to be older (Moore et al. 2007)- they may have adult onset disease, childhood onset disease that has been poorly controlled and progressed to a more severe phenotype or childhood onset disease which improved but then became much worse in later years. Therefore, subjects with severe asthma in this study were much older (mean age 45 years) compared to healthy subjects. Once appreciated, an attempt was made to recruit older healthy volunteers, so the age range does extend up to 54 for healthy subjects (range for severe asthma subjects 21-68).

There was no significant difference in the sex of subjects between the clinical states ($p=0.11$). All the asthma groups had slightly more females than males- mild asthma 57% females, moderate asthma 63% females and severe asthma 69% females. This is in common with other studies of asthma and a female predominance in severe asthma has been noted in several studies including the Severe Asthma Research Program (SARP), the BIOAIR trial and the Wessex Severe Asthma Cohort (Kupczyk et al. 2014; Moore et al. 2007) (personal communication with Professor Peter Howarth). The SARP cohort consists of more than 400 subjects and included the largest group of comprehensively characterised subjects with severe asthma published so far.

Testing of pulmonary function revealed no real difference between healthy subjects and mild asthmatics, while both these groups were significantly different compared to severe asthmatics. Subjects with mild asthma tend to have near normal lung function and so this finding was not unexpected. In the measurement of $FEV_1$, subjects with moderate asthma tended, as a group, to lie

in between mild asthma and severe asthma, but were not statistically significant from either. This is probably due to the small number of subjects in the moderate asthma group and certainly does not exclude a clinically significant difference in symptoms. The recent ERS/ATS guidelines on severe asthma include in their definition of severe asthma the presence of airflow limitation with an $FEV_1$<80% predicted. Over 70% of the severe asthma subjects had an $FEV_1$ that was less than 80% predicted, while almost a third had an $FEV_1$ less than 60% predicted. These figures are not dissimilar to those made in the SARP, which found that 80% of their severe asthma population had an $FEV_1$<80% and 47% had baseline $FEV_1$<60% (Moore et al. 2007).

Subjects with severe asthma also had a significantly higher BMI than healthy subjects and mild asthmatics. Similar observations have been noted in the SARP (Moore et al. 2010). Obesity has been suggested to have a substantial role in the development and severity of asthma (Wenzel 2012), but whether it is a driving component in asthma development or a mere confounder remains controversial. A distinct obesity-related asthma phenotype has been identified within severe asthma and is characterised by adult onset disease, female predominance, and lack of Th2 cytokines. Certainly in our cohort of severe asthma patients, 13 of the 17 patients (76%) with a BMI>30 were female.

Fewer subjects with severe asthma had positive skin prick tests to 1 or more allergens compared to mild asthmatics (68% compared to 100%). However it needs to be pointed out that the presence of atopy was an inclusion criteria used by Dr Grainge and so all the subjects recruited into his study had positive skin prick tests to 1 or more allergens. However, results from the SARP also show that compared to mild and moderate asthmatics, fewer subjects with severe asthma are positive to 1 or more allergens. In fact, using logistic regression, fewer positive skin test responses has been identified as an independent risk factor for severe disease (Moore et al. 2007).

Subjects in the severe asthma group had poor disease control despite being on maximal therapy (as judged by their clinical team) and quite high doses of ICS. The mean ICS dose in this group was 2400 mcg per day (range 1000-4000mcg per day), with 77% of subjects receiving ≥2000mcg BDP equivalent per day. This is similar to findings from the SARP which found that 63-78% of subjects with severe asthma were receiving ≥ 2000mcg BDP/day. Individual doses are

frequently shown as the dose equivalent to BDP so that comparisons between patients and studies can be made. However, this data does not take into account patient technique, which would affect drug delivery into the lungs, or drug formulation. The newer ultra-fine particle inhalers tend to have higher deposition within the lungs, especially the smaller airways than many of the 'older' formulations. Subjects with mild asthma were not included in this analysis because by definition they are all on Step I of the BTS treatment algorithm and therefore not on ICS therapy.

All subjects with moderate asthma recruited into this study were required to have well-controlled disease. This was assessed using the ACQ and all subjects with moderate asthma had an ACQ≤1. As can be seen from the data above, subjects with severe asthma had a much higher ACQ (mean 3.0), reflecting poor symptom control. Having an ACQ>1.5 despite appropriate therapy is also included in the definition of severe asthma in the 2014 ERS/ATS guidelines (Chung et al. 2014). 94% of the severe asthma subjects in our cohort had an ACQ>1.5. The ACQ was measured on the day of the bronchoscopy which is most cases took place at least 4-6 weeks after an exacerbation. Therefore it is more likely that the raised ACQ is reflecting overall poor control rather than temporary poor control due to an exacerbation.

The proportion of AM in BAL fluid was significantly lower in severe asthma compared to healthy subjects (68% vs 87%). This trend has been reported in other studies (St-Laurent et al. 2009; Staples et al. 2012). The literature suggests that BAL cells are 90-95% AM (Contoli et al. 2006; Karta et al. 2014; St-Laurent et al. 2009; Sykes et al. 2012). The mean % of AM if we combined all our subjects was 81% and for the asthmatic subjects 75%, which is considerably lower than the commonly seen value of 95%. In the subgroup of severe asthma subjects, mean BAL AM% was less than 70% and the range was extremely wide. Not only does this support our decision to further purify for AM prior to use, but also suggests that results from other studies that used 'all' BAL cells to represent AM may not necessarily truly represent AM. The reduction in percentage alveolar macrophages in BAL in severe asthma is a reflection of the increase in other cell populations.

Severe asthma subjects had significantly higher numbers of neutrophils in their BAL compared to healthy subjects while the proportion of eosinophils in severe

asthma BAL was not significantly raised compared to healthy subjects. A neutrophil predominant phenotype of severe asthma has been identified which is characterised by significant airflow limitation (Shaw et al. 2007), air trapping (Busacker et al. 2009) and may be associated with obesity in women (Scott et al. 2011). The definition for BAL neutrophilia is unclear, but if a cut off of 50% was used (which is quite high), then 5 of the 35 SA patients (14%) had neutrophilia, but an 'obese-female' predominance was not observed in this small sub-group. We were interested to see that MA subjects, but not MO and SA subjects, had significantly raised BAL eosinophil counts compared to healthy subjects. The MA subjects had well controlled disease not requiring treatment with ICS. However, 5 of the 19 MA subjects (26%) had BAL eosinophils of greater than 4% (compared to 4/35 of the SA subjects, 11%), suggesting asymptomatic eosinophilic inflammation in the lower airways.

In summary the severe asthma patients in our study are older, have a higher BMI, poorer lung function and differences in the inflammatory profile of their BAL compared to healthy subjects. Sub-groups of these patients were used for the different analyses in our study and relevant differences in the clinical characteristics will be discussed in the following chapters.

# 3.2 Characteristics of healthy and severe asthmatic alveolar macrophages

## 3.2.1 Introduction

Macrophages are endowed with a number of receptors for T cell cytokines, and B cell, host and microbial products. They are key modulator and effector cells in the lung immune response and perform important functions including protecting the host through innate immunity and phagocytic clearance of pathogenic and dying cells. In tissues, macrophages mature and are activated in response to these stimuli to acquire specialised functional phenotypes. Traditionally, macrophage activation has been divided into classic vs. alternative, termed M1 and M2 respectively, mirroring the Th1 and Th2 nomenclature. IFNγ , alone or in concert with microbial products (e.g. LPS) or cytokines such as TNFα activate macrophages and these classical or M1 macrophages produce large amounts of TNFα, IL-12, IL-23, IL-6 and IL-8 (Mantovani et al. 2004). These cells also have a high capacity to present antigen and help Th1 and Th17 cell inflammatory responses forward. M2 is used generically to define macrophage activation other than M1 (Mantovani et al. 2004) but typically IL-4 and IL-13 induce this alternative activation phenotype. M2 macrophages are immunomodulators, poorly microbicidal and promote tissue remodelling and repair (Benoit et al. 2008).

Animal models suggest that M2 macrophages take a leading role in asthma and excessive M2 macrophages have been shown to increase inflammatory cell recruitment, mucus secretion and airway hyper-responsiveness (Moreira et al. 2010). However it is unclear if this translates to human asthma and although it has been shown that atopic individuals have higher levels of IL-10 secreting monocytes that differentiate into M2 macrophages *in vitro* (Prasse et al. 2007), AM from subjects with asthma have not been found to have the full M2 phenotype (Staples et al. 2012). In fact, growing evidence supports the role for M1 macrophages in the development of asthma, especially in patients with severe, treatment resistant disease where M1 macrophages produce large amounts of pro-inflammatory mediators including TNF-α, IL-1β and nitric oxide that exacerbate the lung injury and accelerate airway remodelling (Kim et al. 2007). Therefore, we set out to characterise AM from severe asthma subjects. Additionally, we were interested to investigate for any deficiency in the

expression of TLRs and C-type lectins in SA-AM as this would affect their ability to respond to bacterial and viral pathogens.

AM are the predominant cell type in BAL and studies have shown that over 90% of BAL cells are AM (St-Laurent et al. 2009; Sykes et al. 2012). Other immune cells are also present in BAL and these include lymphocytes, neutrophils and eosinophils and the proportion of these cells varies with disease. Finally, epithelial cells can also be found in BAL and it is likely that these cells are sloughed off the bronchial epithelium. As the focus of our study was AM we decided to purify AM from BAL in order to avoid contamination of these other cell populations and to ensure that miRNA and gene expression were reflective of AM.

## 3.2.2 Methods and Results

### 3.2.2.1 Purity assessment of isolated alveolar macrophages

As soon as BAL was obtained at bronchoscopy it was processed (filtered and washed) and cells were resuspended in RPMI medium and placed in an incubator for 2 hours for AM to adhere to the plastic base of the tissue culture plates. Adherent cells were gently detached using Accutase® and then cell surface expression of HLA-DR and CD3 (and appropriate isotype controls) was checked by flow cytometry. HLA-DR is a macrophage marker and expressed by most AM (Popp et al. 1989) while CD3 is a T cell marker. As can be seen in Figure 3-4, 95% of the cells were HLA-DR positive, while none of the adherent cells were CD3 positive indicating that the adherent cells are almost exclusively AM.

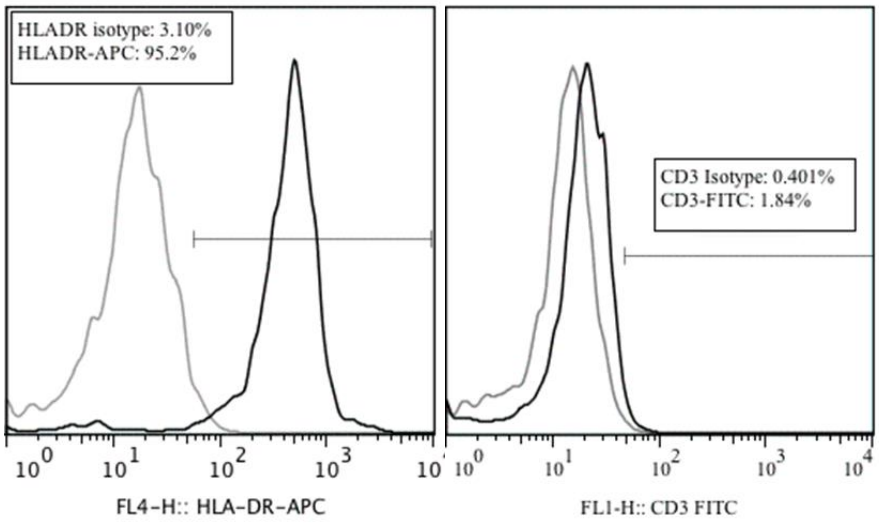


**Figure 3-4 Assessment of alveolar macrophage purity**
Adherent macrophages were stained with anti-CD3 and anti-HLA DR fluorescently labelled antibodies. Analysis using flow cytometry showed that adherent cells were 95% HLA-DR positive (left panel) and CD3 negative (right panel).

We then evaluated the expression of the following genes in AM was using qPCR:

| | | | | |
|---|---|---|---|---|
| TNFα | CD23 | TLR3 | Mannose Receptor (CD206) | FKBP4 |
| IL-6 | | TLR4 | DC-SIGN (CD209) | FKBP5 |
| IL-8 | TGFβ 1 | TLR5 | CLEC 7A (Dectin 1) | |
| IL-1β | TGFβ 2 | TLR6 | CLEC 4E (Mincle) | |
| IL-23A | | TLR9 | CLEC 1A | |
| | | | CLEC 6A | |

**Table 3-2 List of genes evaluated in alveolar macrophages**
TNFα, tumour necrosis factorα; IL, interleukin; TGFβ , transforming growth factorβ ; TLR, toll-like receptor; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; CLEC, C-type lectin; FKBP, FK506 binding protein.

The characteristics of subjects used in this evaluation are shown in Table 3-3. Our analysis includes AM from 29 healthy subjects and 13 severe asthma subjects. We decided to exclude current and ex-smokers with a >10 pack year

smoking history as it is appreciated that smoking can affect macrophage activation (Yuan et al. 2014).

| | Healthy | Severe Asthma | p value |
|---|---|---|---|
| Number | 29 | 13 | |
| Age | 26.6 (18-54) | 41 (22-63) | 0.0006 |
| Sex (M/F) | 16/13 | 5/8 | 0.51 |
| $FEV_1$ | 4.1 (0.1) | 2.2 (0.3) | <0.0001 |
| %predicted $FEV_1$ | 102.7 (2.4) | 71.2 (7.2) | 0.0001 |
| FVC | 4.9 (0.2) | 3.2 (0.3) | <0.0001 |
| %predicted FVC | 102.6 (3.0) | 89.4 (5.3) | 0.046 |
| Body Mass Index | 24.7 (0.6) | 32.1 (1.6) | <0.0001 |
| Atopy (%) | 0 | 8 (67)* | <0.0001 |
| ACQ score | N/A | 2.9 (1.9-4.3) | - |
| ICS dose in mcg (BDP equivalent) | N/A | 2300 (1000-3000) | - |
| Number on daily oral steroids (%) | N/A | 3 (23) | - |
| Number of exacerbations in previous year | N/A | 6.2 (2-12) | - |
| BAL cell differential | | | |
| %macrophages | 85.6 (75-97) | 55.1 (3-90) | 0.0004 |
| %neutrophils | 3.8 (0.3-14) | 22.3 (3-94) | 0.0023 |
| %eosinophils | 0.4 (0-2) | 2.9 (0-30) | 0.051 |

**Table 3-3 Clinical characteristics of healthy and severe asthma subjects used in the analysis of AM gene expression**
Values are means with SEM in parenthesis except for age, ACQ score, ICS dose, number of exacerbations and BAL cell differential for which range is shown and atopy and number on oral steroids for which % is shown. ACQ, asthma control questionnaire; BAL, bronchoalveolar lavage; BDP, beclometasone; $FEV_1$, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroid; N/A, not applicable; * data unavailable for 1 subject. p values calculated using Mann Whitney test except for comparison of sex and atopy for which Chi-square test was used

### 3.2.2.2 Genes showing increased mRNA expression in severe asthma alveolar macrophages

Our analysis shows that the expression of the pro-inflammatory cytokines and chemokines TNF$\alpha$, IL-6, IL-8, which are also typically associated with M1 macrophages was significantly increased in SA-AM.

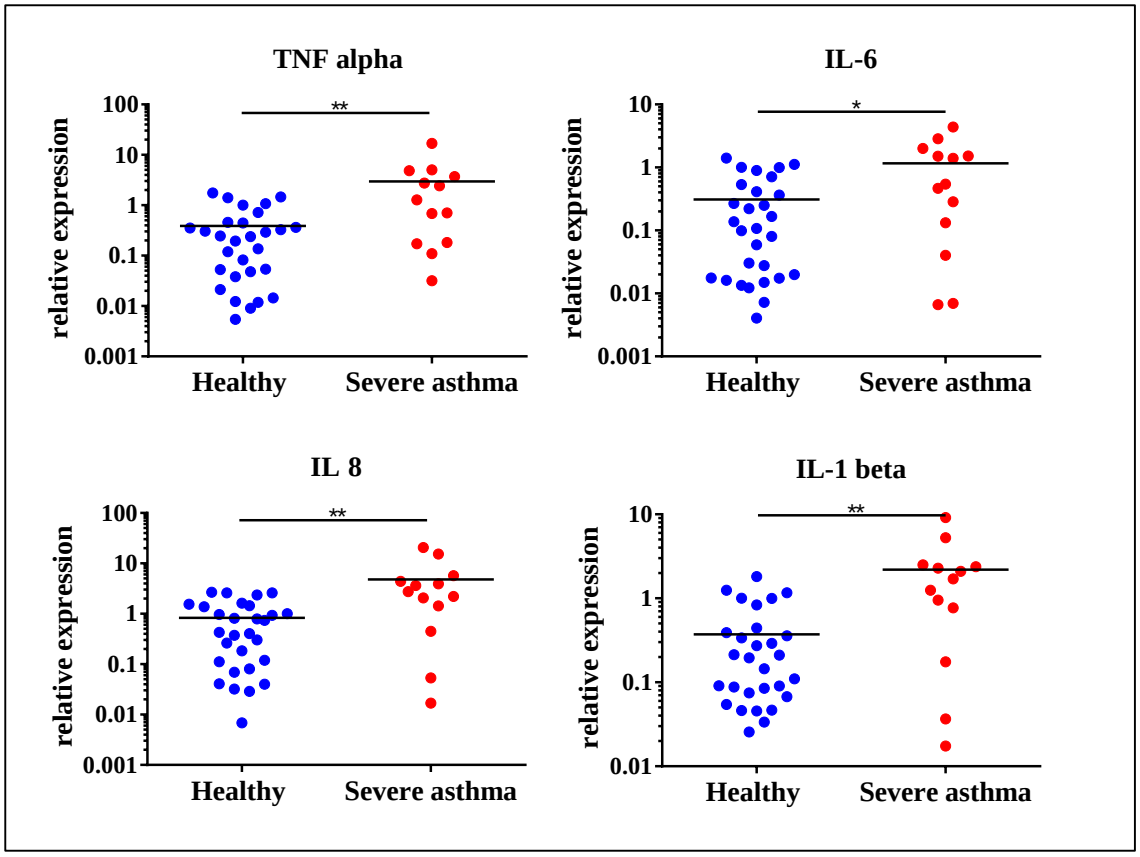


**Figure 3-5 Expression of M1 macrophage associated cytokines is increased in severe asthma AM**
mRNA expression of TNFα, IL-6, IL-8 and IL-1β is increased in SA (n=13) compared to healthy (n=29) AM. Expression is normalised against GAPDH. *p<0.05, **p<0.01 using Mann Whitney test.

The expression of IL-23A mRNA is also significantly increased in SA-AM. IL-23A is a member of the IL-12 cytokine family with pro-inflammatory properties and it is a potent enhancer of Th17 cell differentiation (Haines et al. 2013). In animal models, IL-23 has been shown to modulate Th2 cell differentiation, promote eosinophil infiltration and contribute to neutrophil recruitment (Li et al. 2014).

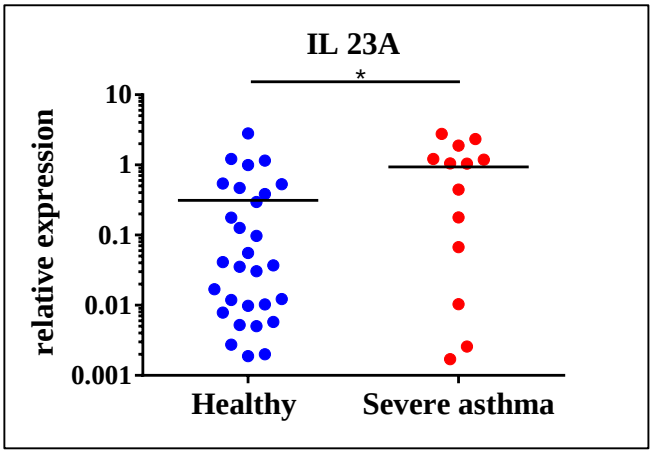


**Figure 3-6 Expression of IL-23A is increased in severe asthma AM**
mRNA expression of IL-23A is increased in SA (n=13) compared to healthy (n=29) AM. Expression is normalised against GAPDH. *p<0.05 using Mann Whitney test.

The expression of a number of TLRs is also increased in SA as shown in Figure 3-7. These TLRs are all broadly thought to exhibit antibacterial responses rather than antiviral responses (which are executed by TLR3, TLR7 and TLR8).

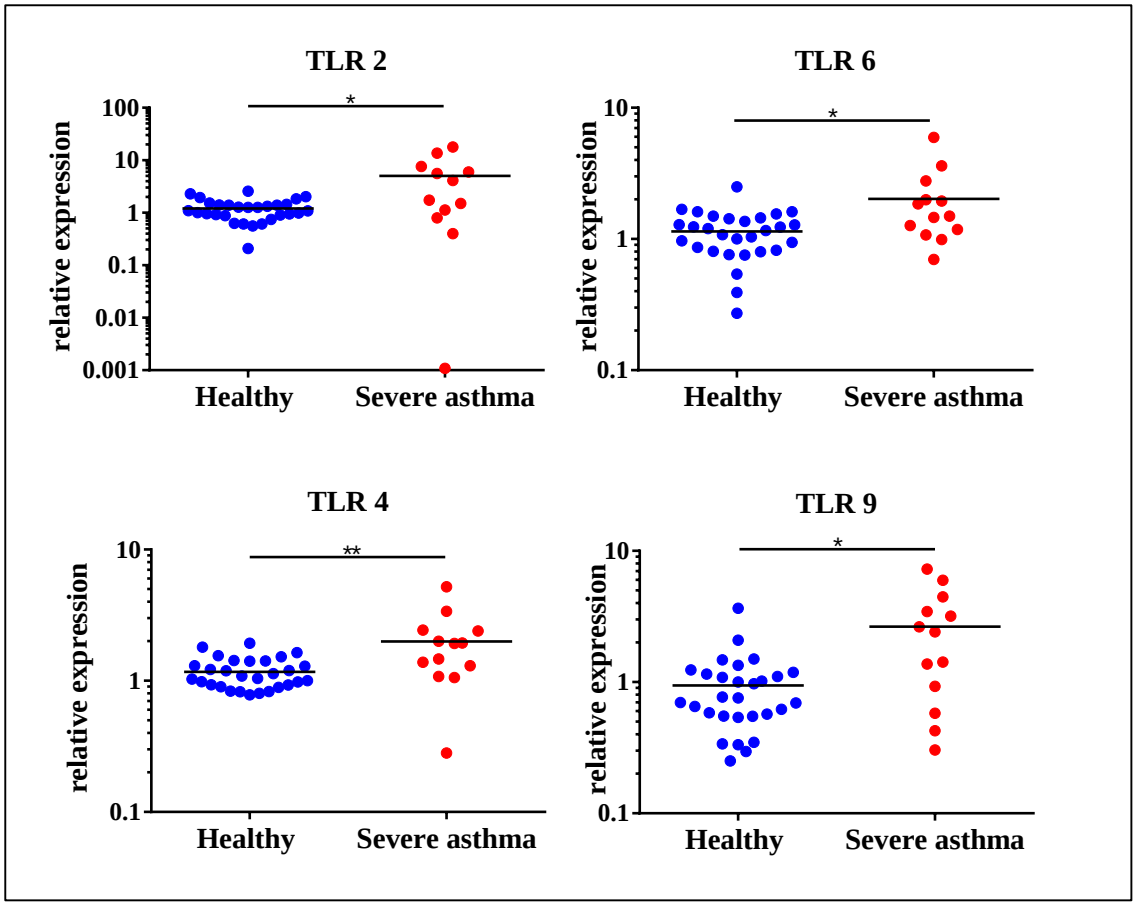


**Figure 3-7 Expression of TLR2, TLR4, TLR6 and TLR9 is increased in severe asthma AM**
mRNA expression of TLR2, TLR4, TLR6 and TLR9 is increased in SA-AM (n=13) compared to healthy (n=29) AM. Expression is normalised against GAPDH. *p<0.05, **p<0.01 using Mann Whitney test.

TLR2 and TLR4 respond to components of the bacterial cell wall. TLR2 usually acts as a heterodimer with TLR1 or TLR6 and responds to lipoproteins and lipoteichoic acid from Gram-positive bacteria (Lien et al. 1999). TLR4 responds primarily and importantly to LPS, a component of Gram-negative bacterial cell walls, but also to other PAMPs such as Streptococcus pneumonia pneumolysin (Malley et al. 2003) and a coat protein from respiratory syncytial virus (Kurt-Jones et al. 2000). TLR9 recognises CpG motifs, which are more prevalent in pathogenic DNA (compared to mammalian DNA) and usually from bacteria although viral DNA may also activate TLR9.

The expression of CD23, the typical M2 macrophage marker was found to be extremely low in both healthy and SA-AM, as evident by high Ct values (over 35

cycles) at qPCR. However, overall the expression of CD23 was higher in SA-AM compared to healthy AM.

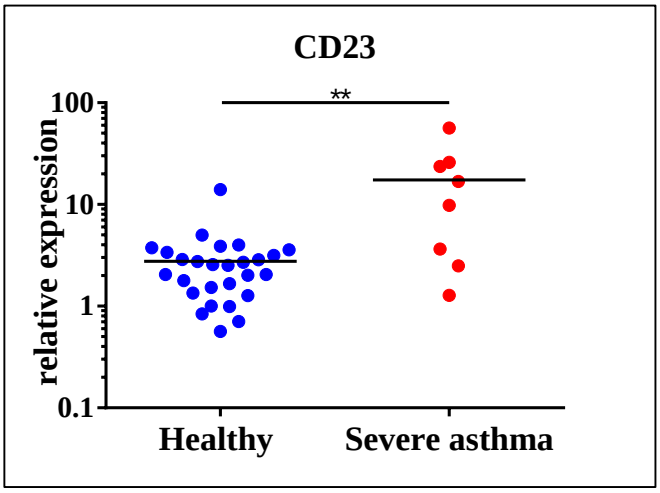


**Figure 3-8 Expression of CD23 is increased in severe asthma AM**
mRNA expression of CD23 in SA-AM (n=8) is increased compared to healthy (n=29) AM. Expression is normalised against GAPDH. **p<0.01 using Mann Whitney test.

As can be seen in Figure 3-8, only 8 SA subjects were used in this evaluation as the expression of CD23 in the remaining 5 was undetectable. Therefore, although this graph has shown that CD23 is increased in SA-AM, *in vivo* the expression of this receptor is extremely low in both clinical states.

Finally we evaluated the expression of FKBP4 and FKBP5. FKBP (FK506-binding protein) is a family of chaperone proteins that interact with the glucocorticoid receptor (GR) and this complex is translocated to the nucleus to activate or suppress specific genes or transcription factors (Zhang et al. 2008). FKBP5 has been shown to be a corticosteroid-induced gene (Kelly et al. 2012) and it was shown to be among the genes most highly induced by ICS in airway epithelial cells of subjects with asthma (Woodruff et al. 2007). However, over-expression of FKBP5 at baseline can inhibit GR signalling and contribute to steroid resistance (Woodruff et al. 2007). The precise effects of steroids on FKBP4 levels are currently unclear but it is likely that they are similar to the effects seen on FKBP5.

Our results show that the expression of both FKBP4 and FKBP5 is significantly increased in SA-AM compared to healthy AM. We were expecting this result because all the patients in the SA group were on treatment with ICS and many

were also on OCS. However, despite this, as can be seen in Figure 3-9 there were a few subjects who had very low expression of FKBP5 in their AM.

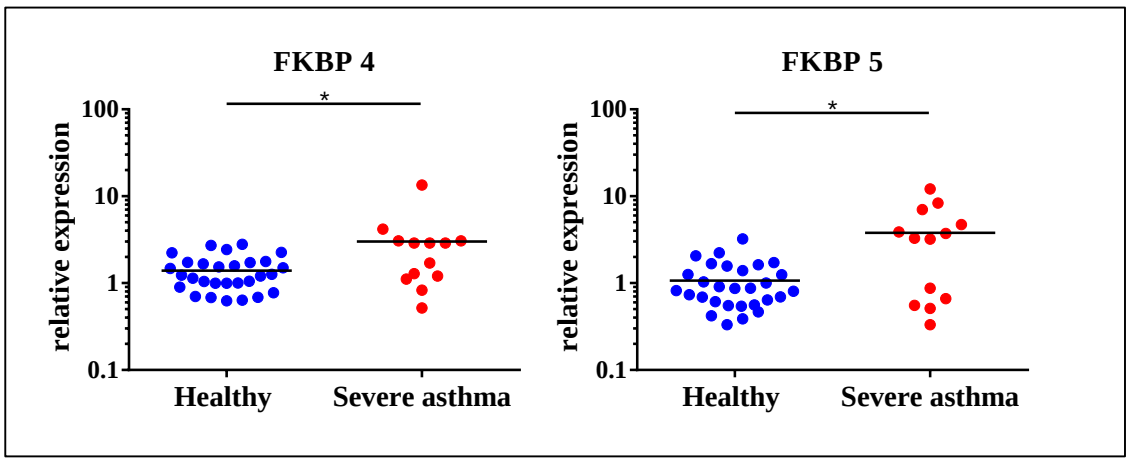


**Figure 3-9 Expression of FKBP4 and -5 is increased in severe asthma AM**
mRNA expression of FKBP4 and FKBP5 in SA-AM (n=13) is increased compared to healthy (n=29) AM. Expression is normalised against GAPDH. *p<0.05 using Mann Whitney test.

### 3.2.2.3 Genes showing similar expression in healthy and severe asthma alveolar macrophages

The expression of all the C-type lectins we evaluated was comparable in healthy and SA-AM. C-type lectins are expressed on the cell surface and are involved in the recognition and internalisation of pathogenic ligands and so they play an important part in the phagocytic role of macrophages. In particular, the mannose receptor, which is a marker of M2 macrophages (Stein et al. 1992) was not raised in SA-AM.

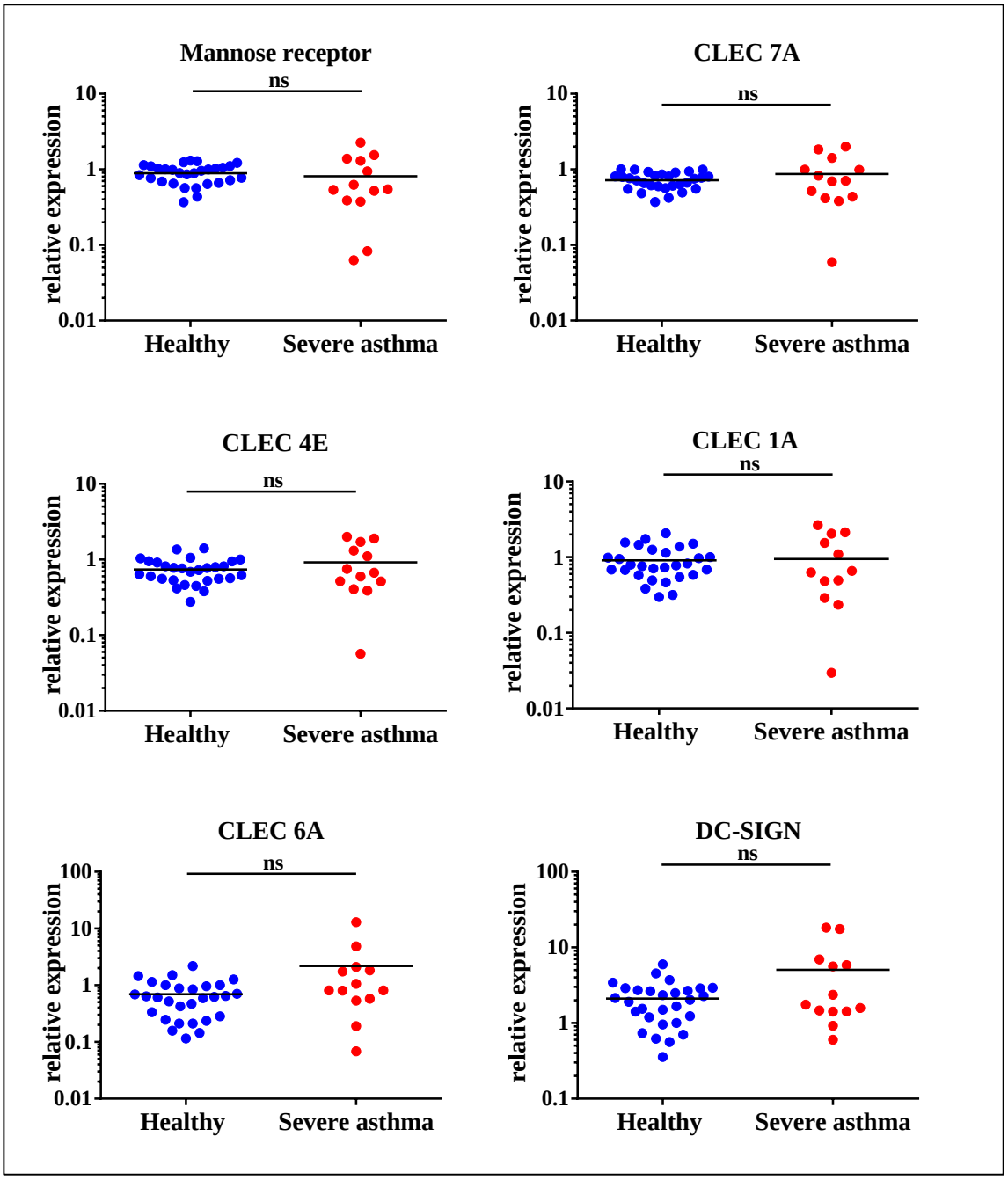


**Figure 3-10 Expression of C-type lectins in healthy and severe asthma alveolar macrophages**
mRNA expression of the Mannose receptor, CLEC 7A, CLEC 4E, CLEC 1A, CLEC 6A and DC-SIGN is not different in healthy (n=29) and severe asthma AM (n=13) AM. Expression is normalised against GAPDH. ns=not significant using Mann Whitney test.

The expression of TLR3 and TLR5 is also comparable in healthy and SA-AM. TLR3 is an endosomal PRR that is activated by viral dsRNA while TLR5 is involved in antibacterial responses as it recognises flagellin, a component of

the flagellae that aid motility of bacteria such as Legionella pneumophila and Pseudomonas aeroginosa (Hayashi et al. 2001).

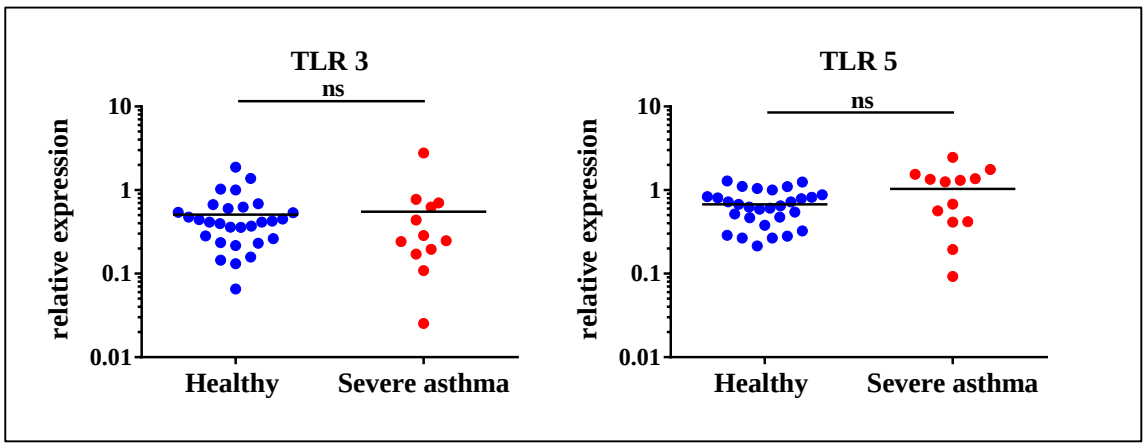


**Figure 3-11 Expression of TLR3 and TLR5 in healthy and severe asthma alveolar macrophages**
mRNA expression of TLR3 and TLR5 is not different in healthy (n=29) and severe asthma AM (n=13). Expression is normalised against GAPDH. ns=not significant using Mann Whitney test.

Finally we found that the expression of TGFβ 1 and TGFβ 2 mRNA is similar in healthy and SA-AM.

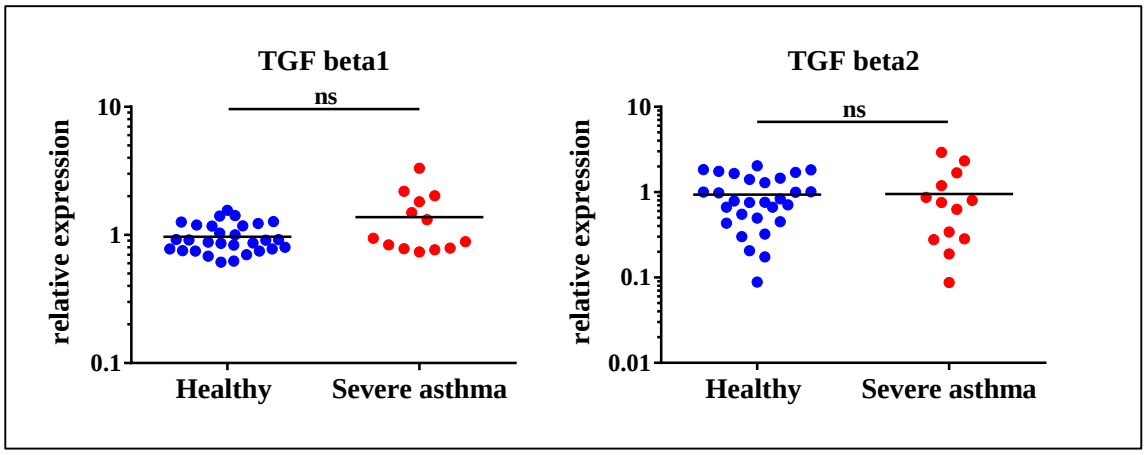


**Figure 3-12 Expression of TGFβ 1 and TGFβ 2 in healthy and severe asthma alveolar macrophages**
mRNA expression of TGFβ 1 and TGFβ 2 is not different in healthy (n=29) and severe asthma AM (n=13). Expression is normalised against GAPDH. ns=not significant using Mann Whitney test.

### 3.2.3 Discussion

Asthma is a complex inflammatory disease of the airways involving several cell types and immune mediators, with most research focusing on eosinophils and CD4+ Th2 cells and the cytokines they secrete including IL-4, IL-5 and IL-13. AM play a key role in promoting the orientation of the adaptive response in a Th1 or Th2 direction and therefore, it has been proposed that AM within the asthmatic airway, where Th2 cytokine levels are increased, are of the M2 phenotype.

Our results show increased mRNA expression of a number of pro-inflammatory cytokines (TNF$\alpha$, IL-6, IL-8, IL-1$\beta$ and IL-23) in SA-AM while the expression of the M2 markers DC-SIGN and the mannose receptor was not increased. This suggests that M1 macrophages are the predominant population within the severe asthmatic airway. CD23, another M2 marker did show increased expression in SA-AM compared to healthy AM, but overall the expression of this receptor was extremely low and at the lower limit of detection by PCR. Therefore, the *in vivo* significance of this finding is unclear.

Traditionally, IL-13 and Th2 cytokines have been accepted as central regulators of the pathophysiologiclal changes associated with asthma. However, it has become apparent that Th2-driven inflammation is prominent in only half of patients with asthma (Woodruff et al. 2009) and non-Th2 driven mechanisms must therefore operate in the remaining half. In persistent and severe asthma, an altered inflammatory profile towards a Th1 type response characterised by an increase in the involvement of neutrophils is seen (Busse et al. 2000). Our patients all had severe persistent asthma with increased numbers of neutrophils in their BAL and therefore it is not completely unexpected that we detected a stronger M1 signal in the AM rather than an M2 signal.

M1 macrophages are usually associated with protection during acute infectious diseases and are induced through LPS, LTA and other microbial product stimulation including intracellular virus (See Figure 1-5 in Introduction). Subjects with severe asthma experience recurrent disease exacerbations (mean 6 exacerbations per year in our cohort) and most of these exacerbations are associated with viral LRT infection and/or bacterial infection (Custovic et al. 2013; Dougherty et al. 2009; Jackson et al. 2011; Kupczyk et al. 2014). RV infection has been shown to increase epithelial cell expression of IL-6 and IL-8

(Corne et al. 1997). Therefore, the increase in M1 signal in SA-AM may be a reflection of the microenvironment they are exposed to, specifically related to the presence of bacteria and virus within the lower airways. However, contrary to this proposal, it has been shown that RV infection of mice with allergic airways disease induces an influx of M2 macrophages (Chung et al. 2014).

IL-23 has the capacity to potently enhance the differentiation of Th17 cells and increased expression of IL-23 in SA-AM may suggest local activation of the IL23-TH17 cell axis. IL-23 production has been shown to be induced in the lung upon viral or bacterial infection and in response to a number of stimuli including TNFα and LPS (Nakajima et al. 2010). Again, this may reflect increased presence of bacterial and viral pathogens within the severe asthmatic airways, which may or may not be associated with disease exacerbation.

TNFα levels in BAL have been shown to be increased in severe asthma subjects and submucosal mast cells are thought to be the primary producer of this cytokine (Howarth et al. 2005). However, as AM are the most predominant immune cell within the lower airway and we have just showed increased mRNA expression of TNFα in AM, it is conceivable that they contribute to the increased TNFα seen within BAL. Increased TNFα would result in enhanced leucocyte migration and activation within the airways, adding to the pathobiology of severe asthma.

Other studies have also failed to identify an M2 signal in asthmatic AM. No increase in the mRNA expression of the M2 markers CCL17, CLEC10A, arginase 1, IL-10, IL-13, CD163, CD206 or CLEC10A was observed in asthmatic AM (Staples et al. 2012) while no difference in the mRNA expression of mannose receptor, TNFα and IL1β was found in asthmatic and healthy AM (Woodruff et al. 2009). This last finding is slightly contrary to ours and is most likely due to differences in the study population. Our patients had severe treatment resistant disease on BTS management step 4/5, while patients in the study by Woodruff *et al* all had mild steroid naïve asthma (BTS management step 1) and it is likely that expression of TNFα and IL1β increases in severe disease.

The expression of a number of TLRs was increased in SA-AM -TLR2, TLR4, TLR6 and TLR9 and all these TLRs are activated by bacterial products. This increased expression may be in response to the local microenvironment i.e.

cytokines and/or bacterial and viral stimuli within the severe asthmatic airway. M1 stimuli, including LPS, IFNγ , IL-6 and TNFα, have been shown to increase the expression of TLR2 and TLR4 in human endothelial cells (Faure et al. 2001) and THP-1 cells (Zarember et al. 2002), a human monocytic cell line, while the Th2 cytokines IL-4 and IL-13 decreased TLR4 expression in intestinal epithelial cells (Mueller et al. 2006). Exposure to Gram-positive and Gram-negative bacteria increased the expression of TLR2 on human monocytes (Beran et al. 2011). Therefore, it is possible that the M1 predominance within the severe asthmatic airway is influencing the expression of these TLRs. However, these M1 cytokines have also been shown to increase the expression of TLR5 in the THP-1 cells and while the expression of this TLR was increased in SA-AM, this was not statistically significant.

A few limitations regarding our characterisation of SA-AM need to be highlighted. Firstly, we only examined the mRNA expression of genes and not the protein expression. Therefore although certain differences exist between healthy and SA-AM at the mRNA stage, these may not necessarily translate to differences in protein expression due to post-trascriptional regulation e.g. due to miRNAs. Secondly, the SA group had fewer subjects compared to the healthy group. The subjects in the SA group were also significantly older than healthy subjects, and more attempts could have been made at age-matching between groups. Finally, our panel of genes investigated could have included a few more M2 markers such as IL-13, IL-10, CD163 and CLEC10A.

We were interested to see that the expression of both FKBP4 and -5 was increased in SA-AM. FKBP5 has been shown to increase with ICS treatment and ours is the first analysis to show a similar trend for FKBP4. However, there were still some SA subjects that showed very low expression of both these genes. This may be due to poor adherence with ICS therapy, which is common in asthma (Heaney et al. 2012) but could also be due to poor deposition of the medication into the distal airways, the site of BAL sampling. The particle size of many commonly used ICS, such as fluticasone and beclomethasone, is too large to enter the distal airways and their overall lung deposition is <20% which could account for an absence in FKBP increase.

In summary, SA-AM appear to be inflammatory and show an increase in several M1 markers. They also show increased expression of a number of TLRs which

may reflect specific stimuli including bacterial presence and a Th1 microenvironment. Increased expression of a number of pro-inflammatory genes would also activate Th17 pathways within the severe asthmatic airways. *In vivo* it is unlikely that M1 and M2 stimuli exist alone and it is more likely that both signatures coexist with the resultant mixed population depending on the balance of signals in the microenvironment. Furthermore, the M1/M2 macrophage polarisation phenotypes are highly plastic to external signals (Davis et al. 2013) and any change in the lung microenvironment could alter their phenotypic features. However the predominance of M1 markers, and lack of significant M2 markers, suggests that the SA-AM are microbicidal and pro-inflammatory. Unfortunately excessive or prolonged M1 activation can be deleterious for the host due to local damage caused by the pro-inflammatory cytokines and other macrophage products e.g. nitric oxide, adding to the pathology within the asthmatic airways.

# Chapter 4 Up-regulation of miRNAs targeting TLR7 in asthma

## 4.1 Up-regulation of miR-150, miR-152 and miR-375 in asthma

### 4.1.1 Introduction

Most of the morbidity and mortality associated with asthma is related to disease exacerbations. These affect quality of life, increase health care use and are even associated with mortality. Additionally, frequent exacerbations have been shown to be associated with disease progression and decline in lung function (O'Byrne et al. 2009). Most exacerbations are associated with viral infections with RV being the most commonly detected virus (Johnston et al. 1995; Wark et al. 2002).

Within the lung, cellular receptors that are part of the innate immune system detect the presence of virus (and other pathogens). Activation of these receptors leads to a series of complex intracellular steps which ultimately lead to the production of IFNs, key anti-viral cytokines. The production of IFN leads to the production of ISGs and together these promote viral clearance and limit viral replication. It is thought that patients with asthma have a deficient innate immune response against viral infection. Both BECs and AMs from patients with asthma have been shown to have a deficient IFN response to RV *in vitro*, compared to healthy subjects (Contoli et al. 2006; Sykes et al. 2012; Wark et al. 2005). The precise mechanism behind this defect is currently unclear.

The treatment of asthma generally includes the use of ICS to suppress inflammation and $\beta_2$-agonists which relieve bronchoconstriction, providing rapid symptomatic relief. This treatment is usually well-tolerated and sufficient for most people with asthma. However, about 10% of patients continue to be symptomatic despite maximal therapy and are termed difficult-to-treat/severe asthma (Wenzel 2005). Due to uncontrolled symptoms and frequent exacerbations this group of patients tend to make the most use of health-care resources and have the highest morbidity (Antonicelli et al. 2004). Frequent

exacerbations lead to loss of work, days missed from school/university and worsening lung function.

A great unmet need exists in our current management of asthma exacerbations, especially for patients who are at the more severe end of the disease spectrum. A greater understanding of the pathophysiology of exacerbations, especially the mechanisms underlying the deficiency in the innate immune system would almost certainly aid in the emergence of novel targeted therapies.

Therefore, to approach this issue, miRNA regulation of genes relative to this area was examined. MiRNAs are small non-coding RNA molecules that exert post-transcriptional control on gene expression. They achieve this by binding to the mRNA and then either cause mRNA degradation or repression of mRNA translation. Over the last decade it has become apparent that deregulation of miRNAs can drive a number of disease states including chronic inflammatory diseases such as rheumatoid arthritis, malignancies such as lung cancer and disorders of the immune system. However the precise role of miRNAs in human asthma, specifically the innate immune response to virus in asthma has not been addressed.

## 4.1.2 Hypothesis and aims

### 4.1.2.1 Hypothesis

MiRNAs targeting key components of the innate immune response to RV are dysregulated in AM from patients with asthma.

### 4.1.2.2 Aims

1. To carry out miRNA microarrays on samples from patients with mild asthma and healthy subjects to highlight disease related alterations in miRNA expression.
2. To use bioinformatic tools to find putative targets of the highlighted miRNAs that may be involved in the innate immune sensing of RV.
3. To confirm deregulation of miRNA (selected in aim 2) in AM from patients with severe asthma using qPCR assays for each individual miRNA.

### 4.1.3 Methods and Results

Subjects were recruited as previously described and they underwent bronchoscopy and BAL. AM were isolated from BAL using the adherence to plastic technique and TRI-Reagent was used to extract the RNA. MiRNA microarrays were performed on RNA from 4 subjects with mild asthma and 4 healthy subjects.

Using Card A and Card B the expression of a total of 745 miRNAs was assessed along with 9 housekeeping genes (3 on Card A and 6 on Card B). These included all the miRNAs that had been discovered and sequenced at the time (2009).

#### 4.1.3.1 Differences in expression of miRNAs in AM from patients with mild asthma and healthy subjects

The clinical characteristics of the subjects used for the microarray are shown in Table 4-1

| | Healthy | Mild Asthma | p value |
|---|---|---|---|
| Number | 4 | 4 | |
| Age | 19.3 (18-21) | 20.5 (18-24) | 0.41 |
| Sex (M/F) | 3/1 | 2/2 | 1.0 |
| $FEV_1$ | 4.7 (0.3) | 4.7 (0.8) | 0.98 |
| %predicted $FEV_1$ | 101.3 (3.4) | 110.8 (8.4) | 0.33 |
| FVC | 5.4 (0.3) | 5.5 (1.0) | 0.94 |
| %predicted FVC | 103.0 (2.6) | 111.3 (8.1) | 0.37 |
| Body Mass Index | 24.0 (0.7) | 21.5 (2.0) | 0.35 |
| Atopy (%) | 0 | 4 (100) | 0.029 |
| BAL cell differential | | | |
| %macrophages | 82.5 (75-92) | 82.7 (69-91) | 0.98 |
| %neutrophils | 4.7 (0.5-13) | 2.6 (1.8-4.5) | 0.51 |
| %eosinophils | 0.6 (0-1.8) | 0.5 (0.3-1.3) | 0.88 |

**Table 4-1 Clinical characteristics of healthy and mild asthma subjects used in the miRNA array**
Values are means with SEM in parenthesis except for age and BAL cell differential for which range is shown and atopy for which % is shown. BAL, bronchoalveolar lavage; $FEV_1$, forced expiratory volume in one second; FVC, forced vital capacity; p values calculated using unpaired t-test except for comparison of sex and atopy for which Chi-square test was used.

The results from the array are displayed as a heat map (Figure 4-1). Only the miRNAs in Card A are shown in this heat map as most of the miRNAs in Card B were undetectable in AM.

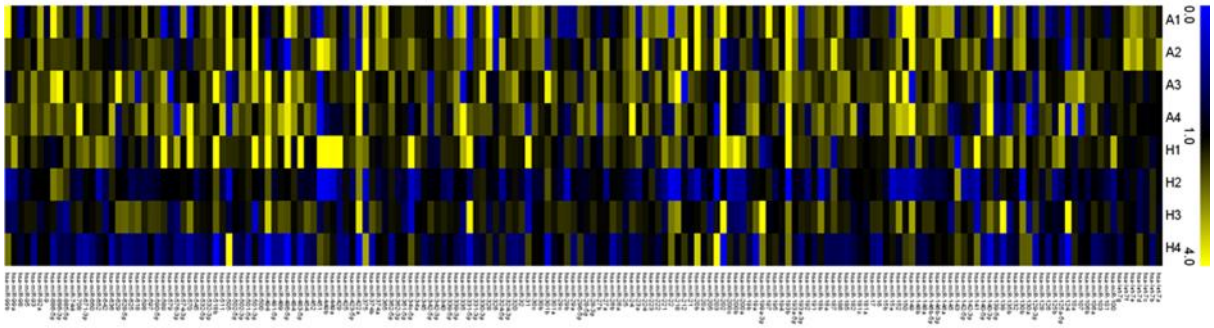


**Figure 4-1 Heat map representing Taqman® Low Density Array expression of miRNAs in healthy and asthmatic AM**
MicroRNA microarray expression of miRNAs (0 to 4 fold, average=1) comparing AM from healthy (H1-4) and mild asthma (A1-4) subjects.

On the heat map, expression of miRNAs varies from 0 (blue) to 4 (yellow) fold and it can be appreciated that more miRNAs show higher expression in asthmatic compared to healthy AM (as indicated by more 'yellow' in the upper four rows representing asthmatic subjects).

Differences in miRNA expression were evaluated by comparing Ct values, which represents the cycle number. For each miRNA, the mean Ct from the 4 samples for mild asthma was compared with the mean Ct from the 4 samples from the healthy group. Of the 745 miRNAs, 29 miRNAs had cycle difference of ≥1 cycle between mild asthma and healthy (Table 4-2). A difference between two Ct values of 1 unit (or 1 cycle) represents a two-fold difference in the expression of the miRNA. This cut-off was used because a 2 fold difference in a miRNA could translate to a biologically relevant difference in the protein expression of the mRNA it targets.

| MicroRNA | Mild Asthma Mean Ct | Healthy Mean Ct | Difference in Ct |
|---|---|---|---|
| miR-10a | 30.80 | 31.85 | 1.05 |
| miR-146b-3p | 29.84 | 31.19 | 1.35 |
| miR-146b-5p | 20.09 | 21.16 | 1.07 |
| miR-149 | 34.15 | 35.27 | 1.12 |
| miR-150 | 24.78 | 26.31 | 1.53 |
| miR-152 | 30.42 | 31.75 | 1.33 |
| miR-186 | 25.68 | 26.94 | 1.26 |
| miR-18b | 35.80 | 36.84 | 1.04 |
| miR-193-b | 31.90 | 34.48 | 2.58 |
| miR-19b | 23.46 | 24.49 | 1.03 |
| miR-20b | 29.13 | 30.83 | 1.7 |
| miR-218 | 29.78 | 31.18 | 1.4 |
| miR-224 | 29.84 | 30.96 | 1.12 |
| miR-24 | 22.54 | 24.07 | 1.53 |
| miR-30c | 23.11 | 24.24 | 1.13 |
| miR-320 | 25.56 | 26.73 | 1.17 |
| miR-326 | 36.04 | 34.56 | 1.48 |
| miR-328 | 27.65 | 28.87 | 1.22 |
| miR-330-3p | 35.38 | 36.50 | 1.12 |
| miR-339-3p | 29.82 | 31.14 | 1.32 |
| miR-375 | 30.48 | 31.57 | 1.09 |
| miR-451 | 33.39 | 31.62 | 1.77 |
| miR-484 | 24.45 | 25.66 | 1.21 |
| miR-502-3p | 31.84 | 32.84 | 1 |
| miR-598 | 30.58 | 31.60 | 1.02 |
| miR-886-3p | 31.58 | 33.15 | 1.57 |
| miR-886-5p | 33.07 | 34.41 | 1.34 |
| miR-92a | 26.90 | 28.01 | 1.11 |
| miR-99b | 34.26 | 35.28 | 1.02 |

**Table 4-2 List of miRNAs highlighted by the microarray**
MiRNAs with at least a two-fold difference in expression in AM from mild asthmatics compared to healthy subjects are listed.

Most of the miRNAs on the table show higher expression in MA compared to healthy. The exceptions are miR-326 and miR-451. This is evident by comparing the mean Ct for each miRNA between the clinical states: the Ct value in MA tends to be lower than the corresponding Ct value in the healthy group. A lower Ct value represents fewer PCR cycles performed before the emitted fluorescence crosses the set threshold and would equate to higher cellular expression. Higher levels of a miRNA tend to lead to lower expression of their protein target. Therefore, in MA, the general trend was for an increase in the expression of a number of miRNAs and this could lead to reduced expression of a range of proteins in AM from patients with asthma.

### 4.1.3.2 Putative protein targets for miRNAs highlighted in microarrays

The next step was to establish the putative protein targets for the miRNAs listed in Table 5-2. This was done using Targetscan version 4.2 (http://www.targetscan.org/vert_42/), a commonly used and widely available bioinformatics resource for miRNAs and genes. This tool is able to provide an extensive list of potential targets for each miRNA that is searched. MiRNAs have several hundred potential targets based on sequence complementarity between the miRNA and the 3'UTR of the target gene (based on Watson-Crick complementarity). Therefore this search tool can only provide 'potential' targets which have to then be confirmed on an individual basis. The list of the potential targets is frequently very long and so PRRs that recognise RV were specifically searched for within the provided lists. These included TLR2, TLR3, TLR7, TLR8, RIG-1 and MDA5.

| | Potential target | |
|---|---|---|
| MicroRNA | TLRs | RLRs |
| miR-150 | TLR7 | - |
| miR-152 | TLR7 | - |
| miR-19b | TLR7 | - |
| miR-218 | TLR2 | - |
| miR-224 | TLR3 | - |
| miR-326 | TLR8 | - |
| miR-328 | TLR2 | - |
| miR-375 | TLR7 | - |

**Table 4-3 List of miRNAs predicted to target virus-relevant pattern recognition receptors**
(Based on Targetscan version 4.2)

Of all the potential targets, TLR7 was highlighted because it appeared to be a target for 4 of the deregulated miRNAs in MA. If all 4 did indeed target TLR7 then synergistically their actions could have clinically significant effects on the expression of TLR7 in AM. An increase in the expression of these miRNAs in asthma would lead to a decrease in the expression of TLR7. This could lead to an impaired ability of the AM to sense the presence of RV and reduced expression of IFN and other anti-viral cytokines. As can be seen in the Table 4-3, none of the miRNAs were predicted to target RIG-1 and MDA5. However, since this search was done a newer version of Targetscan has become available

(Version 6) which predicts that that miR-186 and miR-484 could target MDA5 and RIG-1 respectively.

#### 4.1.3.3 Validation of microarray results

To validate the results from the microarray, individual qPCR assays were carried out for the 4 miRNAs predicted to target TLR7: miR-150, miR-152, miR-375 and miR-19b. This was initially done on AM from 10 subjects with MA and 9 healthy subjects and showed that there was a trend towards higher expression of miR-150, miR-152 and miR-375 in MA, although this was not statistically significant. However, based on the finding that there was a trend towards increased expression of the selected miRNAs in asthma we decided to evaluate the expression of the 4 miRNAs in patients with severe asthma, our group of interest. The clinical characteristics of the subjects used in this evaluation are shown in Table 4-4.

| | Healthy | Severe Asthma | p value |
|---|---|---|---|
| Number | 15 | 15 | |
| Age | 20.1 (18-23) | 43.9 (23-63) | <0.0001 |
| Sex (M/F) | 6/9 | 4/11 | 0.70 |
| $FEV_1$ | 4.1 (0.2) | 2.1 (0.2) | <0.0001 |
| %predicted $FEV_1$ | 99.5 (2.7) | 68.5 (7.0) | 0.0004 |
| FVC | 4.7 (0.3) | 3.1 (0.2) | 0.0011 |
| %predicted FVC | 98.9 (2.8) | 85.9 (4.7) | 0.04 |
| BMI | 23.1 (0.6) | 33.6 (2.1) | 0.0005 |
| Atopy (%) | 0 | 9 (64)* | 0.0002 |
| ACQ score | N/A | 2.8 (1.9-4.9) | - |
| ICS dose in mcg (BDP equivalent) | N/A | 2300 (1000-4000) | - |
| Number on daily oral steroids (%) | N/A | 4 (27) | - |
| Number of exacerbations in previous year | N/A | 6.2 (2-12) | - |
| BAL cell differential | | | |
| %macrophages | 86.3 (75-95) | 61.8 (22-92) | 0.001 |
| %neutrophils | 4.2 (0.5-14) | 13.2 (2.5-68) | 0.008 |
| %eosinophils | 0.4 (0-2) | 3.4 (0-30) | 0.033 |

**Table 4-4 Clinical characteristics of healthy and severe asthma subjects used for evaluation of miRNA expression**
Values are means with SEM in parenthesis except for age, ACQ score, ICS dose, number of exacerbations and BAL cell differential for which range is shown and atopy and number on oral steroids for which % is shown. ACQ, asthma control questionnaire; BAL, bronchoalveolar lavage; BDP, beclometasone; $FEV_1$, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroid; N/A, not applicable; * data unavailable for 1 subject. p values calculated using Mann Whitney test except for comparison of sex and atopy for which Chi-square test was used.

Data from these two comparisons (healthy vs MA and healthy vs severe asthma) are combined and presented in Figure 4-2.

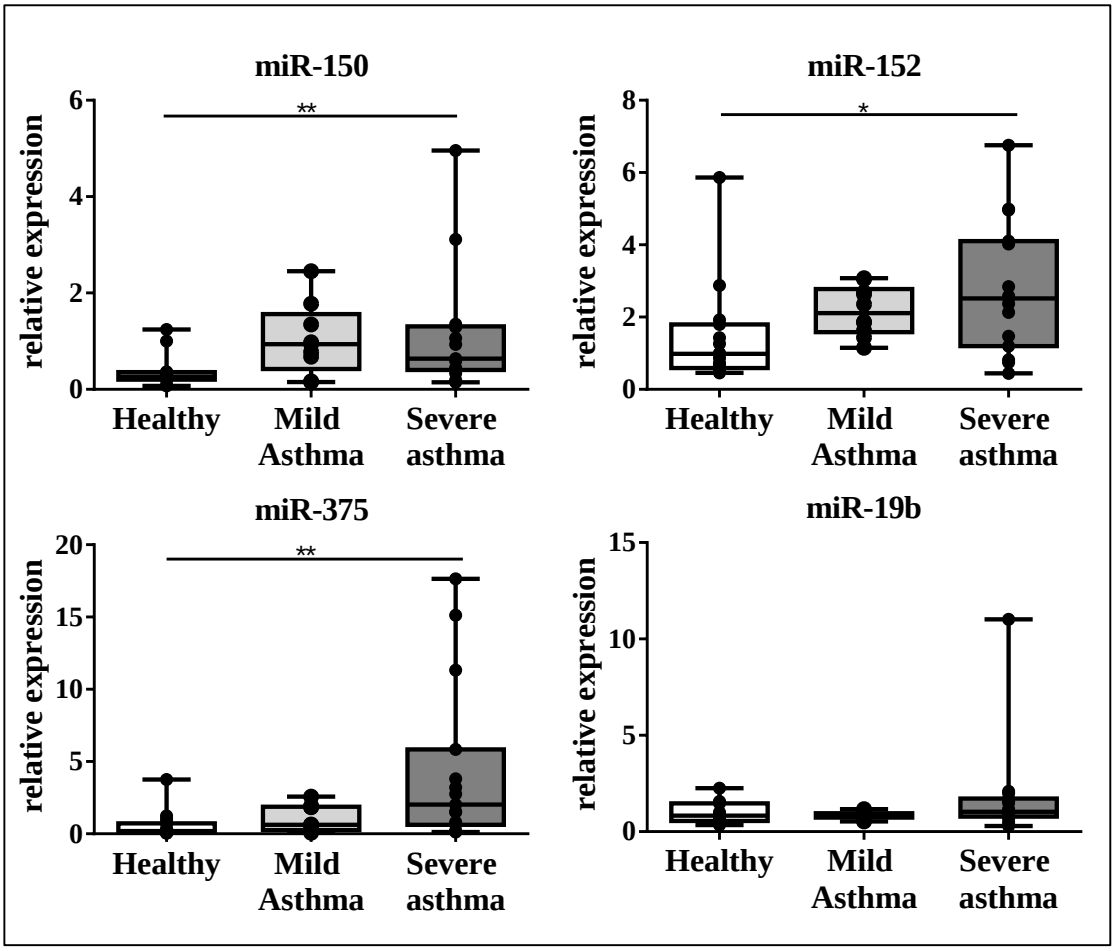


**Figure 4-2 Expression of miR-150, miR-152 and miR-375 is increased in severe asthma compared to healthy AM**
Expression of miR-150, miR-152, miR-375 and miR-19b was evaluated in AM from healthy (n=15), mild asthma (n=10) and SA subjects (n=15). The expression of the miRNAs was normalised against the housekeeping gene RNU44. *p<0.05, **p<0.01, ns=not significant; comparisons done using Kruskal Wallis test with between group testing using Mann Whitney test if results found to be significant.

These results demonstrate that the expression of miR-150, miR-152 and miR-375 is significantly increased in SA-AM, while the expression of miR-19b is comparable in healthy and SA-AM. Based on these results it was decided to focus on miR-150, miR-152 and miR-375.

To ensure that other miRNAs that may be relevant to TLR7 expression, but which had not been highlighted in the original array were not overlooked, we used in silico tools (Targetscan) to searched for miRNAs with binding sites in the 3'UTR of TLR7. This showed that several hundred miRNAs could potential target TLR7 and to simplify the analysis we focused on the top 15 miRNA

families that were broadly conserved among vertebrates. The relative expression of these miRNAs, in AM, was then cross-referenced based on the Ct values obtained in the microarray. It has been shown that in order for efficient repression of target gene expression to occur, a threshold level of miRNA expression is required (Brown et al. 2007) and thus miRNAs that are expressed at low levels within a cell have little or no role in regulating gene expression. A lower Ct value would indicate higher expression, justifying further evaluation, compared to a very high Ct value which would indicate lower expression (Table 4-5). Generally, Ct values of over 35 are thought to indicate extremely low expression in the cell and unlikely to be of clinical significance.

| MicroRNA | Expression in AM (as per microarray) |
|---|---|
| miR-183 | 40 Ct |
| miR-338/338-3p | 35-36 Ct |
| miR-130ac/301ab/301b/301b-3p, 454/721/4295/3666 | 301: 30-31 Ct |
| miR-101/101ab | 30 Ct |
| miR-148ab-3p/152 | 30 Ct |
| miR-19ab | 27 Ct |
| miR-9/9ab | 34/35 Ct |
| miR-150/5127 | 25 Ct |
| miR-216a | Expression undetermined |
| miR-133abc | Expression undetermined |
| miR-219-5p/508/508-3p/4782-3p | Expression undetermined |
| miR-96/507/1271 | Expression undetermined |
| miR-15abc/16/16abc/195/322/424/497/1907 | miR-15: 28-30 Ct |
| miR-182 | 37 Ct |
| miR-144 | Not one of the miRNAs in array |
| miR-375 | 31 Ct |

**Table 4-5 List of other miRNA relevant to TLR7 and their relative expression in AM**
Abundance of other miRNAs relevant to TLR7 in AM (based on their Ct value in the microarray). MicroRNAs highlighted in red are the 3 that we have shown to be increased in severe asthma AM. MicroRNAs highlighted in green are the ones we evaluated further.

Based on this, the expression of miR-301, miR-101 and miR-15a was evaluated in healthy and SA-AM. MiR-144 had not been included in the miRNA array so the relative expression of this miRNA in AM was unknown. Therefore we also evaluated its expression in healthy and SA-AM. Our analysis showed that the expression of miR-144 was below the threshold of detection by qPCR and the

expression of miR-301 and miR-15a was not altered in SA compared to healthy AM. However, the expression of miR-101 was decreased in SA-AM compared to healthy AM which could theoretically lead to an increase in TLR7 expression in SA-AM

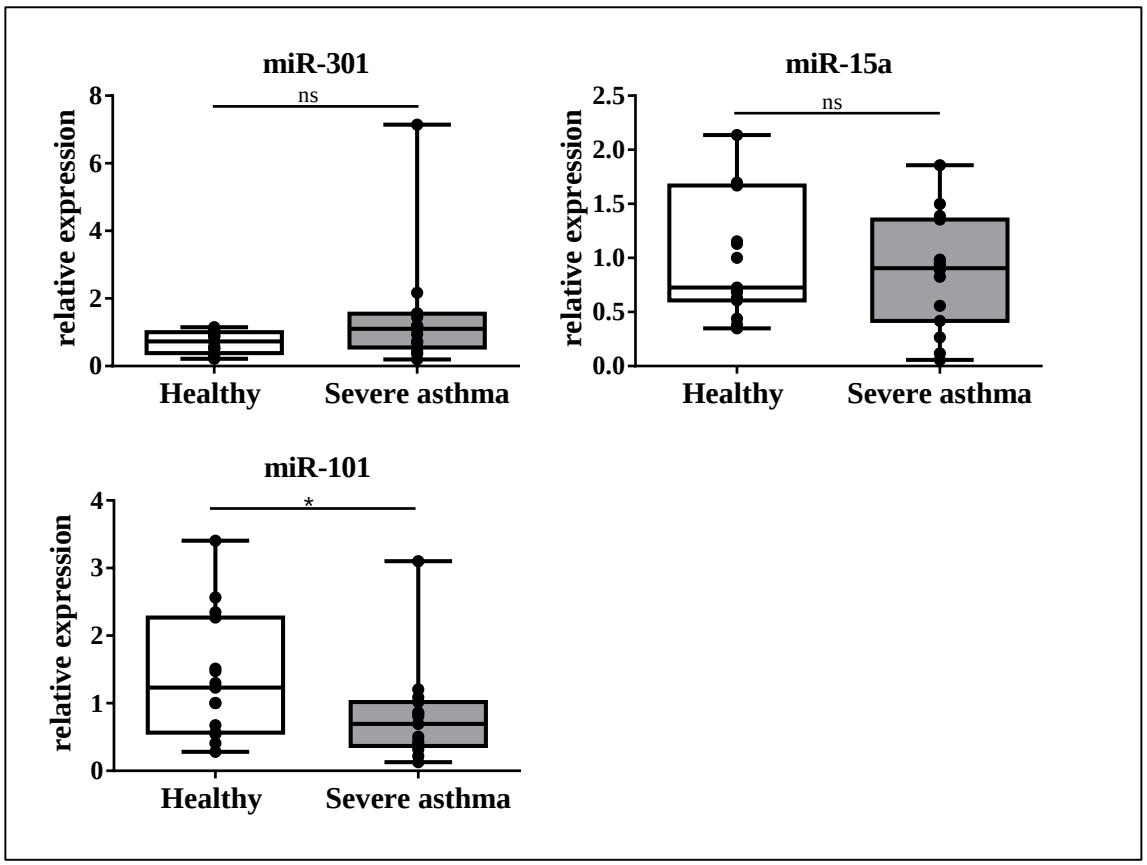


**Figure 4-3 Other TLR7 relevant miRNAs are not increased in severe asthma**
Expression of miR-301 and miR-15a is not increased in severe asthma AM (n=15) compared to healthy (n=15) while the expression of miR-101 is significantly higher in the latter. The expression of the miRNAs was standardised against RNU44. *p<0.05, ns=not significant using Mann Whitney test.

## 4.1.4 Discussion

MiRNAs are key cellular regulators of gene expression and this property endows them an important role in the pathogenesis of several disease conditions. However their role in the pathogenesis of human asthma, especially asthma exacerbations is yet to be clearly defined.

The results from the miRNA microarrays demonstrated increased expression of a number of miRNAs in AM from subjects with mild asthma compared to healthy volunteers. It was decided to carry out the arrays on AM from mild

asthma rather than severe asthma because the former is more likely to represent a more homogeneous group. All the mild asthmatics were on BTS Step I of treatment i.e. not on ICS, which due to their anti-inflammatory properties may influence the expression of miRNAs. This group of subjects were also all atopic and all non-smokers and they were well matched to the healthy group in terms of age, lung function and BMI. In contrast, patients with severe asthma represent a very heterogeneous group. Clinically a number a phenotypes are identified and it is also becoming apparent that cytokine expression varies greatly between patients (Wenzel 2012). In this study, as the patients with SA were recruited from the respiratory clinic, there was a great variability in age, duration of disease, atopic status and other co-morbidities within this group. Any of these factors could potentially influence miRNA expression and because the arrays were only going to be carried out for a small sample size, it was decided against using samples from the severe asthma group.

Employing bioinformatics *in silico* tools it was revealed that these deregulated miRNAs could potentially target a number of TLRs, many of which are important in the innate immune response to virus in asthma. As miRNAs can have several hundred targets, PPRs specifically involved in the innate immune recognition of RV were focused on. In this primary search, members of the IFN signalling pathway were not searched for. The reason for this was because previous reports have shown that exogenous IFNβ restored the deficient innate antiviral response in asthmatic BECs *ex vivo*, inhibiting RV replication to levels similar to those seen in normal BECs (Wark et al. 2005). This suggests that the deficiency in asthma is associated with the production of IFNβ , rather than the actions of IFNβ as the latter pathway appears intact. Therefore it is more likely that the defect is in the pathways leading up to the production of IFNβ i.e. recognition of virus and the intracellular signal transduction activated by PRRs.

The array results also show that the differences in expression of miRNAs between the 2 clinical states are not very large i.e. they are not as high as 5 cycles or 10 cycles (a 5 cycle difference equates to a 32-fold difference in expression, while a 10 cycle difference equates to a 1024-fold difference). This probably reflects a protective mechanism unique to the lung which is required to ensure that the integrity of the respiratory, especially alveolar,

surfaces are maintained to allow for the important function of gas exchange. Large differences in miRNA expression in AM could lead to profound alterations in protein expression which could lead to catastrophic damage to the delicate alveolar surfaces. However, differences such as those detected by the performed arrays should not be ignored because working synergistically a group of miRNAs could profoundly affect proteins in a biologically relevant pathway. A large majority of the miRNAs evaluated in the microarray were shown to be up-regulated in AM from MA compared to healthy subjects. In contrast most, unpublished data from our group has shown that most miRNAs are downregulated in bronchial epithelial cells from MA compared to healthy subjects. Therefore, it is likely that the general increase in miRNA expression in AM (or decrease in bronchial epithelial cells) is a cell specific effect.

Microarrays are a far less sensitive method for measuring miRNA expression compared to individual qPCR assays. Therefore, individual assays were carried out for a selected group of miRNAs, all of which are predicted to target TLR7. MiRNAs targeting TLR7 were selected rather than TLR2, TLR3 or TLR8 for the following reasons:

1. *In silico* analyses revealed multiple miRNAs were predicted to target TLR7 while only one miRNA was predicted to target TLR3. Multiple miRNAs targeting a single gene may act synergistically leading to a larger reduction in gene expression.
2. The miRNA predicted to target TLR8 (miR-326) is down-regulated in mild asthma compared to healthy subjects. This would not cause reduced expression of TLR8 in asthma and if miR-326 expression is indeed significantly reduced in asthma, it could even promote increased expression of this TLR in AM in asthma.
3. TLR2 has been shown to be activated by components of the viral capsid leading to the production of pro-inflammatory cytokines (Saba et al. 2014; Triantafilou et al. 2011). At present it is unclear if TLR2 signalling contributes significantly to IFN production.

However, the expression of miR-101, another TLR7 relevant miRNA was significantly reduced in SA-AM compared to healthy and so could theoretically counteract some of the inhibitory effects of miR-150, miR-152 and miR-375 on TLR7 expression.

TLR7 was also an attractive and biologically important receptor to focus on because it recognises ssRNA. RV is a ssRNA virus which undergoes minimal to no replication within AM (Gern et al. 1996; Saba et al. 2014). However, the AM does recognise the presence of RV and produces anti-viral cytokines that have important protective effects on nearby epithelial cells (Korpi-Steiner et al. 2010). Therefore, it is most likely that TLR3, RIG-1 and MDA5 play a negligible role in AM as they all are usually activated by dsRNA formed during viral replication. *In vitro* studies with BECs have shown that only 5-10% of these cells get infected with RV (Chen et al. 2006; Mosser et al. 2002) and so only this small percentage of cells will be producing protective IFN. Therefore paracrine IFN produced by AM would be paramount in protecting and creating an 'anti-viral' state in these non-infected cells.

As a group, the severe asthma subjects were older and had a higher BMI than the healthy subjects. Almost 50% of the severe asthma subjects (n=304) in SARP had a BMI over 30 while an interim analysis of 200 subjects in the Wessex Severe Asthma Cohort showed that the mean BMI was 32, not too dissimilar from a mean BMI of 33 in our group of severe asthma subjects. In retrospect we should have attempted to age-match the healthy subjects we recruited for this part of the analysis as age could theoretically affect miRNA expression. However, during normal lung aging miRNA expression has been shown to remain unchanged (Williams et al. 2007).

The above presented results could have important clinical implications. An increase in the expression of 3 miRNAs targeting TLR7 could lead to significant reductions in the expression of this protein in AM. Reduced expression of TLR7 would impair the ability of AM to sense the presence of RV and lead to reduced production of IFN. The downstream effect of this would be increased viral replication and reduced viral clearance. This would lead to worsening LRT symptoms, more prolonged illness, and impairment in lung function and clinically manifest as an asthma exacerbation. Therefore the obvious next step was to confirm that the three miRNAs do indeed directly target TLR7.

# 4.2 MicroRNA-150, -152 and -375 directly target TLR7

## 4.2.1 Introduction

Each miRNA has hundreds of evolutionary conserved targets and several times that number of non-conserved targets (Bentwich et al. 2005). Bioinformatics tools can be used to generate a list of potential target genes for a miRNA. The algorithms they use are based on complementarity of bases between the miRNA and 3'UTR of the target gene. Mature miRNAs are embedded within a larger RNA-protein complex known as the RISC. Within the RISC, the miRNA selects its target, largely based on complementary base-pairing between the miRNA and the mRNA transcript. This brings the mRNA transcript within range of the RISC effector proteins and this interaction usually leads to either translational inhibition or mRNA degradation. In both cases the end result is reduced protein expression. Multiple miRNAs can bind within the 3'UTR of a single mRNA and it is likely that it is the balance between these associations that determines the expression of the gene and the biological significance (Peter 2010). In addition to base-pair complementarity, other factors are involved in the regulation that miRNAs exert on gene expression (described in Section 1.5.4). It is also important to be aware that computational algorithms can yield false positive (miRNA:mRNA predictions that are statistically significant but cannot be verified) and false negative (miRNA:mRNA predictions that are true pairs, but missing from the results) interactions. Therefore, in this section we set out to validate the prediction that miR-150, miR-152 and miR-375 can bind to sites within the 3'UTR of TLR7 to reduce the expression of this gene.

## 4.2.2 Hypothesis and objectives:

### 4.2.2.1 Hypothesis

MiR-150, miR-152 and miR-375 directly target the 3'UTR of TLR7 and work synergistically to reduce the translation of TLR7.

#### 4.2.2.2 Objectives

1. Clone the 3'UTR of TLR7 in the reporter vector pRL-TK and co-transfect this vector with or without vectors that overexpress each of the 3 miRNAs. Additionally, compare these results with the effect of a vector over-expressing all three miRNAs together.
2. Perform site-directed mutagenesis of the predicted binding sites for the three miRNAs within the 3'UTR of TLR7. Co-transfect these constructs with or without vectors over-expressing each of the three miRNAs.

### 4.2.3 Methods and Results:

In addition to using the bioinformatics tool TargetScan, we also checked other bioinformatics databases (miRanda and miRecords) to ensure that the seed sequences for miR-150, miR-152 and miR-375 all have binding sites in the 3'UTR of TLR7. Figure 4-4 shows the predicted binding sites for the 3 miRNAs in the 3'UTR of TLR7 as generated by miRanda (http://www.microrna.org/microrna/getMirnaForm.do). MiR-150 has 3 predicted sites while miR-152 and miR-375 bind at one site each. Only 2 of the 3 miR-150 sites were studied as the interaction of miR-150 with the third site was thought to be much weaker.

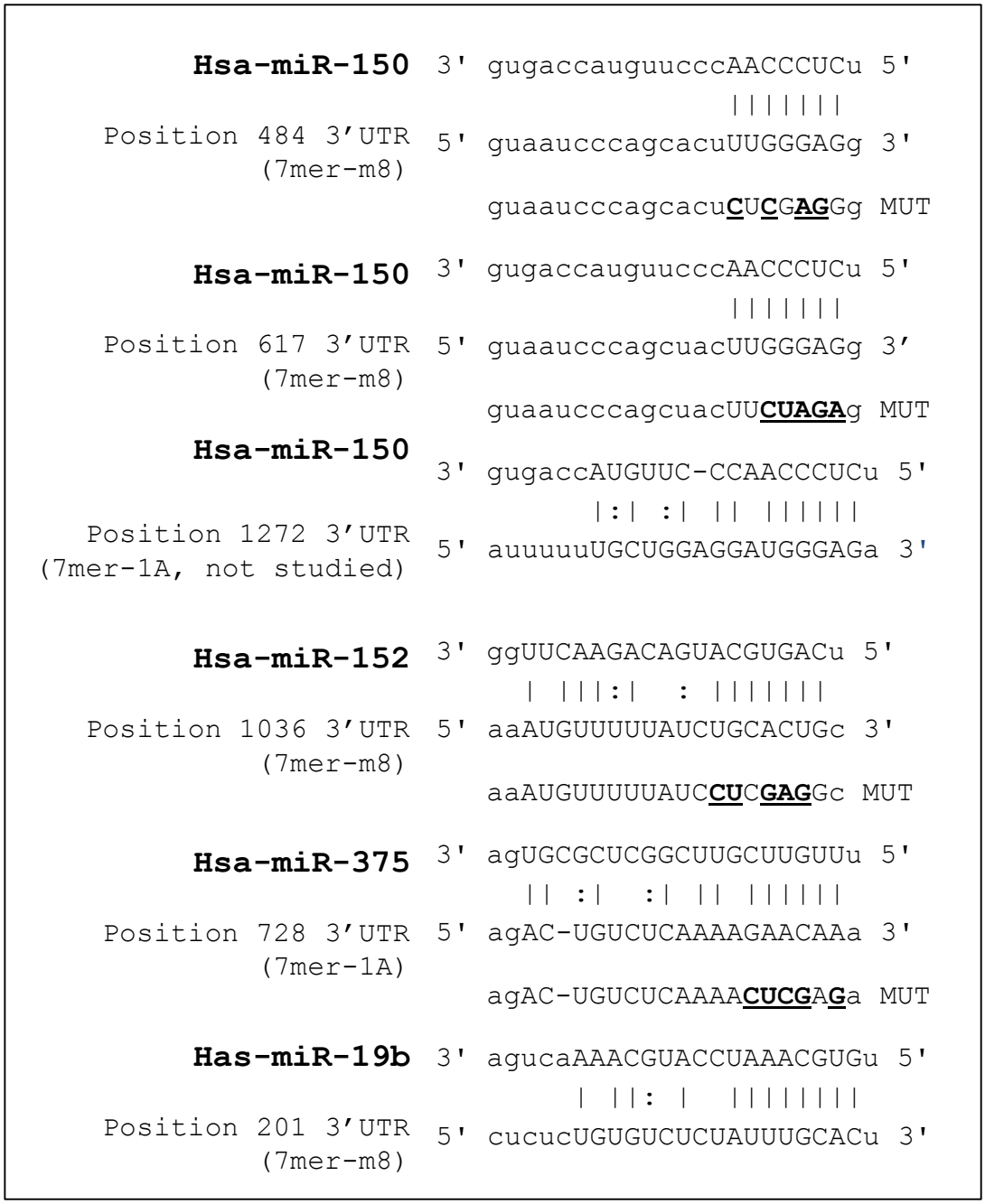


**Figure 4-4 Alignments of target binding sites on the 3'UTR of TLR7 with seed sequences of miR-150, miR-152 and miR-375**
miRanda-generated alignments of target sites on the 3'UTR of TLR7 with seed sequences of miR-150, miR-152 and miR-375. Also shown are sequences of mutants generated (MUT). The mutated nucleotides in the target sites are underlined.

To confirm a direct molecular link between miR-150, miR-152 and miR-375, the 3'UTR of TLR7 was cloned at the 3' end of the Renilla luciferase reporter gene harboured in the pRL-TK vector. This recombinant plasmid (pRLTK_3'UTR_TLR7) was termed WT (wild-type) and was co-transfected into

HeLa cells along with pCDNA3.1_150, pCDNA3.1_152 or pCDNA3.1_375. As a control, pCDNA3.1 (-) was also co-transfected into cells. The results from this transfection were compared to a set in which the 3'UTR of TLR7 was mutated at the binding sites for miR-150 (pRLTK_MUT_3'UTR_TLR7_150_1 and pRLTK_MUT_3'UTR_TLR7_150_2), miR-152 (pRLTK_MUT_3'UTR_TLR7_152) and miR-375 (pRLTK_MUT_3'UTR_TLR7_375). These vectors were designed as described in section 2.10.2.

As an additional control a pRL-TK empty vector i.e. not containing the 3'UTR of TLR7 was co-transfected into cells along with pCDNA3.1_150, pCDNA3.1_152 or pCDNA3.1_375 to ensure that the actions of the miRNAs were specifically on the 3'UTR of TLR7. The transfections were done 3 times in duplicate and measurements were made using the Dual-Luciferase® assay system. The results are shown in Figure 4-5.

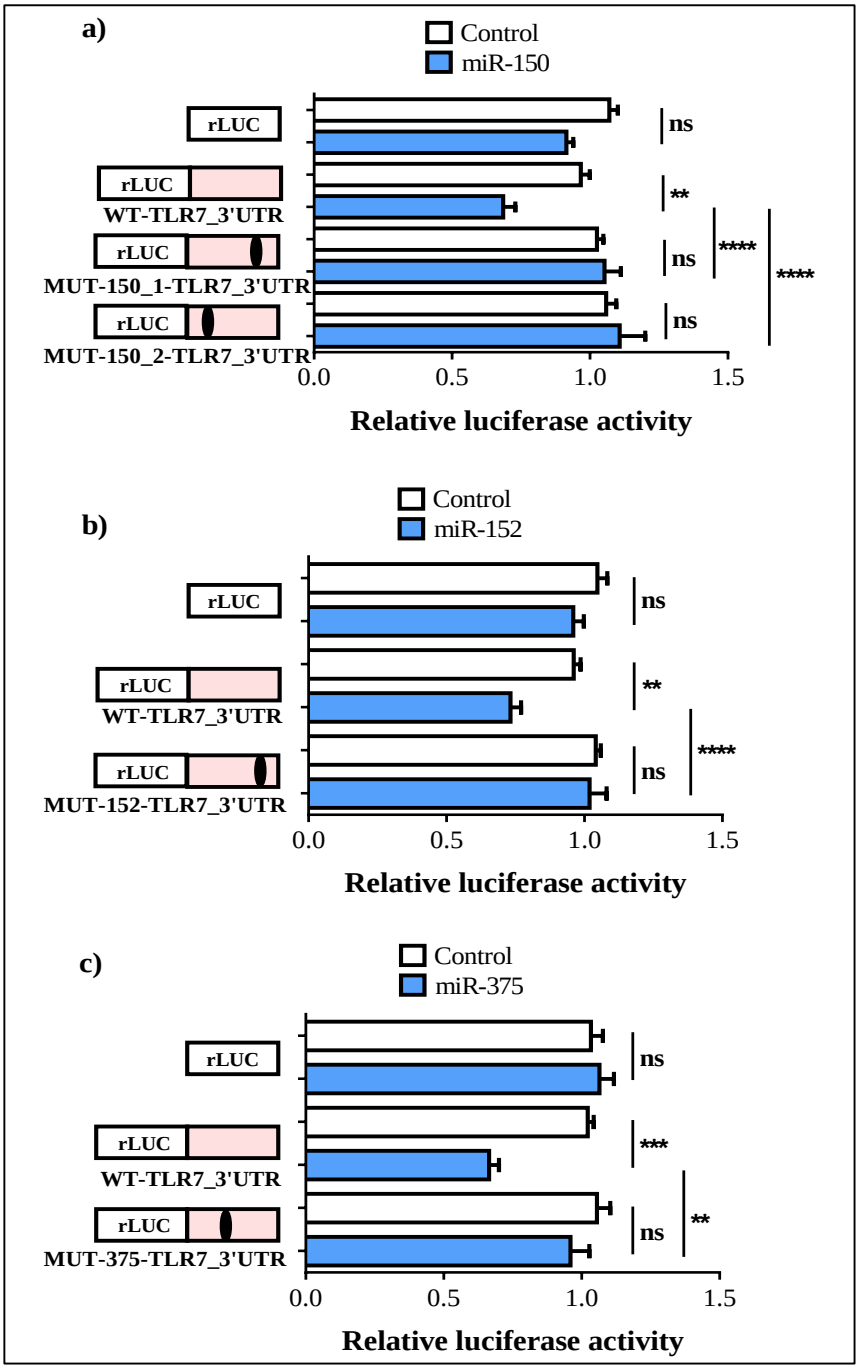


**Figure 4-5 Mir-150, miR-152 and miR-375 directly target the 3'UTR of TLR7**
Luciferase assay results showing the action of miR-150 (a), miR-152 (b) and miR-375 (c) on the 3'UTR of TLR7. Transfection of each miRNA with the pRL-TK empty vector (containing renilla luciferase, rLUC only) has no effect on luciferase activity. Transfection of each miRNA reduces rLUC activity of the TLR7 reporter (WT-TLR7). The effect is abrogated when the predicted binding sequences for the respective miRNAs are mutated- MUT-150_1-TLR7 and MUT-150_2-TLR7 for miR-150, MUT-152-TLR7 for miR-152 and MUT-375-TLR7 for miR-375. ns=not significant, **p<0.01, ***p<0.001, ****p<0.0001; p values were calculated using 1-way ANOVA with Sidak multiple comparison's test. Data is shown as mean+SEM.

As can be seen from Figure 4-6 adding each of the miRNAs to a pRL-TK empty vector has no effect on luminescence. When the miRNA is added to a vector containing the 3'UTR of TLR7 a significant reduction in luminescence is observed indicating that the miRNA is binding to the 3'UTR to reduce luciferase translation. When the specific binding site for each of the miRNAs is mutated, addition of the miRNA has no effect on luminescence i.e. NO reduction in luminescence is seen. This implies that the miRNAs are unable to bind to each of their specific sites and therefore unable to exert their inhibitory effects on translation.

In order to investigate if together miR-150, miR-152 and miR-375 had a synergistic effect on the expression of TLR7, pCDNA3.1_mix i.e. containing all 3 miRNAs was co-transfected into Hela cells along with pRLTK_3'UTR_TLR7. The effect of this was compared to co-transfecting pCDNA3.1_150, pCDNA3.1_152 or pCDNA3.1_375 on their own. As a control, pCDNA3.1 (-) was co-transfected into cells. All transfections were normalised using pGL3 (Firefly), done 3 times in duplicate and measurements were made using the Dual-Luciferase® assay system. The results can be seen in Figure 4-6.

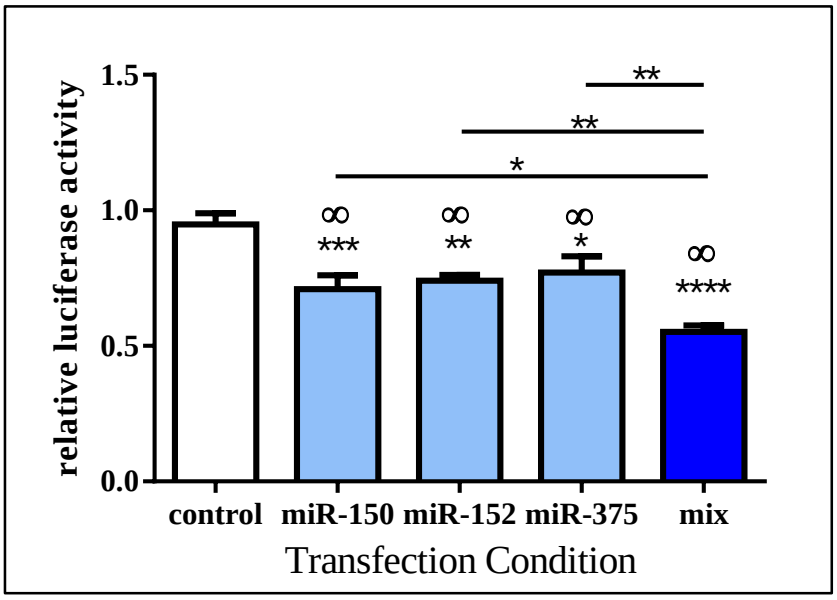


**Figure 4-6 Luciferase Assay showing the three miRNAs together have a greater inhibitor effect on the expression of TLR7**
pRLTK_3'UTR_TLR7 was co-transfected with either pCDNA(-) (control), pCDNA3.1_150, pCDNA3.1_152, pCDNA3.1_375 or pCDNA_mix and luminescence production was measured. *p<0.05, **p<0.01, **p<0.001, ****p<0.0001, ∞ compared to control; p values were calculated using 1-way ANOVA with Sidak's multiple comparisons test. Data are presented as mean+SEM.

As can be seen from Figure 4-6, compared to when pCDNA(-) is co-transfected with pRL-TK_3'UTR_TLR7 (the control sample) co-transfecting each miRNA in isolation leads to significant reductions in luciferase activity but all 3 miRNAs (mix) together leads to a much greater reduction in luciferase activity (about 50%). As the reduction in luciferase activity caused by the 'mix' is significantly greater than the reduction observed when each miRNA is co-transfected in isolation it suggests that the miRNAs may be operating in a coordinated fashion.

### 4.2.4 Discussion

Computational algorithms can be used to predict targets for a miRNA. In addition to sequence complementarity between the miRNA and target mRNA, other parameters used by these algorithms include thermodynamic stability of the miRNA:mRNA duplex, accessibility of the target binding site(s), clustering and position of the binding sites within the 3'UTR of the mRNA and interaction of other RNA-binding proteins. We initially used the TargetScan Database (version 4.2) but cross-referenced our finding using other in silico databases to ensure that a database that may prioritise different parameters when predicting miRNA targets also predicted that miR-150, miR-152, and miR-375 could target TLR7. Given the complexity of any potential interaction between miRNA and mRNA target, computational algorithms cannot be solely relied on and any predicted target needs to be further verified.

The results presented above confirm that miR-150, miR-152 and miR-375 bind to the 3'UTR of TLR7 to reduce its expression. Figure 4-5 shows significantly less luminescence when vectors over-expressing miR-150, miR-152 or miR-375 are co-transfected with pRLTK_3'UTR_TLR7. Reduced luminescence equates to reduced translation of the renilla gene due to inhibitory effects of the miRNAs acting within 3'UTR of TLR7 fused to this gene. Importantly when the putative binding site for each miRNA is mutated, the miRNAs are unable to exert their inhibitory action on translation and no change in luminescence is observed. This implies that no reduction in the translation of luciferase has occurred due to the inability of the miRNA to bind to the 3'UTR.

We next explored if the 3 miRNAs could act synergistically in inhibiting the translation of TLR7. Figure 4-6 shows that when the vector containing all 3 miRNAs was over-expressed in the cell, far greater reduction in luminescence

was observed compared to over-expression of each miRNA singly. This suggests that the 3 miRNA may be working together, leading to additive effects on gene expression. These findings are consistent with the concept that miRNAs frequently operate in a coordinated fashion to have considerable greater biological effect than individual miRNAs alone (Peter 2010). Figure 4-4 shows the position of the binding sites for the 3 miRNAs within the 3'UTR of TLR7. The 2 binding sites for miR-150 and the site for miR-375 are in fairly close proximity to each other (miR-150_1 position 498-504, miR-150_2 position 631-637, miR-375 position 742-748). It has been shown that tandem binding sites have a synergistic effect on the expression of the target, even when the binding sites correspond to different miRNAs (Hon et al. 2007; Krek et al. 2005).

Taken together these results confirm a new role for miR-150, miR-152 and miR-375 in the regulation of the expression of TLR7. The inhibitory action of these three miRNAs on 3'UTR of TLR7 has not been described previously and our results clearly demonstrate that TLR7 is an actual target of miR-150, miR-152 and miR-375, not just a putative target. Furthermore, we have been able to demonstrate that together these three miRNAs are likely to behave synergistically and have a more significant effect on the expression of TLR7 than each working individually.

As was shown in Figure 4-3 miR-101 could theoretically oppose some of the inhibitory effects that these 3 miRNAs have on TLR7 expression. In retrospect we should have evaluated if miR-101 could directly target the 3'UTR of TLR7 with the luciferase assay as we have done for these 3 miRNAs. However at the time we decided to proceed with the evaluation of TLR7 expression in SA-AM in the first instance.

# Chapter 5 Reduced expression and function of TLR7 in severe asthma

## 5.1 Severe Asthma AM have a reduced IFN response to rhinovirus

### 5.1.1 Introduction

RV is a ssRNA virus belonging to the *Picornavirus* family. Within host cells a dsRNA intermediary is formed during viral replication and both the single stranded and dsRNA genomes activate cellular PRRs to initiate the innate immune response to infection. This anti-viral response is orchestrated by IFNs and the subsequent rapid induction of ISGs (Khaitov et al. 2009; Schoggins et al. 2011). Together they limit viral replication, limit viral budding from infected cells and activate the adaptive immune system (Le Bon et al. 2002; Schoggins et al. 2011). Until recently, the predominant airway cell type shown to be infected by RV and support its replication was the airway epithelial cell. However AM are the most numerous immune cell within the lower airways (Ancochea et al. 1993) and it is conceivable that RV directly infects AM. It has been shown that RV co-localises with the macrophage marker CD68 in biopsies of experimentally infected humans with asthma (Bentley et al. 2013). Additionally, these immune cells release cytokines that have paracrine effects on nearby BECs activating them to participate in the anti-viral response (Korpi-Steiner et al. 2010).

Previous work has suggested that the vulnerability of asthmatic subjects to RV is due to a defective IFN response to the virus. Studies have shown that structural and BAL cells from mild-to-moderate asthmatic subjects (on BTS treatment stages 1-3) have an impaired IFN response to RV *ex vivo* (Contoli et al. 2006; Sykes et al. 2012; Wark et al. 2005). Clinically, this deficiency translated to a more prolonged respiratory illness and significant worsening in lung function (Contoli et al. 2006). However, more recently, cultured epithelial cells from well-controlled mild-to-moderate asthmatic subjects were found to have comparable RV-induced IFN responses to healthy subjects. Therefore, it remains uncertain whether asthmatic airway cells do indeed have impaired IFN

responses to RV. Furthermore, IFN responses of airway cells from adults with severe asthma have not been studied.

About 10-20% of patients with asthma have severe or difficult-to-treat disease (Bateman et al. 2004; Bousquet et al. 2010). These patients tend to suffer with regular disabling symptoms despite maximal medical therapy and get recurrent exacerbations. As a result, quality of life is significantly impacted, numerous comorbidities develop and baseline lung function is low. Exacerbations are also associated with a greater decline in lung function, which in most cases will cause greater disability. As mentioned previously these exacerbations are frequently caused by viruses, most commonly RV. Therefore, in the first instance we decided to investigate the IFN responses to RV mounted by AM from subjects with severe asthma.

## 5.1.2 Hypothesis and aims

### 5.1.2.1 Hypothesis

AM from patients with severe asthma have an impaired IFN response to RV

### 5.1.2.2 Aims

1. Investigate the IFNβ and IFNα response to RV by SA-AM

## 5.1.3 Methods and results

AM were isolated from BAL using the adherence to plastic technique. AM from healthy (n=10) and severe asthma subjects (n=10) were treated with RV-16 (MOI 0.6) and the expression of IFNβ and IFNα mRNA and protein was evaluated 24 hours later. As negative controls cells were treated with medium alone. We also treated cells with UV-inactivated RV as an additional control. For IFNα, 2 primers were used: IFNα1 (detects subtypes 1, 6 and 13) and IFNα2 (detects subtypes 2, 4, 5, 8, 10, 14, 17 and 21) (Khaitov et al. 2009). Protein expression was measured using Meso Scale Discovery System. The lower limits of detection were 0.7pg/ml and 34pg/ml for IFNα2 and IFNβ respectively. A Meso Scale Discovery System assay was not available for IFNα1 and the volume of supernatant available (200µl) did not allow for standard ELISA testing.

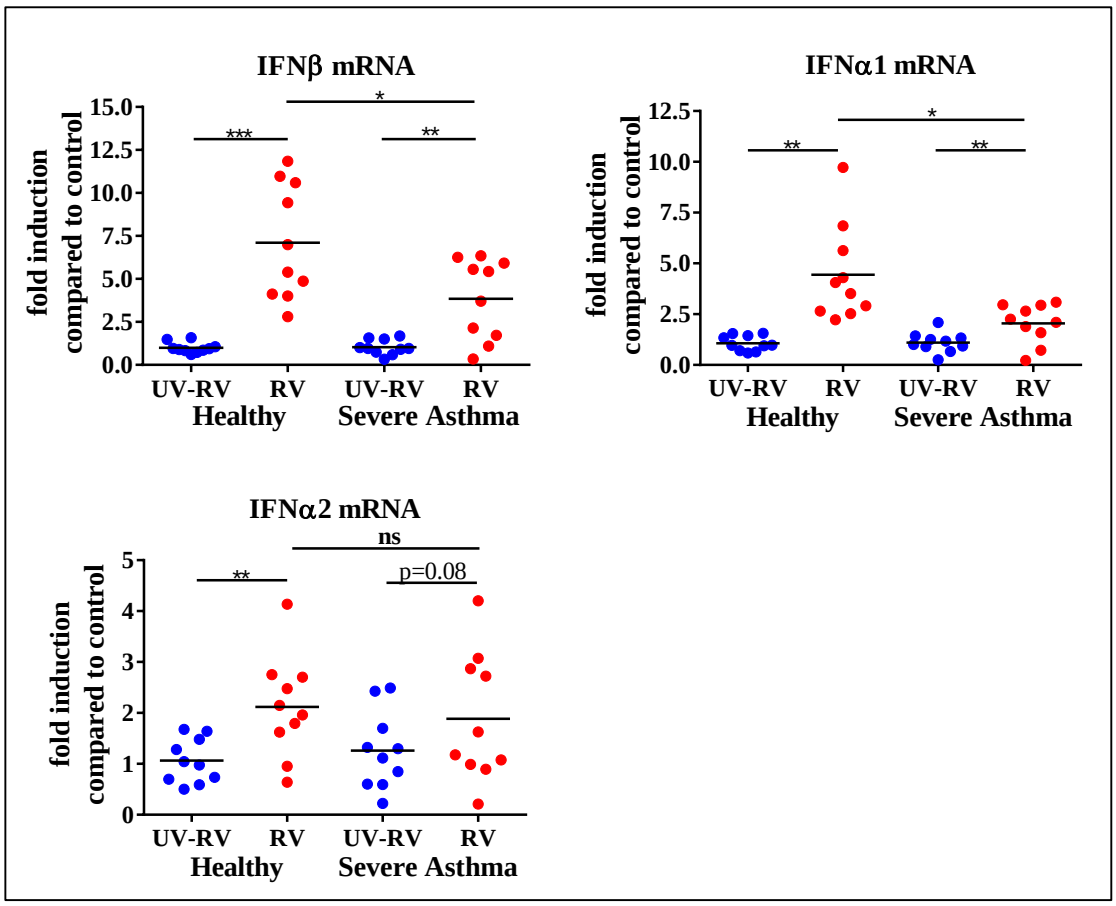


**Figure 5-1 Rhinovirus-induced expression of IFNβ and IFNα mRNA is significantly reduced in severe asthma AM compared to healthy**
AM from 10 healthy and 10 SA subjects were exposed to RV-16 for 24 hours after which IFNβ, IFNα1 and IFNα2 expression was assessed by qPCR. Expression is relative to medium alone and standardised against GAPDH. UV-RV, UV-inactivated RV; RV, RV-16. *p<0.05, **p<0.01, ***p<0.001; comparisons made using Kruskal-Wallis test with between group testing with Wilcoxon test or Mann Whitney as appropriate.

These results show that AM from healthy and SA subjects are able to produce significant amounts of IFNβ and IFNα1 when exposed to RV but the induction by SA-AM is significantly less than that by healthy AM. UV-inactivated RV does induce some IFNβ and IFNα1 production, but this is minimal and not significantly different from treatment with medium alone (data not shown). The induction of IFNα2 mRNA is far less than IFNβ and IFNα1 and only healthy AM show significant induction with RV compared to UV-inactivated RV and at 24 hours the induction by SA-AM is not deficient compared to healthy AM.

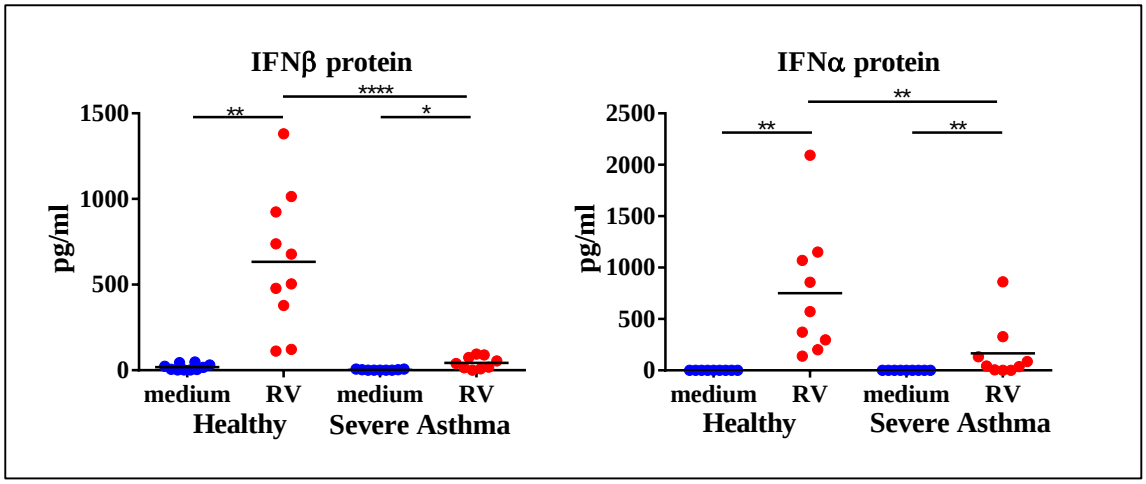


**Figure 5-2 Rhinovirus-induced expression of IFNβ and IFNα protein is significantly reduced in severe asthma compared to healthy subjects**
AM from 10 healthy and 9 SA subjects were exposed to RV-16 for 24 hours after which IFNβ and IFNα was assessed by ELISA (MSD). *p<0.05, **p<0.01, ****p<0.0001; comparisons made using Kruskal-Wallis test with between group testing with Wilcoxon test

Again, these results show that both healthy and SA-AM produce significant amounts of IFNβ and IFNα in response to RV but the induction by SA-AM is significantly less than healthy. There was no significant induction of IFNβ or IFNα protein with UV-irradiated RV (data not shown). The supernatant from one SA subject was lost during processing.

## 5.1.4 Discussion

Asthma exacerbations and severe asthma are linked with high morbidity, significant mortality and high treatment costs (Custovic et al. 2013). Viral infections account for approximately 80% of exacerbations (Grissell et al. 2005; Wark et al. 2002) and RV is consistently the most frequent pathogen identified. However the precise mechanisms underlying RV-induced asthma exacerbations are not known. Studies have shown that structural and BAL cells form mild-to-moderate asthmatic subjects have an impaired IFN-dependent anti-viral response to RV *ex vivo* (Contoli et al. 2006; Sykes et al. 2012; Wark et al. 2005). However, there is lack of consistency in the literature, with some studies failing to find any defect in IFN production in asthmatic BECs (Sykes et al. 2014), fibroblasts (Bedke et al. 2009) and PBMCs (Sykes et al. 2012).

Bronchial epithelial cells are the primary site of RV replication within the airways and the secretion of anti-viral and pro-inflammatory cytokines by BECs

is dependent on the internalisation and replication of viral RNA, as UV-inactivated RV does not elicit a response from these cells (Chen et al. 2006; Schroth et al. 1999). However *ex vivo* work has shown that only 5-10% of BECs get infected with RV (Chen et al. 2006; Lopez-Souza et al. 2004; Mosser et al. 2002) and only a very small proportion of cells in epithelial biopsies from human volunteers inoculated with RV appear to be actively infected (Arruda et al. 1995). Yet, during *in vivo* RV infection there are marked elevations in the concentrations of numerous mediators suggesting that non-infected epithelial cells must also be producing these cytokines as must be cells other than BECs. It is most likely that these paracrine signals to un-infected BECs are coming from infected AM and in support of this it has been shown that epithelial cytokine release during RV infection is significantly augmented by monocytic IFN release (Korpi-Steiner et al. 2010).

Our results show that in patients with severe asthma AM have significant impairment of virus-induced IFN expression. SA-AM are able to mount a robust IFNβ and IFNα response to RV but the magnitude of this response is significantly inferior to that of healthy AM. Less macrophage derived IFN on nearby infected and un-infected BECs would reduce the magnitude of the overall anti-viral response and lead to increased viral replication, budding and infection of nearby cells. This would lead to persistence of the virus within the lower airways, delayed viral clearance and predispose the individual to worsening of their asthma symptoms and development of an asthma exacerbation.

Although the immune responses of SA-AM have not been previously studied, it has been shown that BECs from children with severe treatment-resistant asthma have reduced IFN responses to RV (Edwards et al. 2013). A similar defect could not be detected in BECs from mild-to-moderate well-controlled asthma subjects (50% of subjects in this study were on BTS Step 1) (Sykes et al. 2014) which could indicate that the IFN deficiency may be secondary to processes related to asthma severity and our results would support that.

We were interested to see that UV-irradiated RV also induced some IFN release, albeit very small amounts. Due to the UV treatment the virus would be unable to replicate but it may still be able to signal through TLR7 and TLR8 although it is highly probable that the viral genome itself has been damaged and no

longer able to activate these PRRs. TLR2 is present on the surface of host cells and shown to be activated by components of the viral capsid. UV treatment of RV (unless extremely high doses are used) does not directly affect the viral capsid (Nuanualsuwan et al. 2003) and TLR2 would be able to recognise PAMPs within this structure and be activated (Triantafilou et al. 2011). Although TLR2 does not generally induce the production of IFNs (Doyle et al. 2002; Kawai et al. 2011; Toshchakov et al. 2002), there have been reports that TLR2 activation by viruses can sometimes lead to the production of small amounts of type I IFN (Barbalat et al. 2009). But it is unclear how significant this contribution is to the cell's overall production of IFN.

A major limitation of our work is that we only evaluated IFN expression at 24 hours. Work in PBMCs has shown that following RV induction, levels of IFNβ and IFNα increase to peak at 8 hours but remain significantly elevated at 24 hours (Khaitov et al. 2009). Therefore the 8 hour time point may have been more appropriate, especially for mRNA measurements. This may explain why measurements of IFNα2 were quite low and no significant difference was found between healthy and SA-AM. However, due to the timing of most of our bronchoscopies (mornings), processing time and time for AM adherence, infection with RV would not usually take place until 2-3 pm in the afternoon making the 8 hour time point a little impractical. Work done on BAL cells (which are predominantly AM) shows that protein expression of IFNβ and IFNα peak between 8 and 24 hours (Sykes et al. 2012) and therefore for protein measurements the 24 hour time point was appropriate.

Another limitation of our data is that while the mRNA measurements were done for IFNα1 and IFNα2, the protein measurements were only done on IFNα2. An MSD assay is not available for IFNα1 and due to limited supernatant available (200 µl only) we were unable to use a standard ELISA platform for this measurement. However although the levels of IFNα2 mRNA were not significantly different between healthy and SA-AM, the measurement of IFNα2 protein clearly shows that SA-AM are have a deficient IFNα2 response to RV.

Our previous results have demonstrated that miR-150, miR-152 and miR-375 directly target the 3'UTR of TLR7 to reduce its expression and the expression of all 3 miRNAs is increased in AM from patients with severe asthma compared to healthy subjects. Clinically, this could lead to a reduction in the protein

expression of TLR7 in the cell which in turn could hinder the cell's ability to sense the presence of virus, leading to reduced production of IFN, which would lead to increased viral replication and disease exacerbation. Given our results with miRNAs and that we have just established that SA-AM have a defective IFN response to RV, we now decided to focus on TLR7.

# 5.2 The expression and function of TLR7 is reduced in severe asthma

## 5.2.1 Introduction

The ssRNA genome of RV activates cellular PRRs including membrane bound and endosomal TLRs- TLR2, TLR3, TLR7 and TLR8 along with cytosolic RIG-1 and MDA5. TLR2 recognises and responds to molecular patters on the virus capsid, TLR3 recognises dsRNA, TLR7 and TLR8 recognise ssRNA and the cytosolic receptors recognise dsRNA (RIG-1 can also recognise ssRNA containing a terminal 5-triphosphate). Among these PRRs, TLR2 is unique in that its activation generally leads to the production of pro-inflammatory cytokines and it is not a major inducer of type I IFNs (Barbalat et al. 2009; Doyle et al. 2002; Kawai et al. 2011; Toshchakov et al. 2002). Activation of the remaining receptors by RV is the main trigger for cellular IFN production. IFNs are secreted and act in a paracrine and autocrine manner to induce a diverse array of genes which have direct antiviral properties and promote an antiviral state in cells.

Since a deficiency or reduced function of any of these PRRs could lead to deficient IFN production, others have examined their expression in asthma. In mild-to-moderate asthmatic subjects no defect in the mRNA levels of these receptors and their intracellular signalling molecules was found in BAL cells (Sykes et al. 2012). With regard to TLR7 specifically, no deficiency was found in its expression in PBMC from mild-to-moderate asthmatic subjects (Lun et al. 2009; Roponen et al. 2010). Recently, the expression of TLR7 was shown to be reduced in bronchial biopsies from patients with severe asthma (Shikhagaie et al. 2014), suggesting that perhaps defects in TLR7 expression could be related to disease severity. However, adequate TLR7 expression and function is much more relevant for AM than epithelial cells. The reason for this is that IFN production from BECs is replication dependent i.e. it requires the production of dsRNA, while minimal or no replication of the RV genome occurs in AM and so PRRs activated by ssRNA play a much more prominent role. At present the expression of TLR7 in AM has not been studied.

## 5.2.2 Hypothesis and aims

### 5.2.2.1 Hypothesis

Increased expression of miR-150, miR-152 and miR-375 leads to reduced expression of TLR7 in AM from patients with severe asthma leading to reduced anti-viral function of TLR7 in these cells

### 5.2.2.2 Aims

1. Demonstrate reduced expression of TLR7 in SA-AM at the mRNA level
2. Demonstrate reduced expression of TLR7 in SA-AM at the protein level
3. Demonstrate reduced function of TLR7 in SA-AM

## 5.2.3 Methods and results

The clinical characteristics of healthy and severe asthma subjects used for baseline measurement of TLR7 (and other PRRs) are shown in Table 5-1.

| | Healthy | Severe Asthma | p value |
|---|---|---|---|
| Number | 23 | 23 | |
| Age | 28.1 (19-54) | 43.4 (22-63) | 0.0001 |
| Sex (M/F) | 11/12 | 7/16 | 0.3651 |
| $FEV_1$ | 4.1 (0.2) | 2.0 (0.2) | <0.0001 |
| %predicted $FEV_1$ | 104.0 (2.9) | 65.2 (5.6) | <0.0001 |
| FVC | 5.0 (0.2) | 3.0 (0.2) | <0.0001 |
| %predicted FVC | 104.7 (3.8) | 84.3 (4.2) | 0.001 |
| Body Mass Index | 24.9 (0.7) | 33.0 (1.5) | <0.0001 |
| Atopy (%) | 0 | 15 (68)* | 0.0002 |
| ACQ score | N/A | 3.2 (1.9-4.9) | - |
| ICS dose in mcg (BDP equivalent) | N/A | 2300 (1000-4000) | - |
| Number on daily oral steroids (%) | N/A | 8 (35) | - |
| Number of exacerbations in previous year | N/A | 6.9 (2-12) | - |
| BAL cell differential | | | |
| %macrophages | 83.6 (53-97) | 62.9 (3-97) | 0.0158 |
| %neutrophils | 3.1 (0.25-11) | 18.5 (0.5-95) | 0.0014 |
| %eosinophils | 0.4 (0-1.8) | 2.7 (0-30) | 0.092 |

**Table 5-1 Clinical characteristics of healthy and severe asthma subjects used for evaluation of PRR expression**
Values are means with SEM in parenthesis except for age, ACQ score, ICS dose, number of exacerbations and BAL cell differential for which range is shown and atopy and number on oral steroids for which % is shown. ACQ, asthma control questionnaire; BAL, bronchoalveolar lavage; BDP, beclometasone; $FEV_1$, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroid; N/A, not applicable; * data unavailable for 1 subject. p values calculated using Mann Whitney test except for comparison of sex and atopy for which Chi-square test was used.

The measurement of TLR7 (and other PRRs) was done at baseline i.e. on the day that AM were recovered from BAL. RNA extraction was performed using TRI-Reagent.

### 5.2.3.1 TLR7 expression is reduced in severe asthma AM

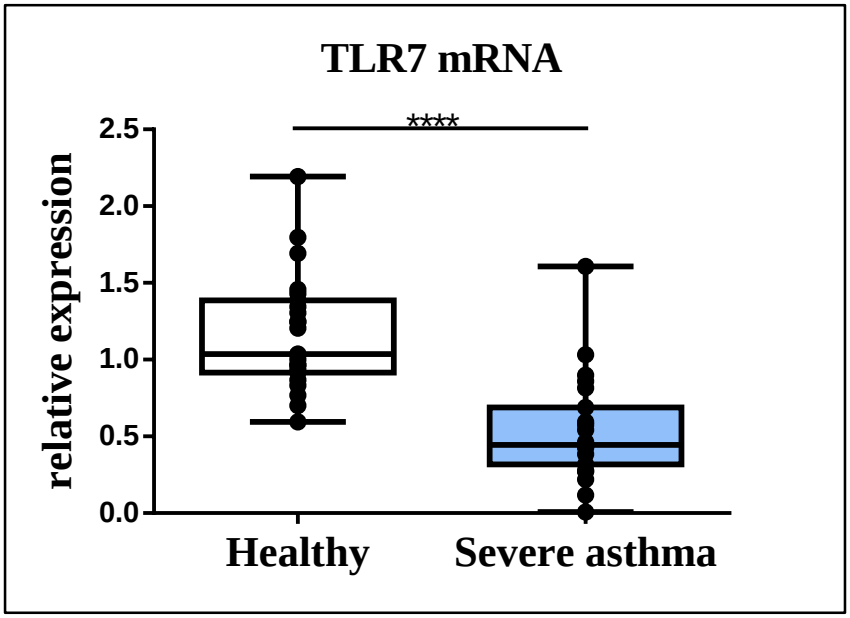


**Figure 5-3 Expression of TLR7 mRNA is significantly reduced in AM from severe asthma patients compared to healthy subjects**
Expression of TLR7 was measured by qPCR from 23 healthy subjects and 23 severe asthma subjects. Expression is relative to GAPDH. ****p<0.0001 using Mann Whitney test.

As can be seen in Figure 5-3, the expression of TLR7, at the mRNA level is significantly reduced in AM from subjects with severe asthma compared to healthy volunteers. Sub-group analysis showed that within the severe asthma group there was no difference in TLR7 expression between atopic (n=15) and non-atopic subjects (n=7).

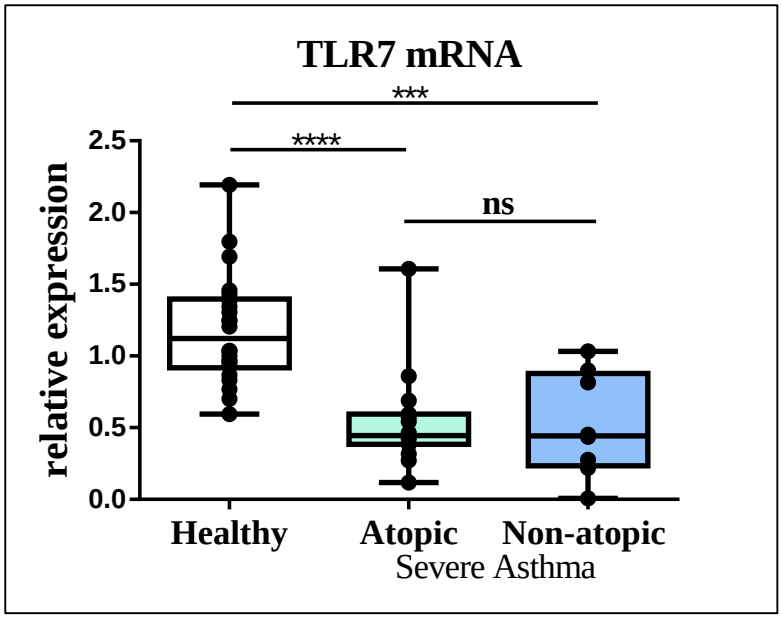


**Figure 5-4 Expression of TLR7 mRNA is not affected by atopy in severe asthma AM**
Expression of TLR7 mRNA is reduced in AM from atopic (n=15) and non-atopic (n=7) subjects with severe asthma, compared to healthy AM (n=23) while there is no difference between atopic and non-atopic individuals. Expression is relative to GAPDH. ***p<0.001, ****p<0.0001, ns=not significant, using Kruskal-Wallis test with between group testing with Mann Whitney test.

In order to investigate if TLR7 deficiency may be related to asthma severity, as has been suggested by the literature, we evaluated the expression of TLR7 in subjects with mild asthma who were on BTS treatment step 1 (prn β-2 agonist only). The clinical characteristics of these subjects are shown in Table 5-2.

| | Mild Asthma |
|---|---|
| Number | 19 |
| Age | 21.3 (0.8) |
| Sex (M/F) | 6/13 |
| $FEV_1$ | 3.8 (0.3) |
| % predicted $FEV_1$ | 101.0 (3.5) |
| FVC | 4.7 (0.3) |
| % predicted FVC | 108.8 (2.8) |
| Body mass index | 22.2 (0.6) |
| Atopy (%) | 19 (100) |
| $PC_{20}$ methacholine | 2.6 (0.6) |

**Table 5-2 Clinical characteristics of mild asthma subjects**
Values are means with SEM in parenthesis unless otherwise indicated. $FEV_1$, forced expiratory volume in one second; FVC, forced vital capacity; $PC_{20}$, dose of methacholine that induces a 20% drop in $FEV_1$ compared to baseline.

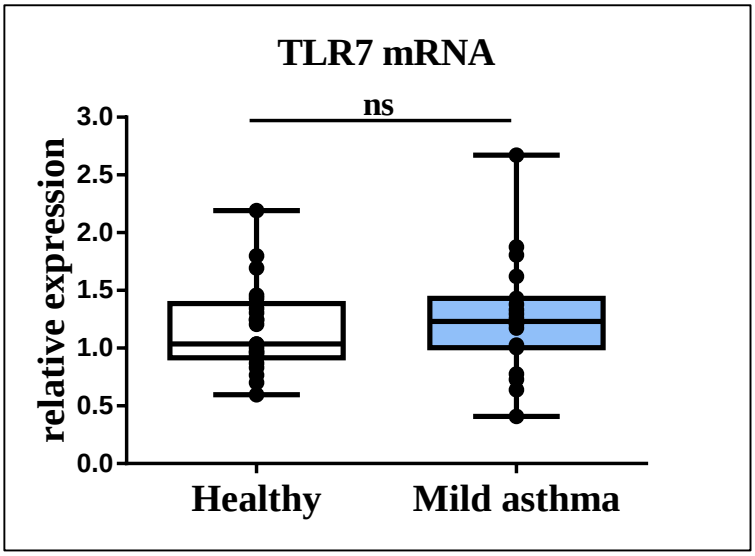


**Figure 5-5 Expression of TLR7 is not deficient in mild asthma AM compared to healthy AM**
Expression of TLR7 mRNA was evaluated in 23 healthy and 19 mild asthma subjects and no difference in its expression between the two clinical states was found. Expression is relative to GAPDH. ns=not significant; comparison done using Mann Whitney test.

The BAL from these subjects was provided by Dr Grainge and all subjects were atopic and had bronchial hyperreactivity on methacholine challenge testing. This analysis confirmed that there was no difference in the expression of TLR7 in mild asthma (n=19) compared to healthy subjects (n=23).

We then evaluated TLR7 protein expression. AM recovered from BAL were subjected to lysis using the protein lysis buffer NP-40. Expression of TLR7 protein was assessed by Western blotting and densitometric analyses. Briefly, 50µg of protein was separated in a 4-12% gel and transferred to a PVDF membrane. After blocking the membrane was incubated overnight at 4˚C with anti-TLR7 antibody (1:500). Subsequently it was incubated with anti-mouse-HRP and bands were visualised using an ECL-Advance system. Normalisation was done against β-actin. Densitometric analysis of the western blots confirmed reduced expression of TLR7 protein in severe asthma compared to healthy subjects (Figure 5-6). The western blot also confirmed that the expression of TLR7 was not reduced in MA-AM compared to healthy.

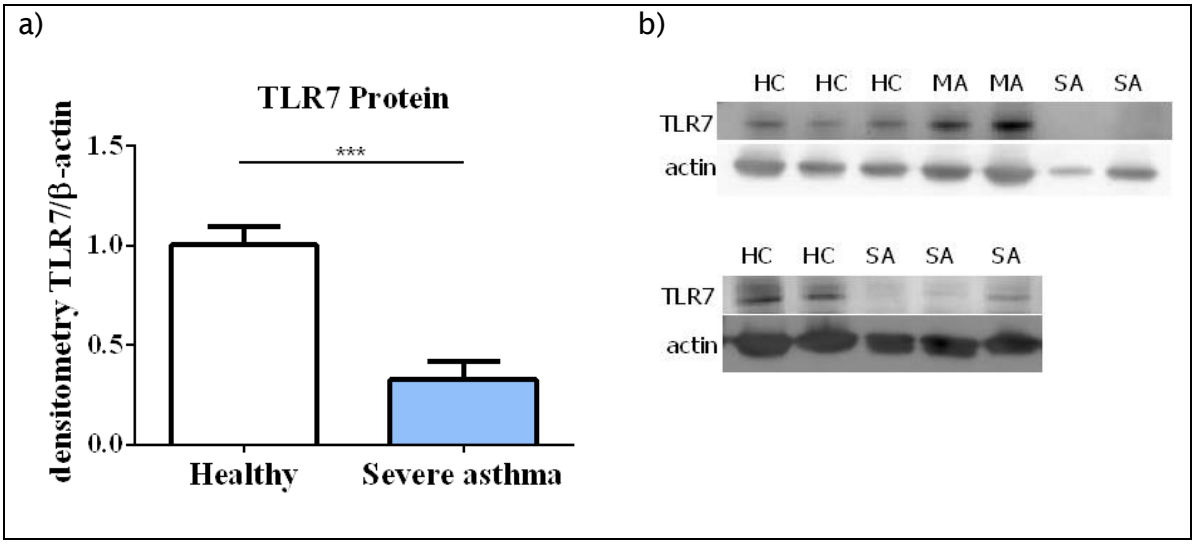


**Figure 5-6 Expression of TLR7 protein is significantly less in severe asthma AM compared to healthy**
(a) Densitometric analysis of TLR7 protein expression was calculated relative to β-actin. Healthy n=5, severe asthma n=8. Graph displays mean+SEM. ***p<0.001; p value calculated using unpaired t-test.
(b) 2 representative western blot images showing reduced expression of TLR7 protein in severe asthma compared to healthy AM. HC=healthy control, MA=mild asthma, SA=severe asthma, actin=β-actin

### 5.2.3.2 Expression of other PRRs relevant to RV is not deficient in severe asthma AM

Since the ssRNA genome of RV can also signal via TLR8 and the dsRNA intermediary formed during RV replication signals via other PRRs in the cell (TLR3, RIG-1 and MDA5) we also evaluated the expression of these receptors in healthy and SA-AM. Figure 5-7 shows that there is no significant difference in the expression of these receptors between the healthy and SA-AM. However, there is a trend towards reduced expression of TLR8 in SA-AM.

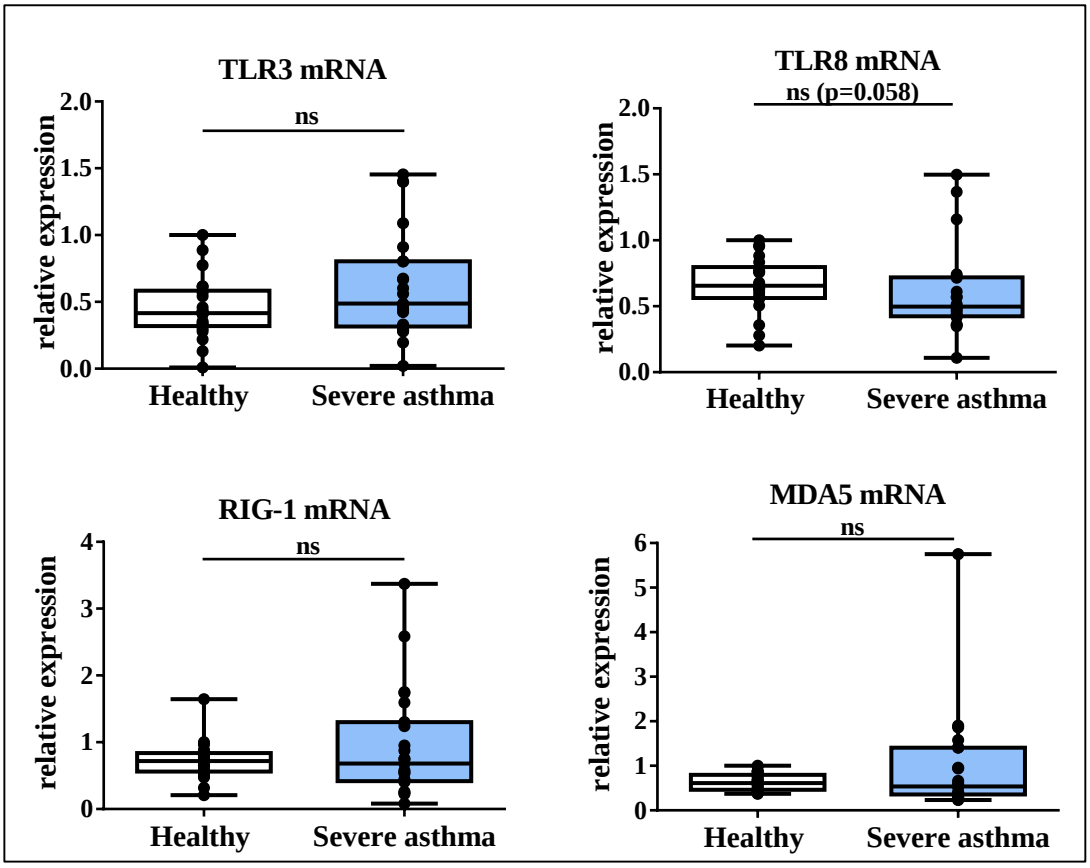


**Figure 5-7 The expression of TLR3, TLR8, RIG-1 and MDA5 is not deficient in severe asthma**
Expression of TLR3, TLR8, RIG-1 and MDA5 was evaluated in healthy (n=23) and severe asthma AM (n=23). Expression is relative to GAPDH. ns=not significant; comparisons made using Mann Whitney test.

We have previously assessed the expression of TLR2, the PRR that recognises motifs in the viral capsid, and shown increased expression in SA-AM compared to healthy (Figure 3-7). It is thought that signalling through this receptor predominantly leads to the production of pro-inflammatory cytokines and is unlikely to contribute much to the overall IFN production from the cell.

As TLR7 signals via the adaptor protein MyD88 (Takeda et al. 2004) we evaluated whether the expression of this cytoplasmic signal transducer was altered in SA-AM. Additionally, bioinformatics analysis suggested that miR-150, which we found to be significantly elevated in severe asthma, can also target MyD88. However, as can be seen in Figure 5-8, MyD88 expression is not significantly reduced in SA-AM.

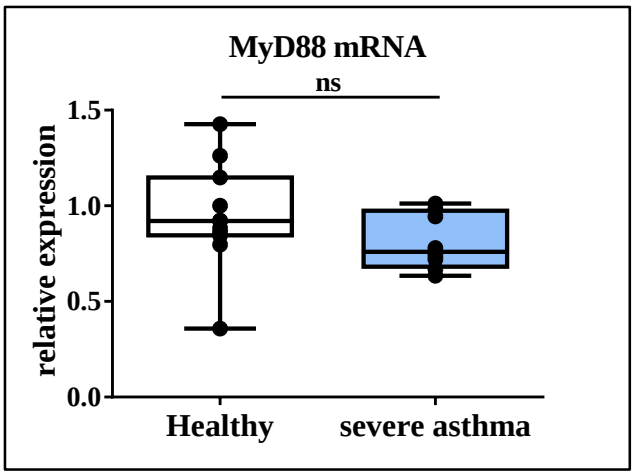


**Figure 5-8 Expression of MyD88 mRNA is comparable in healthy and severe asthma subjects.**
Expression of MyD88 was measured by qPCR in 11 healthy and 8 severe asthma subjects. Expression is relative to GAPDH. ns=not significant; comparison done using Mann Whitney test.

### 5.2.3.3 Reduced function of TLR7 in severe asthma

Our results have shown that TLR7 expression is significantly reduced in AM from patients with severe asthma compared to healthy subjects. In order to investigate if this deficiency leads to reduced function of TLR7, AM from healthy and severe asthma subjects were stimulated with the TLR7 specific agonist imiquimod (5µg/ml) and IFN responses evaluated 24 hours later. Imiquimod (R837) is an imidazoquinoline and a specific TLR7 agonist (Lee et al. 2003), unlike R848 (resimiquod) which has been shown to activate both TLR7 and TLR8.

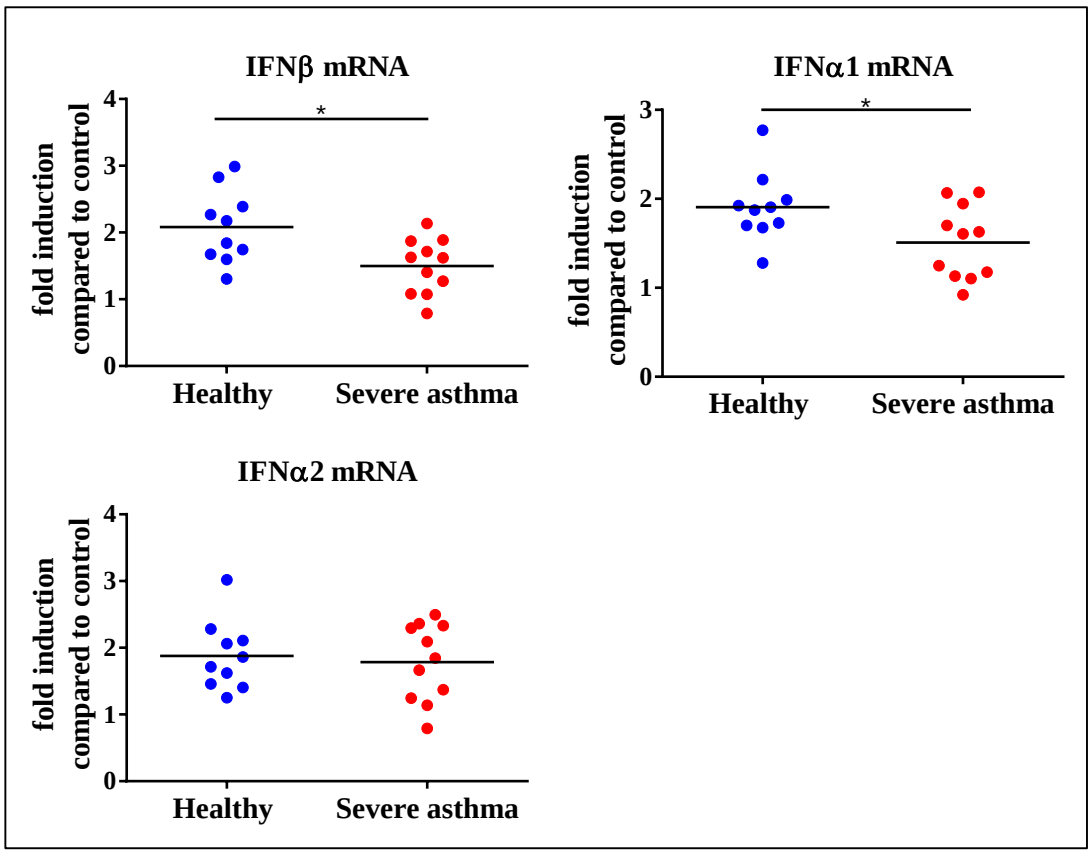


**Figure 5-9 Imiquimod-induced expression of IFN in severe asthma and healthy alveolar macrophages**
AM from healthy (n=10) and severe asthma (n=11) subjects were treated with imiquimod for 24 hours after which IFNβ , IFNα1 and IFNα2 mRNA expression was evaluated. Expression is relative to treatment with medium alone and standardised against GAPDH. *p<0.05 by Mann Whitney Test.

This shows that imiquimod induced production of IFNβ and IFNα1 mRNA is significantly reduced in SA-AM compared to healthy AM. Again, as was the finding with RV, the induction of IFNα2 is comparable in healthy and SA-AM. However, measurements of IFNα2 protein (Figure 5-10) confirmed reduced imiquimod-induced induction in SA-AM compared to healthy AM.

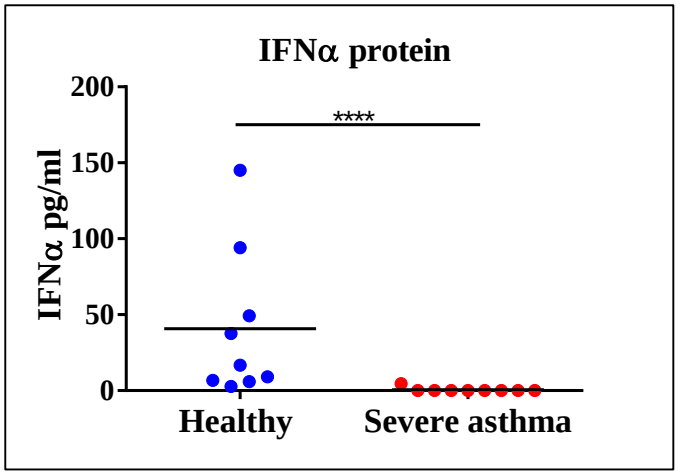


**Figure 5-10 Imiquimod-induced expression of IFNα protein is significantly reduced in severe asthma AM compared to healthy**
AM from 9 healthy and 9 SA subjects were exposed to imiquimod for 24 hours after which IFNα was assessed by ELISA (MSD). ****p<0.0001 using Mann Whitney test.

The release of IFN signals to nearby cells to induce the expression of a set of ISGs that amplify interferon signalling, activate the adaptive immune response and directly inhibit virus replication (Liu et al. 2011). Therefore we evaluated if TLR7-induced production of ISGs was also deficient in SA-AM. The mRNA expression of two ISGs was measured- MxA and OAS and was found to be significantly reduced in SA-AM compared to healthy AM.

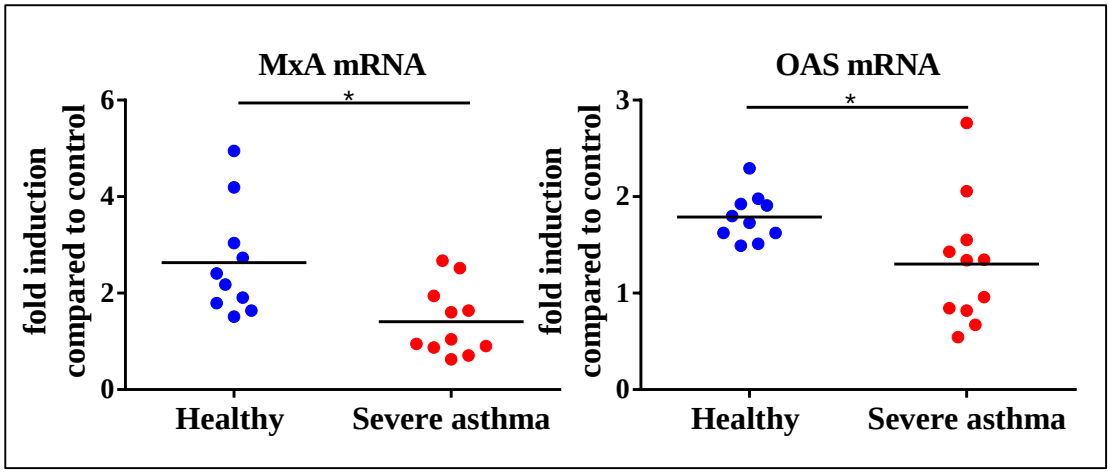


**Figure 5-11 Imiquimod-induced expression of ISGs is significantly reduced in severe asthma AM compared to healthy**
AM from 10 healthy and 11 severe asthma subjects were exposed to imiquimod for 24 hours after which MxA and OAS mRNA was measured. Expression is relative to treatment with medium alone and standardised against GAPDH. *p<0.05 using Mann Whitney test.

Finally, to confirm that impaired IFN production was related to reduced TLR7 expression we also measured the expression of IFNβ when AM were stimulated with Polyinosinic-polycytidylic acid (PolyI:C) (10μg/ml), a synthetic analog of dsRNA which stimulates TLR3, RIG-1 and MDA5. Poly:IC induced IFN production by SA-AM was not deficient compared to healthy AM confirming that the function of these 3 other PRRs is intact in SA-AM.

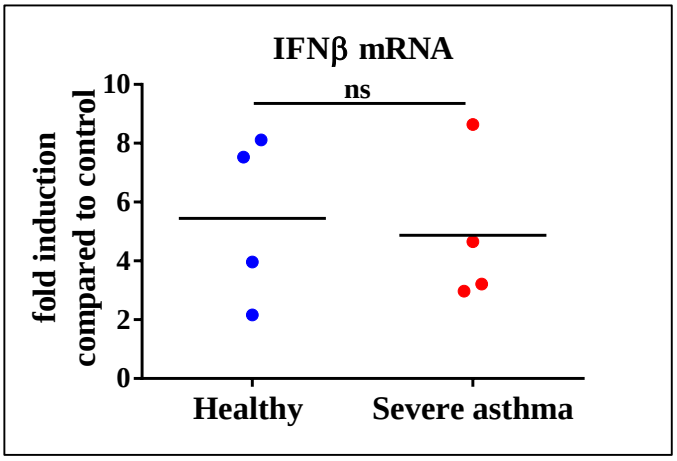


**Figure 5-12 Expression of Poly:IC induced IFNβ mRNA is not deficient in severe asthma AM**
Healthy (n=4) and severe asthma (n=4) AM were incubated with Poly:IC for 24 hours after which IFNβ mRNA expression was measured. Expression is relative to treatment with medium alone and standardised against GAPDH. ns=not significant using unpaired t-test.

## 5.2.4 Discussion

Asthmatic bronchial epithelial cells and BAL cells have been shown to have an impaired anti-viral IFN response to RV infection and it has been proposed that this IFN deficiency drives the vulnerability of asthmatic subjects to recurrent lower respiratory tract RV infection. The mechanisms underlying the defective IFN response are unclear. PRRs play an important role in the innate immune response to viral infection- TLR2, TLR3, TLR7, TLR8, RIG-1 and MDA5 are the PRRs activated by RV and their activation leads to the production of anti-viral and proinflammatory cytokines. Reduced expression or function of these receptors could lead to reduced production of anti-viral cytokines which would result in increased viral replication and reduced viral clearance.

Our results show that the expression of TLR7 is significantly reduced in SA-AM compared to healthy AM. This is associated with significantly reduced function of TLR7 in AM from patients with severe asthma and we have demonstrated that *ex vivo*, SA-AM produced significantly less IFNβ and IFNα when exposed to the specific TLR7 agonist imiquimod compared to healthy subjects. The expression of the downstream ISGs is also deficient in SA-AM suggesting that reduced expression of TLR7 also affects the amplification of the IFN response. The expression of other PRRs relevant to the IFN responses to RV was not found to be deficient in SA-AM indicating that the defect was specific for TLR7. Furthermore, the function of these receptors, namely TLR3, RIG-1 and MDA5, as evaluated by stimulating cells with PolyI:C, was comparable in healthy and SA-AM. Taken together, these results show that the TLR7 signalling pathway is reduced in SA-AM and that this is due to reduced expression of TLR7.

Reduction in TLR7 mRNA levels suggests that miR-150, miR-152 and miR-375 are primarily exerting their effects through degradation or decay of the TLR7 mRNA, rather than translational repression. This is consistent with the recent finding that over 80% of miRNA mediated target repression is due to decreased mRNA levels (Guo et al. 2010). However, the reduction in TLR7 protein levels seem greater than reduction in mRNA levels, suggesting that even though miR-150, miR-152 and miR-375 have caused degradation of the TLR7 mRNA they also inhibit its translation to further reduce its overall protein expression in SA-AM.

It has been shown that RV undergoes only minimal replication in AM (Gern et al. 1996; Laza-Stanca et al. 2006). This would suggest that PRRs that recognise ssRNA (TLR7 and TLR8) would be central to the IFN response mounted by AM. Given the importance of TLR7, other groups have evaluated its expression in asthmatic immune cells. No defect in the expression of TLR7 was found in BAL cells (Sykes et al. 2012), monocytes (Roponen et al. 2010) or PBMCs (Lun et al. 2009) from mild-to-moderate asthma subjects. We believe that the TLR7 deficiency that we have detected is likely to be caused by processes related to asthma severity and control. The subjects in our severe asthma group were on BTS step 4-5 of asthma treatment and all had poorly controlled disease (mean ACQ: 3.2, range: 1.9-4.9) characterised by recurrent exacerbations (an average of 6 in the preceding 12 months) while the subjects studied by Sykes *et al* had well-controlled asthma on BTS step 1-3 (mean ACQ

0.8) and those studied by Roponen *et al* and Lun *et al* were on BTS step 1-2 of asthma treatment. In support of this it has recently been demonstrated that TLR7 immunohistochemical staining was significantly reduced in severe asthmatic epithelial cells but not epithelial cells from MA subjects (Shikhagaie et al. 2014). Similarly, we also found that MA-AM, unlike SA-AM, had normal expression of TLR7. Work done in our group looking at miRNA degregulation in asthmatic BECs did not find altered expression of miR-150, miR-152 and miR-375 in severe asthma BECs (unpublished work) suggesting that mechanisms other than miRNAs are reducing the expression of TLR7 in severe asthmatic BECs.

Using imiquimod, we showed that reduced expression of TLR7 in SA-AM is associated with reduced function of this receptor and a reduction in the overall IFN response by the cell. TLR7 function has been shown to be reduced in peripheral monocytes from adolescents with atopic asthma (Roponen et al. 2010). In keeping with our findings, when these asthmatic monocytes were stimulated with PolyI:C, no defect in ISG production was detected, suggesting the defect was specific to TLR7. However, as mentioned above, no reduction in the expression of TLR7 was found in asthmatic monocytes in this study suggesting that reduced function of TLR7 in monocytes was not due to defects in its expression. Different mechanisms may cause reduced function of TLR7 in peripheral blood immune cells and AM. It has been shown that TLR expression profiles differed significantly between autologous monocytes and AM (Juarez et al. 2010) suggesting that TLR expression patterns may be tissue-specific. Therefore, results obtained when studying asthmatic peripheral blood cells may not accurately reflect AM and even though immune cells in the lung are probably recruited from the systemic circulation, it is likely that their expression of immune receptors changes.

In contrast to the findings by Roponen *et al* which studied monocytes from adolescents with asthma, imiquimod induced IFN production by PBMCs from well-controlled adult asthmatic subjects was not found to be impaired (Sykes et al. 2013). The reason for these contrasting findings is not clear but may be related to differences in cells studied (monocytes vs PBMCs) and the age of subjects (adolescents vs adults).

Once activated by ssRNA, TLR7 signals through a MyD88 dependent pathway which ultimately leads to the nuclear translocation of IRF3, IRF7 and NF-κ B. The expression of MyD88 was not found to be different between healthy and severe asthma subjects. However, if the one healthy subject who had extremely low expression of MyD88 was excluded then the analysis would show that SA-AM do have lower MyD88 than healthy AM. MiR-150 has been shown to directly target and reduce the expression of MyD88 in monocyte derived macrophages and reduce signalling via TLR2 (Ghorpade et al. 2013). Although the effect of reduced MyD88 on signal transduction via other TLRs was not examined, it is extremely likely that signalling via the other MyD88 dependent TLRs (which include all TLRs except TLR3) would also be negatively affected. Our sample numbers in this analysis was relatively small however there is a trend towards reduced MyD88 expression in severe asthma which could lead to impaired innate immune activity against a range of pathogens.

We were interested to see that although the expression of TLR8 was not significantly reduced in SA-AM there was a trend towards reduced TLR8 expression in these cells, compared to healthy AM. We looked back at the list of miRNAs our arrays had identified as being differentially expression in asthmatic AM to see if any could potentially target TLR8. MiR-326, which is predicted to target TLR8, was one of the miRNAs highlighted as being differentially expressed in asthma but the expression of this miRNA was lower in asthmatic AM compared to healthy. If indeed it did regulate TLR8 expression, then reduced miR-326 expression is likely to lead to increased TLR8 expression, not reduced, as our results have shown. However, overall the expression of miR-326 was extremely low (i.e. Ct values over 35) indicating that the biological relevance of this miRNA in AM is likely to be negligible.

Because TLR8 was initially thought to be non-functional in mice, much less is known about it, compared to TLR7, which it is genetically and functionally related to (Cervantes et al. 2012). The genes which code for TLR7 and TLR8 are both located in the X chromosome and it has been shown that single nucleotide polymorphisms and haplotypes in both genes may confer susceptibility to asthma and other atopic disorders such as rhinitis and atopic dermatitis (Moller-Larsen et al. 2008). Like TLR7, TLR8 is also involved in the recognition of ssRNA of viral origin and reduced monocyte expression of TLR8 has been reported in infants with RSV bronchiolitis compared to healthy infants

(Bendelja et al. 2010). TLR8 expression has also been shown to down-regulated by RV infection in PBMCs (both asthmatic and healthy) (Pritchard et al. 2014). It could be speculated that recurrent viral infection in the severe asthma group may have led to reduction in TLR8 expression in the SA-AM but confirmation of reduced TLR8 protein expression (in addition to our current mRNA data) is needed before further inferences are made. However, any reduction in TLR8 would certainly contribute to the reduced IFN responses to RV mounted by SA-AM.

We have previously shown that the expression of TLR2 is significantly higher in SA-AM compared to healthy AM (Figure 3-7). It could be argued that increased expression of TLR2 in SA-AM could compensate for reduced TLR7 expression but TLR2 activation has been shown to predominantly activate NF-κ B, leading to the production of pro-inflammatory cytokines. Therefore, increased expression of this TLR may in fact exacerbate the pro-inflammatory immunopathology in the asthmatic airways.

In summary, we have shown that the expression and function of TLR7 is reduced in severe asthma and this leads to reduced RV-induced IFN production by SA-AM. AM-derived IFN has an important impact on the overall antiviral response and it appears to boost BEC-derived IFN production (Korpi-Steiner et al. 2010) and helps crease an anti-viral state in the lower airways even in the absence of extensive viral replication. Furthermore, IFN release from AM is crucial because it is likely to occur before activation of PRRs in BECs, as the latter requires viral replication to occur. In the preceding sections we have shown that the expression of miR-150, miR-152 and miR-375 is increased in asthma and that they are able to inhibit TLR7 expression. We have now confirmed that this has biologically relevant effects as it leads to reduced expression and function of TLR7 in SA-AM.

# 5.3 Reduced TLR7 expression in severe asthma correlates with clinical parameters of asthma control

## 5.3.1 Introduction

According to well established literature, reduced virus-induced IFN production would increase the susceptibility of patients to lower respiratory tract RV infection (Contoli et al. 2006; Message et al. 2008). In patients with SA this is likely to lead to an asthma exacerbation. Recurrent disease exacerbations are associated with higher use of inhaled and oral corticosteroids, lower quality of life and worse asthma control (Kupczyk et al. 2014).

Having shown that TLR7 expression and function is reduced in SA-AM we wished to explore if the expression of this PRR correlated with clinical parameters relevant to asthma exacerbations including ACQ, number of exacerbations, ICS and OCS use and lung function.

## 5.3.2 Results

The ACQ is a well-validated measure of asthma control (Juniper et al. 1999) and takes into account symptoms, quality of life and need for symptomatic treatment (short acting β 2-agonist use). Our results show that TLR7 mRNA expression in SA-AM correlated inversely with the patient's ACQ score at the time of the bronchoscopy. It also correlated inversely with the number of exacerbations the patient had experienced in the previous 12 months. This suggests lower expression of TLR7 is associated with more frequent exacerbations and also a higher ACQ score i.e. poorer asthma control.

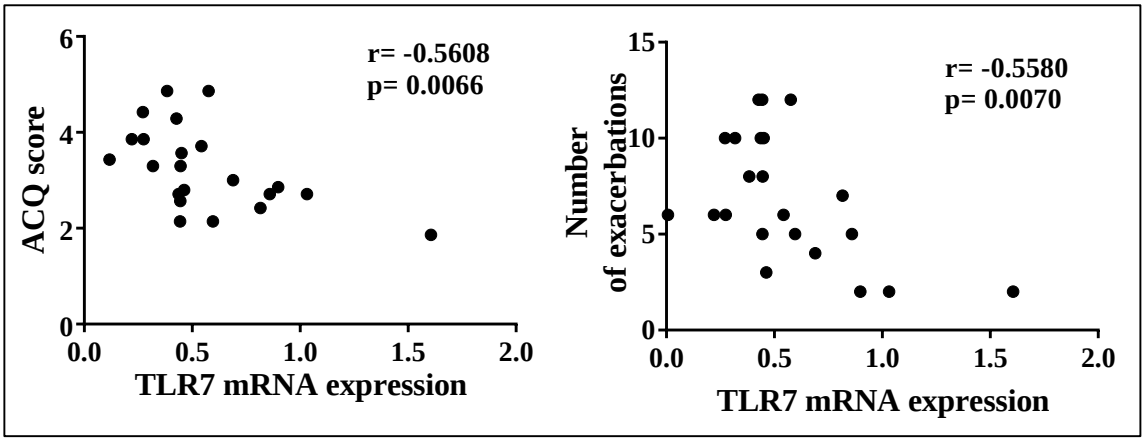


**Figure 5-13 TLR7 expression inversely correlates with clinical parameters of asthma control**
Expression of TLR7 mRNA in severe asthma AM inversely correlated with the patients' ACQ scores and the number of exacerbations experienced in the previous 12 months. Analysis done using Spearman rank correlation.

All the patients in the severe asthma group were on BTS Step 4 or 5 of asthma treatment and were receiving high doses of ICS therapy (mean BDP equivalent dose 2300 mcg). TLR7 expression did not correlate with dose of ICS that the patient was receiving. However TLR7 expression did correlate inversely with the total amount of oral corticosteroid (OCS) therapy that the patient had received in the previous 12 months.

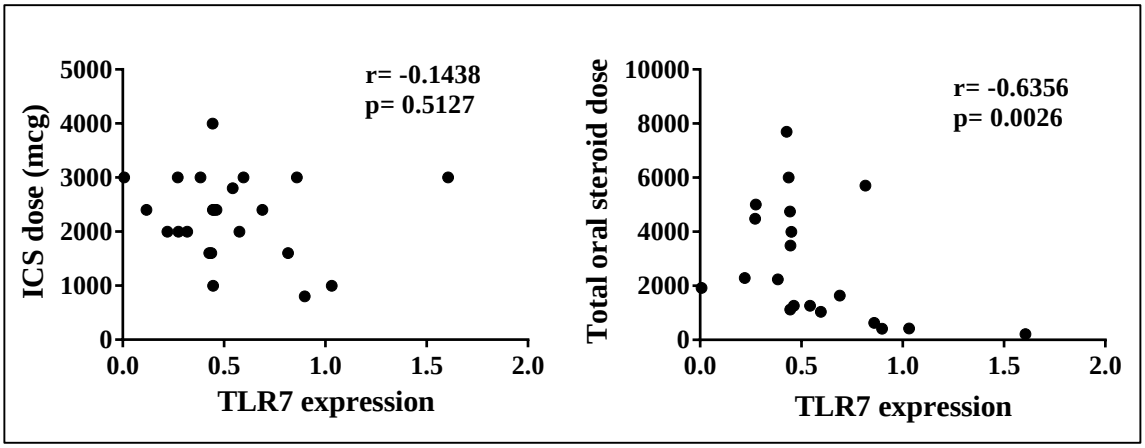


**Figure 5-14 TLR7 expression inversely correlates with total oral steroid dose but does not correlate with ICS dose**
Expression of TLR7 expression mRNA in severe asthma AM inversely correlated with the total amount of OCS the patient had received in the previous year but not with the daily ICS dose (BDP equivalent, in mcg) that the patient was taking. Analysis done using Spearman rank correlation.

Finally we explored if TLR7 expression correlated with airflow obstruction. In the SA patients, TLR7 expression in AM did not correlate with % predicted $FEV_1$.

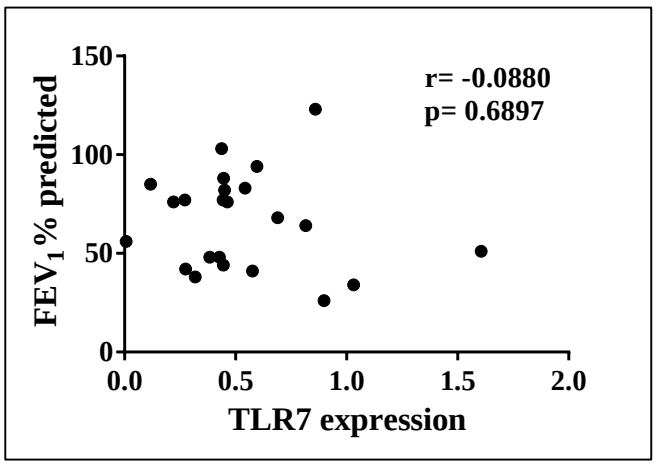


**Figure 5-15 TLR7 expression does not correlate with %predicted $FEV_1$**
Expression of TLR7 mRNA in severe asthma AM does not correlate with the patient's % predicted $FEV_1$. Analysis done using Spearman rank correlation.

## 5.3.3 Discussion

Within the airways, AM act as the first-line sensor of invading viruses and produce IFN at the site of infection. This IFN acts in a paracrine manner on nearby un-infected BECs activating anti-viral pathways within these cells and facilitating the induction of a robust innate anti-viral response. Our previous results show that SA-AM have a reduced IFN response to RV and that deficient expression of TLR7 is the likely cause of this. Subjects with severe asthma experience recurrent disease exacerbations and we propose that reduced IFN responses form SA-AM drives the vulnerability of these subjects to not just lower respiratory tract viral infection but also recurrent asthma exacerbations and poorer disease control. To support this, we found a clear correlation between the level of TLR7 expression in SA-AM and the number of exacerbations the patient had experienced in the previous year. During and in the period after an exacerbation asthma symptoms increase and this is reflected by an increased ACQ score, suggesting poorer control. We also found that TLR7 expression in SA-AM inversely correlated with the patient's ACQ score. We usually waited 4-6 weeks after an exacerbation to perform a bronchoscopy and therefore the increased ACQ score of our patients is unlikely to reflect an ongoing exacerbation.

Our severe asthma subjects reported a mean of 6 exacerbations in the previous year and it is unlikely that all were virus-driven. However, reduced overall IFN would lead to increased and persistent viral replication within airway cells which has been shown to be associated with more severe symptoms, poorer lung function, increased pro-inflammatory cells recruitment and an exaggerated local inflammatory state (Contoli et al. 2006; Message et al. 2008; Zhu et al. 2014) which would contribute to the immunopathology of an asthma exacerbations. RV infection has been shown to predispose to bacterial and allergy driven asthma exacerbations. In children, there is considerable evidence that RV can predispose to secondary bacterial infection (Cheuk et al. 2007; Louie et al. 2009; Miller et al. 2007) and in adults, virus and bacterial co-infection increase the severity of the exacerbations (Wark et al. 2013). The molecular mechanisms underlying this clinical association are thought to include RV-induced reduction in the anti-bacterial activity of AM (Oliver et al. 2008) and delay in bacterial clearance (Unger et al. 2012). Even after resolution of viral infection, virus-induced desensitisation of TLRs persists, decreasing pro-inflammatory cytokine responses to bacteria (Didierlaurent et al. 2008) and immunosuppressive cytokines produced during the viral infection linger, enhancing susceptibility to bacterial pneumonia (van der Sluijs et al. 2004). The risk of allergen induced exacerbation is also increased after RV infection, even after resolution of the acute infection via mechanisms including RV induced predisposition to late asthmatic reactions to sensitising allergen, increased histamine release and eosinophil recruitment (Calhoun et al. 1994; Green et al. 2002; Lemanske et al. 1989). Therefore, once infected with RV within the lower airways, asthmatic individuals are at an increased risk of bacterial and allergen driven exacerbations.

TLR8 is another intracellular PRR that recognises ssRNA and once activated, like TLR7, it induces the production of type I IFNs (and NF-κ B cytokines). As yet, TLR8 deficiency has not been identified in asthma but infants with RSV bronchiolitis have been shown to have reduced PMBC TLR8 compared to healthy infants (Bendelja et al. 2010). Interestingly, the degree of TLR8 deficiency negatively correlated with severity of illness. It is interesting that like our results which show TLR7 expression correlates inversely with number of exacerbations, the expression of another closely related PRR has also been shown to correlate to clinical parameters of virus-induced airways disease.

TLR7 expression did not correlate with the dose of ICS that the patient was receiving. Although this suggests that increasing doses of ICS are not associated with reduced TLR7 expression, it does not exclude a steroid related effect on expression. TLR7 expression did inversely correlate with the total OCS dose the patient had received in the previous year. However, patients with more frequent exacerbations would require more frequent courses of OCS. Therefore the correlation between TLR7 expression and cumulative OCS dose may just reflect increased frequency of exacerbations.

Patients with severe asthma tend to have poorer lung function and a greater decline in lung function over time than those with mild-to-moderate asthma (Witt et al. 2014). TLR7 expression did not correlate with lung function in our patient cohort.

In summary, these results link the molecular effects of aberrations in miRNA expression i.e. reduced TLR7 expression to clinician-derived (number of exacerbations in the previous year) and patient derived (ACQ score) measures of asthma control, strengthening the clinical relevance of our findings. This places TLR7 and AM as key players in the pathogenesis of asthma exacerbations and valid targets in the search for novel treatments for virus-induced disease exacerbations.

# Chapter 6 Role of the asthmatic environment and steroid therapy on the expression of miRNAs and TLR7

## 6.1 Introduction

MiRNA expression is regulated at multiple levels- during their transcription, during nuclear and cellular processing and during interaction with their target mRNA (Breving et al. 2010; Bronevetsky et al. 2013). It is likely that local stimuli are able to influence miRNA expression at each of these levels. The promoter regions of miRNA genes are controlled by transcription factors similar to protein-coding genes (Krol et al. 2010). For example, TNFα and Poly:IC have been shown to induce the expression of miR-155 by influencing the AP-1 transcription factors (O'Connell et al. 2007) and LPS and TNFα induction of miR-146a is mediated by NF-κ B binding to its promoter (Taganov et al. 2006). In fact, activation of TLRs leads to alterations in the expression of a number of miRNAs and in many cases this is likely to be mediated by NF-κ B signalling pathways (Foster et al. 2013). LPS has also been shown to enhance the post-transcriptional processing of miR-155 precursors (Ruggiero et al. 2009) and therefore influence miR-155 expression at the level of cellular processing. Other stimuli, which may be present within the airways such as Th1 and Th2 cytokines, cigarette smoke and pollutants have also been shown to affect miRNA expression but the precise mechanisms involved are currently unclear (Chiba et al. 2009; Fry et al. 2014; Schembri et al. 2009).

Having shown that miR-150, miR-152 and miR-375 are increased in SA-AM and that their target, TLR7, is significantly reduced in these cells resulting in biologically relevant deficiencies in the IFN response to RV, we next explored what may be influencing the expression of these miRNAs and TLR7 in SA-AM. A number of factors may be influencing miRNA expression in SA-AM including the local cytokine milieu, presence of bacterial or viral pathogens and drug treatment. Asthma is traditionally thought to result from inflammation driven by Th2 responses and mediated by cytokines including IL-4, IL-5 and IL-13. However, there is increasing evidence that a significant proportion of human asthma may be driven by alternative forms of inflammation and it has been

shown that non-Th2 driven mechanisms operate in about half of patients with asthma (Woodruff et al. 2009). The expression of IFNγ , a Th1 associated cytokine has been shown to be increased in bronchial biopsies from SA subjects (Shannon et al. 2008) while its expression in sputum has been shown to be related to asthma severity (Cho et al. 2005; Truyen et al. 2006). This suggests that Th1 cytokines may have a predominant presence within the severe asthmatic airway. In addition to cytokines, the asthmatic airways also contain bacterial species and viruses and the composition of the different species differs compared to healthy subjects (Huang 2013). In particular, members of the Proteobacteria phylum (which includes *Haemophilus* spp.), were more commonly identified in BAL from asthmatic subjects (Hilty et al. 2010). LPS pre-treatment of AM has been shown to reduce RV-induction of chemokines that would promote viral clearance (Karta et al. 2014) suggesting that endotoxin exposure within asthmatic airways could alter AM immune responses to RV.

ICS therapy is recommended as maintenance daily therapy in all forms of asthma, except mild asthma treated on Step 1 of the BTS algorithm. It aims to suppress airway inflammation and through this reduce airway hyperresponsiveness and control asthma symptoms. ICS act via the cellular glucocorticoid receptor (GR) which translocates to the nucleus to influence gene transcription (Barnes 2006). Most miRNA genes would be under transcriptional control of a promoter. Glucocorticoids are able to interact with a number of transcription factors and reports have shown that they are also able to repress the transcription of some miRNAs (Molitoris et al. 2011).

Therefore, we decided to investigate the miRNA-related effects of:

1. The local microenvironment (which would include cytokines and bacterial and viral pathogens)
2. Steroid treatment

Although we are proposing that changes in TLR7 expression within SA-AM are the result of increased expression of miR-150, miR-152 and miR-375 within these cells, it is possible that external stimuli are also directly influencing TLR7 expression, without affecting miRNA levels. Therefore, we also evaluated for changes in TLR7 in both these models.

# 6.2 Effect of the local microenvironment on miRNA and TLR7 expression

## 6.2.1 Methods and results

AM recovered from BAL from 6 healthy individuals were cultured with BAL fluid supernatant previously obtained from 10 SA subjects. BAL from the SA subjects had been filtered and spun in order to obtain cells for AM isolation (as previously described) and the supernatant stored at -80˚C until use. As a control, the healthy subject's own BAL supernatant was used. To simulate a Th1 and Th2 environment, AM from the healthy subjects were also treated with IL-4, IL-13, TNFα and IFNγ (all 10ng/ml). After 24 hours AM were collected in TRI-Reagent for RNA extraction. As a control AM were treated with medium alone.

### 6.2.1.1 Effect of BAL components on miRNA and TLR7 expression

The expression of miR-150, miR-152 and miR-375 after exposure of healthy AM to severe asthmatic BAL fluid was measured using qPCR and is shown in Figure 6-1.

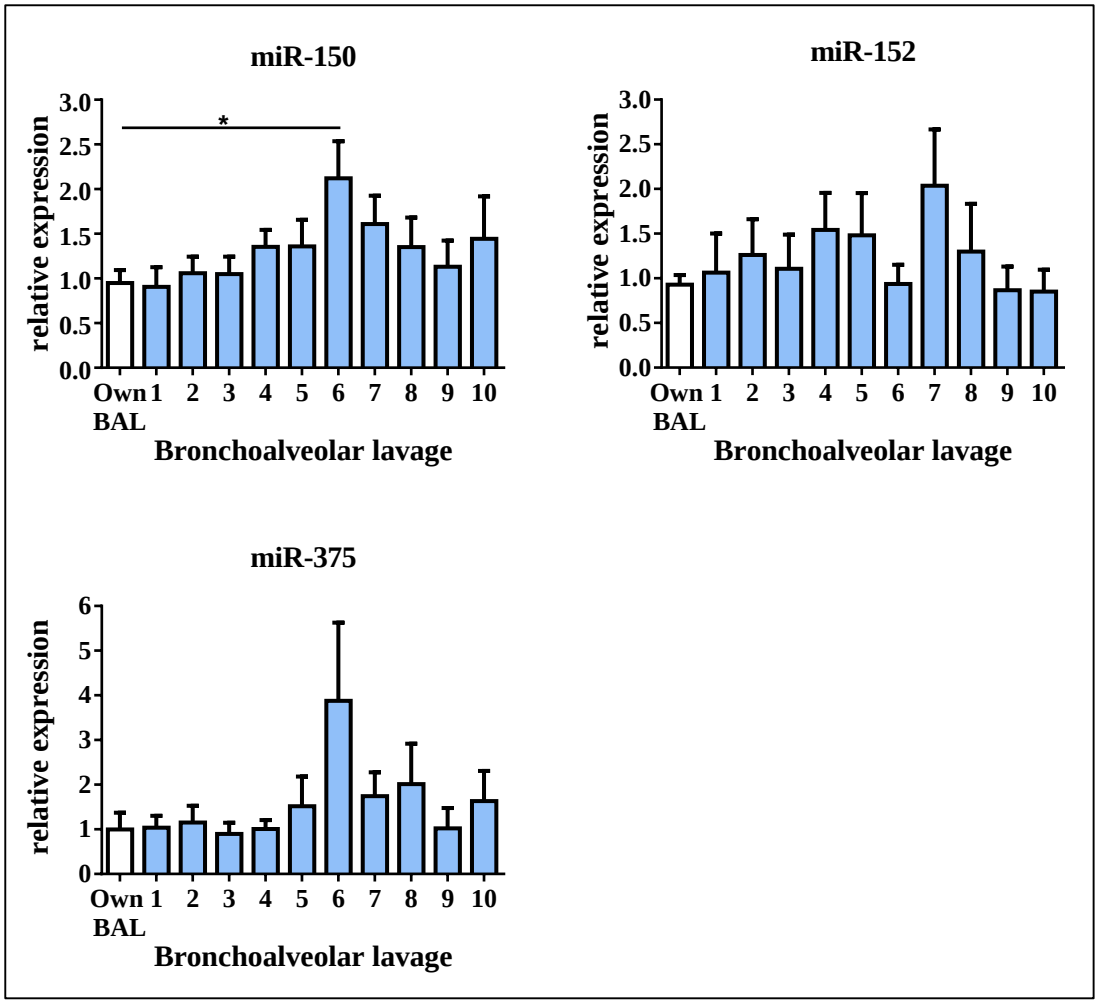


**Figure 6-1 Expression of miR-150, miR-152 and miR-375 in healthy AM exposed to bronchoalveolar lavage fluid from severe asthma subjects**
AM from 6 healthy subjects were exposed to BAL fluid from 10 SA subjects (numbers 1-10 on graphs) and their own BAL for 24 hours. Expression is relative to treatment with medium alone and is standardised against RNU44. Graphs show mean+SEM. *$p<0.05$ using one-way ANOVA with Dunnett's multiple comparison test and between group testing with t-test if results significant.

This analysis showed that exposure to BAL fluid did not induce significant changes to the expression of miR-150, miR-152 and miR-375, except for BAL6 which significantly increased the expression of miR-150. BAL6 also increased the expression of miR-375, but this was not statistically significant. However, what is apparent in these graphs is that exposure to severe asthma BAL did not reduce the expression of any of these miRNAs and it may be that longer exposure periods would lead to significantly increased expression. We then looked at the expression of TLR7 in these cells and this is shown in Figure 6-2.

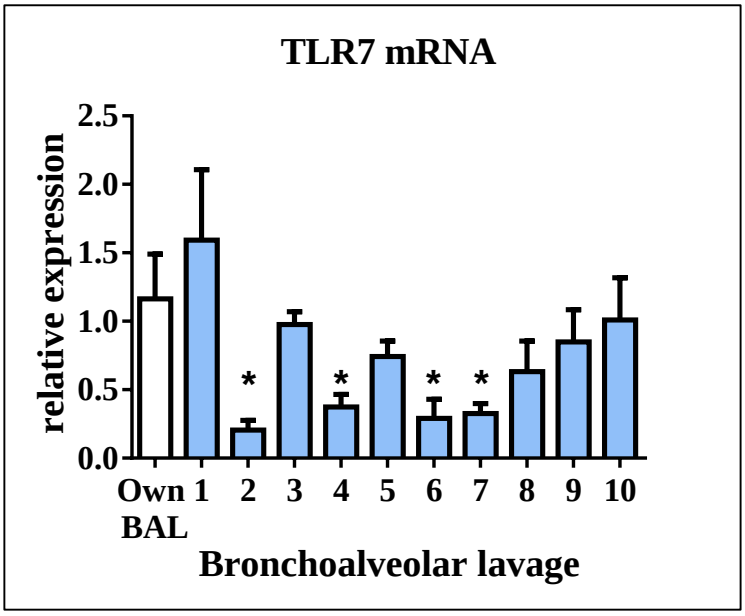


**Figure 6-2 Expression of TLR7 mRNA in healthy AM exposed to bronchoalveolar lavage from severe asthma subjects**
AM from 6 healthy subjects were exposed to BAL fluid from 10 SA subjects (numbers 1-10 on graph) and their own BAL for 24 hours. Expression is relative to treatment with medium alone and is standardised against GAPDH. Graph shows mean+SEM. *p<0.05 compared to Own BAL using one-way ANOVA with between group testing using t-test if results significant.

This shows that exposure to severe asthma BAL (BAL2, BAL4, BAL6 and BAL7) significantly reduces the expression of TLR7 in healthy AM. This suggests that there is likely to be common features in the BAL fluid of these 4 subjects. Again, this analysis shows that SA BAL fluid (collectively) does not contain anything that significantly increases the expression of TLR7 in healthy AM. In order to explore if the changes in the expression of the miRNAs and TLR7 may be related we combined the data onto a single graph (Figure 6-3). Specifically, we were looking for decreases in the expression of TLR7 coinciding with increases in the expression of miR-150, miR-152 and miR-375. As a guide, a dotted line is drawn on the graph at relative expression of 1 to help highlight changes compared to the control sample.

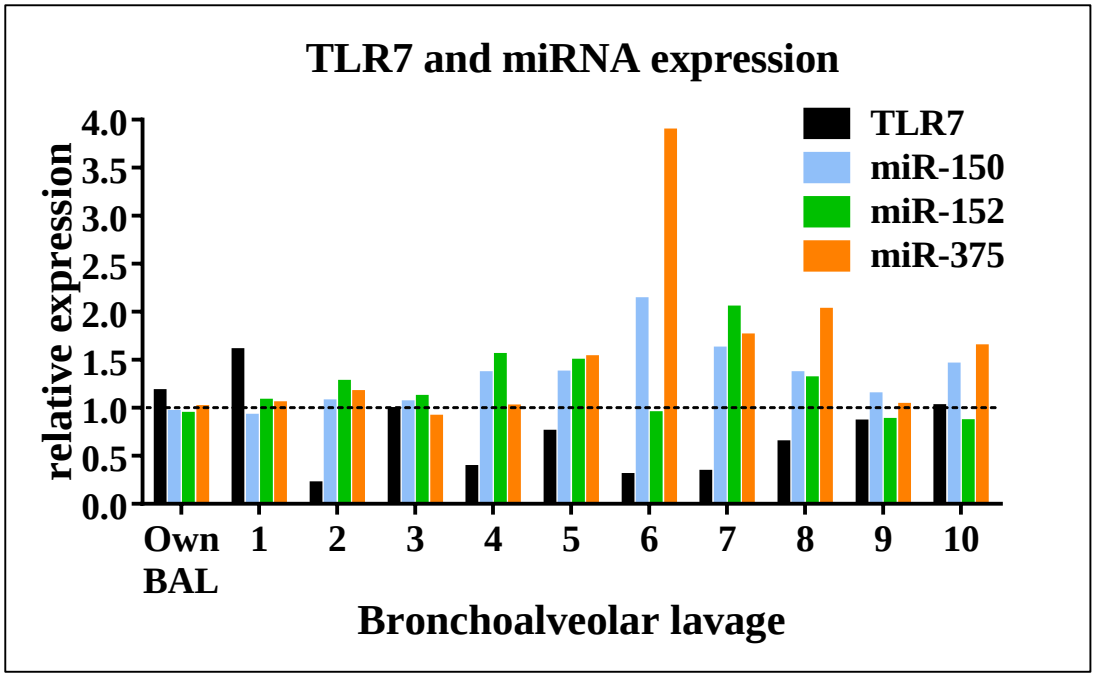


**Figure 6-3 Expression of miRNAs and TLR7 in healthy AM exposed to bronchoalveolar lavage from severe asthma subjects**
AM from 6 healthy subjects were exposed to BAL fluid from 10 SA subjects (numbers 1-10 on graph) and their own BAL for 24 hours. Expression is relative to treatment with medium alone and standardised against RNU44 (for miRNAs) and GAPDH (for TLR7). Graph shows mean expression.

This analysis shows that reductions in TLR7 mRNA expression are accompanied by relatively increased miRNA expression with 6 of the 10 severe asthma BAL samples- 4, 5, 6, 7, 8 and 10 although the changes in miRNA expression are not statistically significant (as shown in Figure 6-1). In 2 cases (BAL 3 and 9) there is no change in TLR7 or miRNA expression.

### 6.2.1.2 Effect of Th1 and Th2 cytokines on miRNA and TLR7 expression

The expression of miR-150, miR-152 and miR-375 after exposure of healthy AM to Th1 and Th2 cytokines was measured using qPCR and is shown in Figure 6-4. This shows that IL-4, IL-13, TNFα and IFNγ do not alter the expression of miR-150, miR-152 and miR-375 after 24 hours of stimulation.

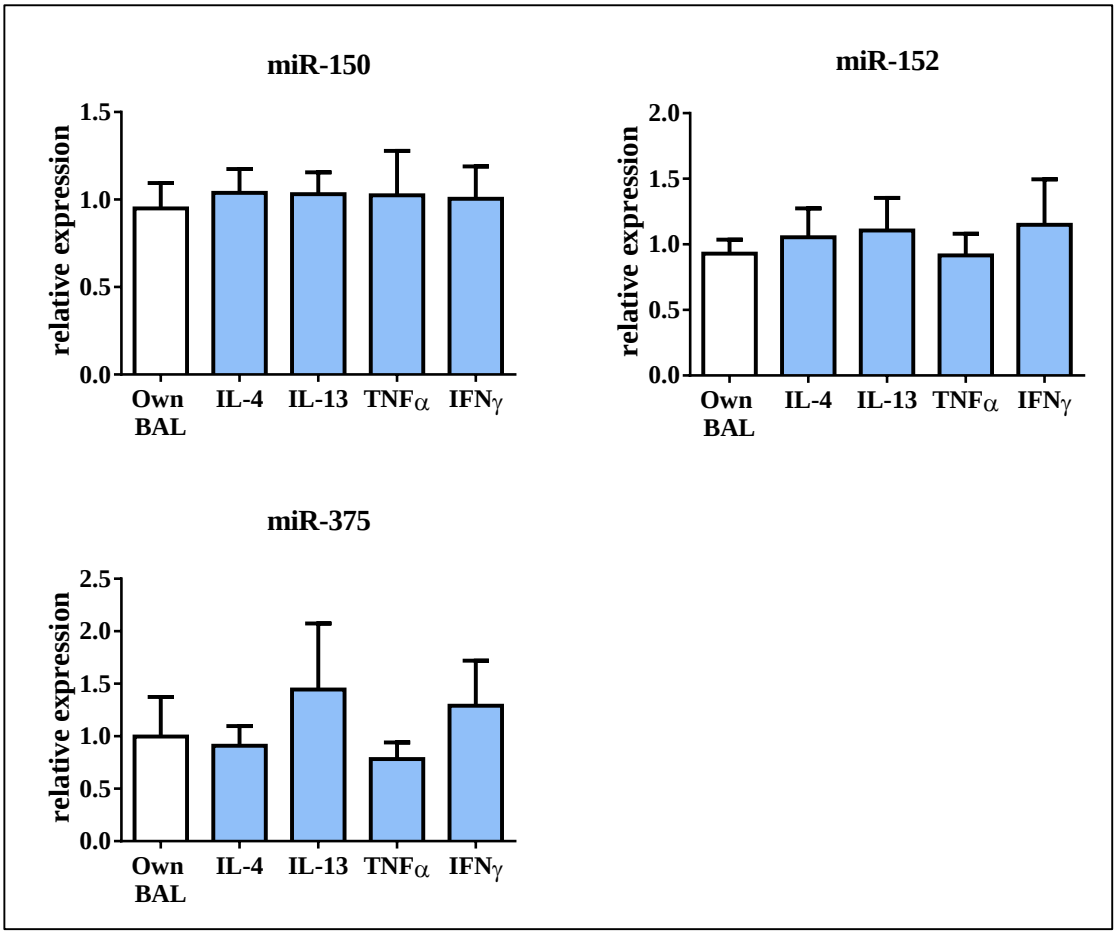


**Figure 6-4 Expression of miR-150, miR-152 and miR-375 in healthy AM exposed to Th1 and Th2 cytokines**
AM from 6 healthy subjects were stimulated with IL-4, IL-13, TNFα and IFNγ for 24 hours. As a control they were also exposed to their own lavage fluid. Expression is relative to treatment with medium alone and standardised against RNU44. Graphs show mean+SEM.

We then explored if these pro-inflammatory cytokines could affect the expression of TLR7 (Figure 6-5) and although this showed that there were no significant changes in TLR7 expression IL-4 induced a robust decrease in TLR7 expression ($p<0.05$ using a paired t-test).

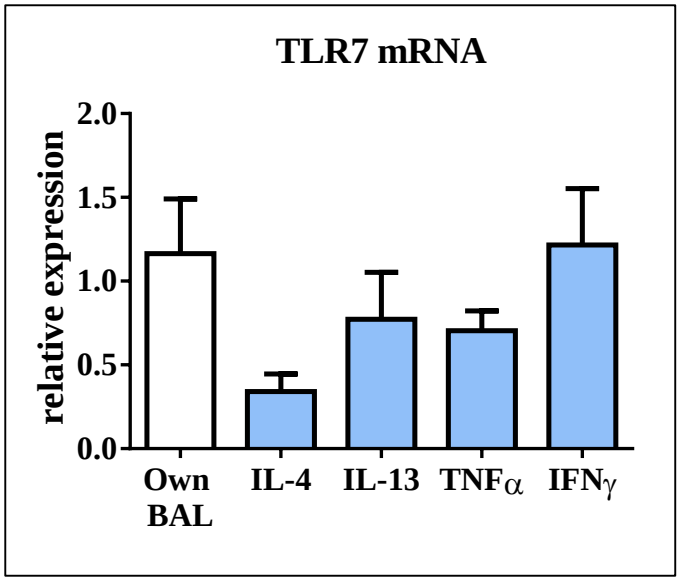


**Figure 6-5 Expression of TLR7 in healthy AM stimulated with Th1 and Th2 cytokines**
AM from 6 healthy subjects were stimulated with IL-4, IL-13, TNFα and IFNγ for 24 hours. As a control they were also exposed to their own lavage fluid. Expression is relative to treatment with medium alone and standardised against GAPDH. Graph shows mean+SEM. No significant changes in the expression of TLR7 was found using 1-way ANOVA.

Again we combined the results from the miRNA and TLR7 evaluation onto a single graph (Figure 6-6) and this confirmed that the Th1 and Th2 cytokines were not affecting the expression of miR-150, miR-152 and miR-375 or TLR7 (except for IL-4).

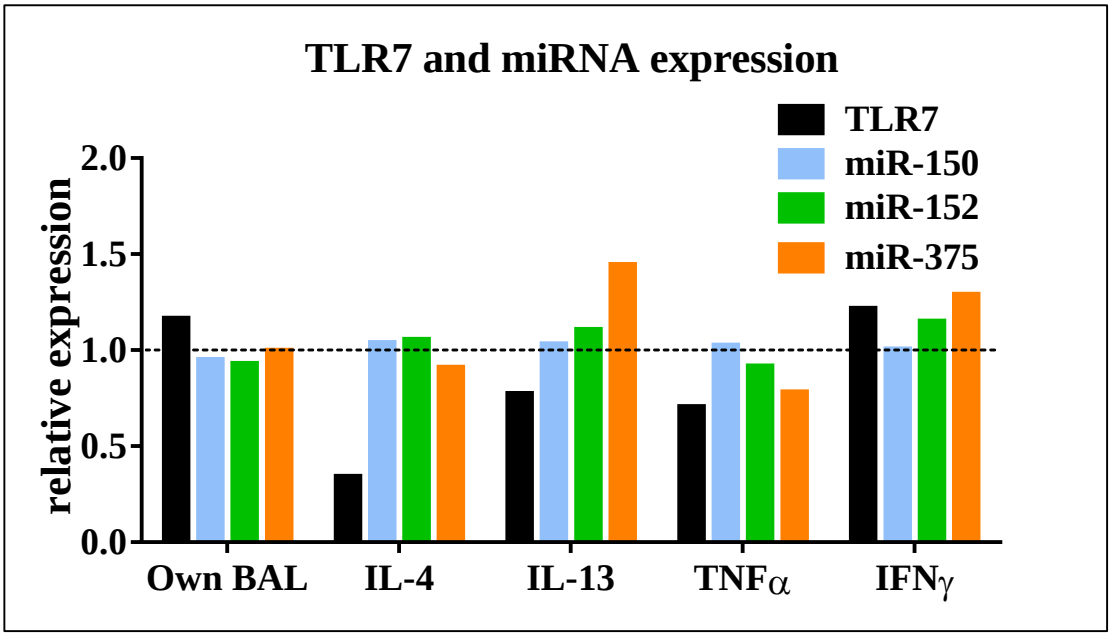


**Figure 6-6 Expression of miRNAs and TLR7 in healthy AM stimulated with Th1 and Th2 cytokines**
AM from 6 healthy subjects were stimulated with IL-4, IL-13, TNFα and IFNγ for 24 hours. As a control they were also exposed to their own lavage fluid. Expression is relative to treatment with medium alone and standardised against RNU44 (for miRNAs) and GAPDH (for TLR7). Graph shows mean.

#### 6.2.1.3 TLR7 expression is reduced in AM from patients with chronic obstructive pulmonary disease

*Ex vivo* RV-induced IFNβ production by BAL cells from patients with COPD has been shown to be significantly impaired compared to healthy subjects (Mallia et al. 2011) while CF bronchial epithelial cells have be shown to be severely deficient in their IFN response to RV (Vareille et al. 2012). Like asthma, COPD and CF are chronic inflammatory respiratory diseases but while asthma is thought to result from a gene-environment interaction, COPD is usually due to smoking (environment) and CF is due to mutations in the cystic fibrosis transmembrane regulator gene (genetic). However, all three conditions are characterised by recurrent lower respiratory tract infections. Therefore, we hypothesised IFN (and TLR7) deficiencies may be caused by processes related to chronic inflammation and infection with bacterial and viral organisms.

Therefore we evaluated the expression of TLR7 in AM from patients with COPD (Figure 7-8). BAL was provided by another study within the department and AM isolated as previously described.

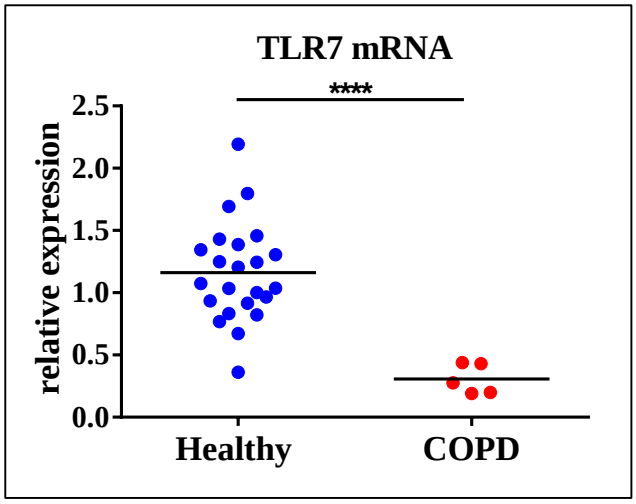


**Figure 6-7 Expression of TLR7 mRNA is significantly reduced in COPD AM**
Expression of TLR7 was measured using qPCR in healthy (n=23) and COPD (n=5) AM. Expression is relative to GAPDH. ****p<0.0001 using Mann Whitney test.

This showed that compared to healthy subjects, TLR7 expression in COPD AM was significantly reduced. Unfortunately as the samples were provided from another study there was insufficient RNA to also evaluate miRNA expression in these cells. However, this preliminary data is exciting because it supports the theory that processes related to chronic inflammation and recurrent infections are likely to be responsible for reduced RV-induced IFN responses in asthma (and COPD).

# 6.3 Effect of steroid therapy on miRNA and TLR7 expression

## 6.3.1 Methods and Results

The effect of steroids on miRNA and TLR7 expression was evaluated in an *in vivo* and an *ex vivo* model. For the *in vivo* model we recruited a group of well-controlled moderate asthma (MO) subjects. These patients were on Step 3 of the BTS treatment algorithm and their disease was severe enough to require treatment with an ICS and a LABA. With the use of this treatment, their disease was well-controlled as judged by the ACQ. The reason this group was chosen was because all patients with severe asthma were also on similar treatment (ICS + LABA), but their disease was uncontrolled. Therefore, any differences in miRNA expression between these two groups would theoretically be due to processes related to disease severity and not ICS therapy as both groups were receiving ICS. For the *in vitro* model we evaluated the expression of the 3 miRNAs in AM from healthy subjects after they had been treated with to dexamethasone *ex vivo*.

### 6.3.1.1 Expression of miR-150, miR-152 and miR-375 is not affected by steroids

The clinical characteristics of these MO subjects are shown in the Table 6-1.

| | Moderate Asthma |
|---|---|
| Number | 8 |
| Age | 39.8 (4.3) |
| Sex (M/F) | 3/5 |
| $FEV_1$ | 2.9 (0.2) |
| %predicted $FEV_1$ | 86.8 (4.6) |
| FVC | 4.3 (0.2) |
| %predicted FVC | 106.5 (3.9) |
| Body mass index | 28.7 (1.4) |
| Atopy (%) | 5 (60) |
| ACQ score | 0.7 (0.1) |
| ICS dose in mcg (BDP equivalent) | 725 (75) |
| Number on daily oral steroids (%) | 0 |
| Number of exacerbations in previous year | 0 |
| BAL cell differential | |
| %macrophages | 81.2 (4.6) |
| %neutrophils | 7.8 (3.6) |
| %eosinophils | 0.8 (0.3) |

**Table 6-1 Clinical characteristics of moderate asthma subjects**
Values are means with SEM in parenthesis unless otherwise indicated. ACQ, asthma control questionnaire; BAL, bronchoalveolar lavage; BDP, beclometasone; $FEV_1$, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroid; N/A, not applicable.

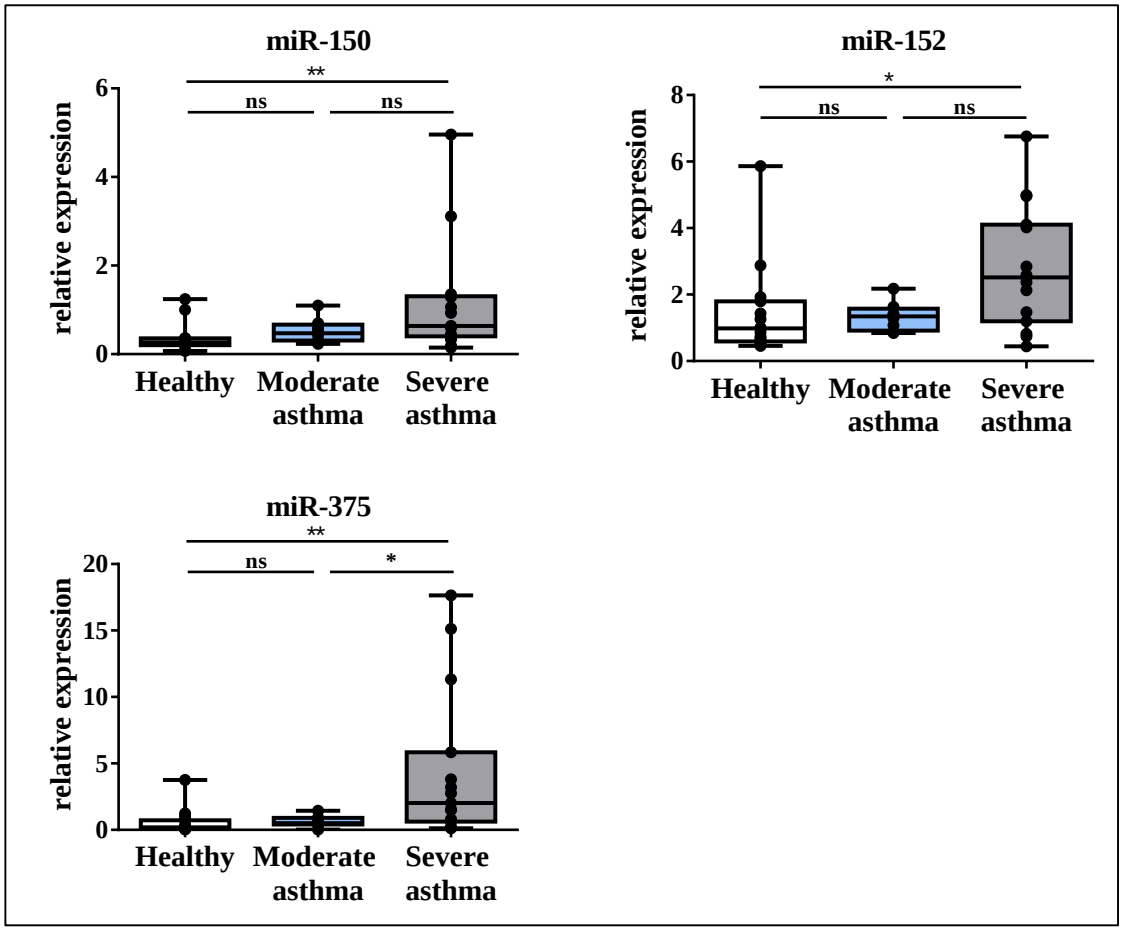


**Figure 6-8 Expression of miR-150, miR-152 and miR-375 in moderate asthma AM, compared to their expression in healthy and severe asthma AM.**
Expression of miR-150, miR-152 and miR-375 was assessed by qPCR in 15 healthy, 8 moderate asthma and 15 severe asthma subjects. Expression is relative to the housekeeping gene RNU44. *p<0.05, ** p<0.01, ns=not significant; comparisons made using Kruskal-Wallis test followed by between group testing with the Mann Whitney test if results were significant.

These results show that the expression of these miRNAs in not significantly different in MO-AM compared to healthy and SA-AM. However, the expression of all 3 miRNAs appears to be lower in MO-AM compared to SA-AM although this is statistically significant only in the case of miR-375.

We then proceeded to evaluate the expression of miRNAs in healthy AM exposed to dexamethasone. AM were cultured for 24 hours in the presence of 3 concentrations of dexamethasone (10nM, 100nM and 1000nM). The expression of miR-150, miR-152 and miR-375 was measured by qPCR and compared to control cells treated with medium alone. This evaluation showed

that dexamethasone treatment had no effect on the expression of miRNAs in AM.

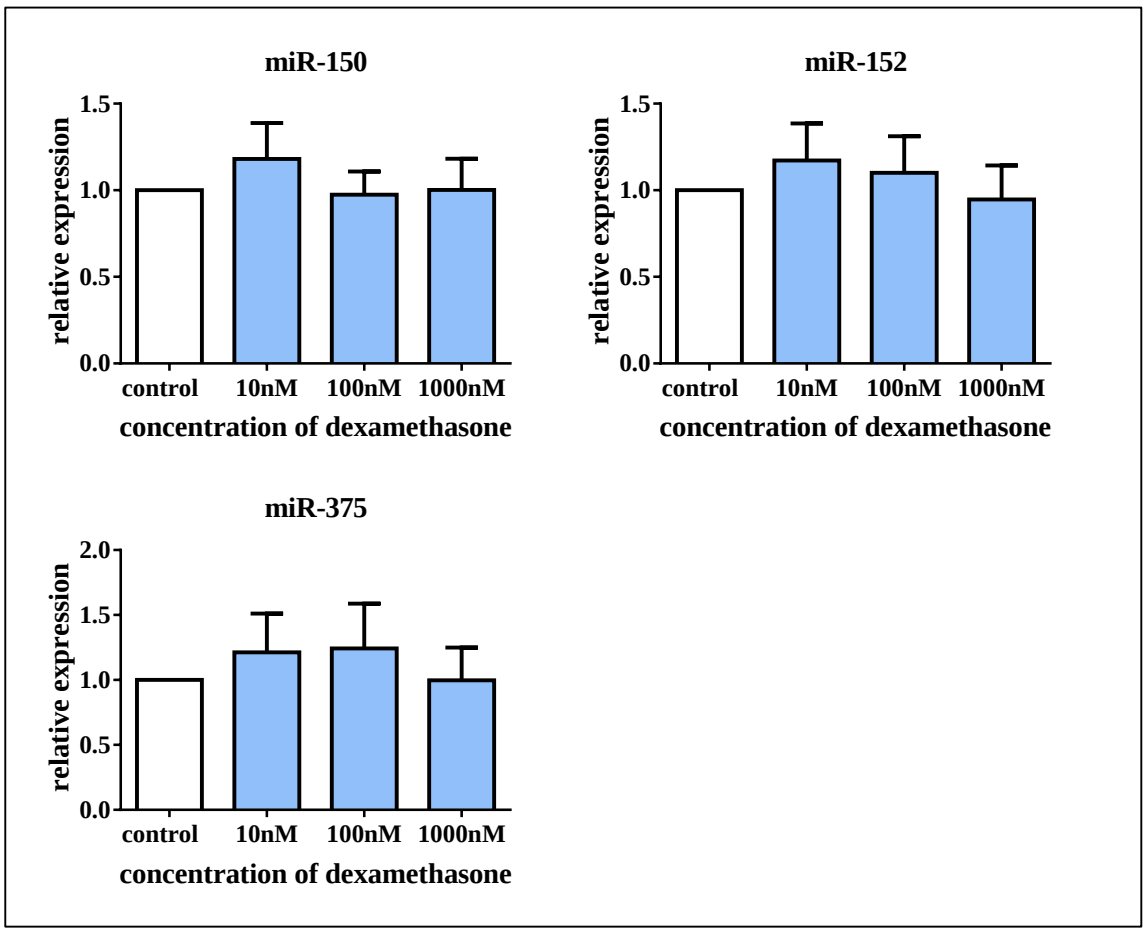


**Figure 6-9 Dexamethasone has no effect on the expression of miR-150, miR-152 and miR-375 in AM at 24 hours.**
AM from seven healthy subjects were treated *ex vivo* with dexamethasone at concentrations of 10nM, 100nM and 1000nM for 24 hours after which miR-150, miR-152 and miR-375 expression was measured. Expression is relative to the housekeeping gene RNU44. Each concentration was compared to the expression of the control sample and the mean and SEM are shown. Statistical analyses were carried out using 1-way ANOVA with Dunnett's multiple comparison test.

### 6.3.1.2 The effect of steroids on TLR7 expression

We then measured TLR7 expression in AM from MO subjects. This identified that the expression of TLR7 was significantly higher in MO-AM compared to SA-AM while it was not significantly different from that in healthy AM.

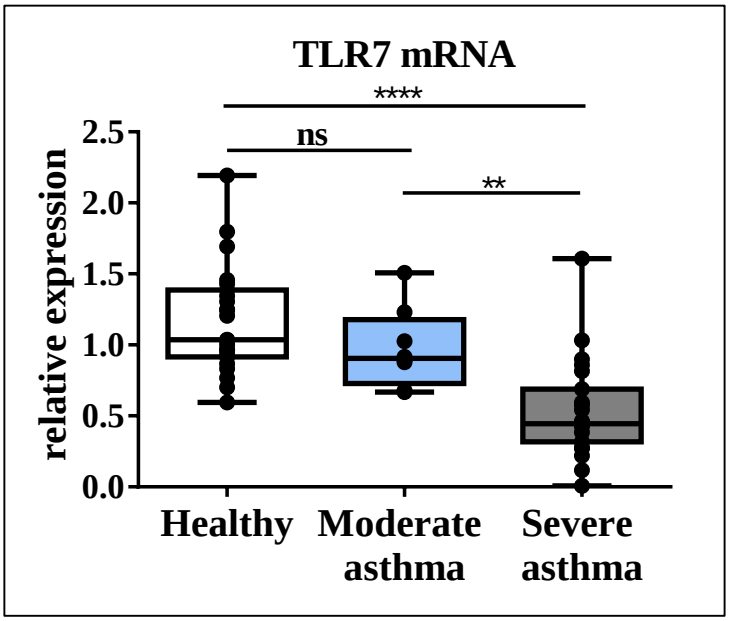


**Figure 6-10 Expression of TLR7 mRNA is reduced in severe asthma AM compared to healthy and moderate asthma AM**
Expression of TLR7 was assessed by qPCR in 23 healthy, 8 moderate asthma and 23 severe asthma subjects. Expression is relative to the housekeeping gene GAPDH. ** p<0.01, ****p<0.0001, ns=not significant; comparisons made using Kruskal-Wallis test followed by between group testing with the Mann Whitney test if results were significant

*Ex vivo* exposure of healthy AM to dexamethasone showed that at all three concentrations tested (10nM, 100nM and 1000nM) dexamethasone reduced the expression of TLR7 by 40-60%.

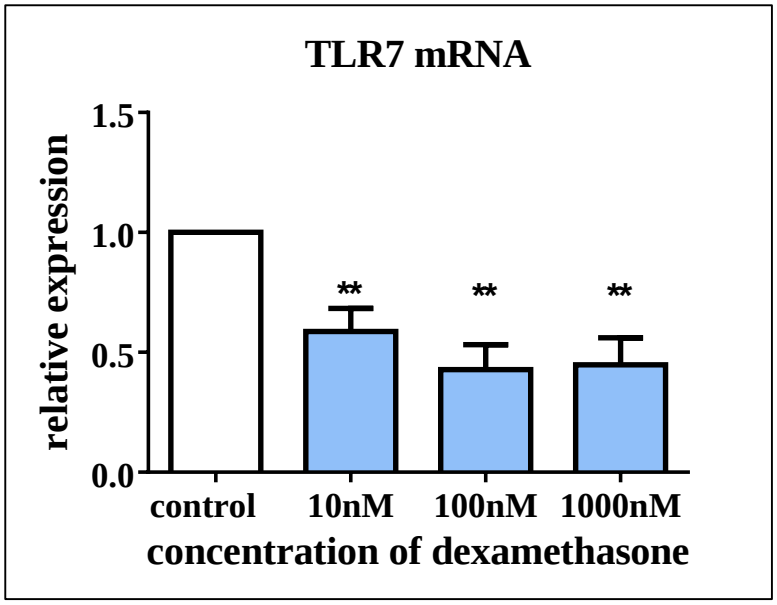


**Figure 6-11 Dexamethasone reduces the expression of TLR7 in AM**
AM from seven healthy subjects were treated *ex vivo* to dexamethasone at concentrations of 10nM, 100nM and 1000nM for 24 hours after which TLR7 mRNA expression was measured. Expression is relative to the housekeeping gene GAPDH. Each concentration was compared to the expression of the control sample and the mean and SEM are shown. ** $p<0.01$ compared to control sample. Statistical analyses were carried out using 1-way ANOVA with Dunnett's multiple comparison test and between group testing with paired t-test if results significant.

In summary, our results in this chapter suggest that it is unlikely that increased expression of miR-150, miR-152 and miR-375 in SA-AM is due to steroid treatment. Rather, it is more likely that the milieu within the lower airways of patients with SA is affecting miRNA related changes in TLR7 expression.

## 6.4 Discussion

We have previously shown that the expression of miR-150, miR-152 and miR-375 are significantly increased in SA-AM, leading to reduced TLR7 expression. In this section we explored if these changes were related to the microenvironment within the airways of patients with severe asthma or to steroid treatment, which is received by all patients with severe asthma. Therefore, we exposed healthy AM to BAL fluid from severe asthma subjects to try and replicate the local microenvironment within the lower airways and to dexamethasone. Exposure to severe asthma BAL fluid showed that while the expression of TLR7 was significantly reduced by exposure to some of the severe asthma BAL samples, in most cases this was associated with increased miRNA expression, although this was not always statistically significant. Treatment with dexamethasone did not affect miRNA expression in healthy AM but did reduce TLR7 expression at all the concentrations tested. The lack of corresponding miRNA and TLR7 changes in these *ex vivo* models may be due to:

1. MiRNA expression changes tend to precede changes in target mRNA levels and as we are able to detect reductions in TLR7 mRNA levels, the changes in miRNA expression would have occurred earlier (for example at 8 hours). Therefore at 24 hours, when levels were evaluated, we may have missed the peak in miRNA expression and levels may already be returning to baseline.
2. Changes in TLR7 expression in this setting may be unrelated to miR-150, miR-152 and miR-375.

Exposure to 4 of the severe asthma BAL fluid samples caused significant reductions in TLR7 expression and we propose that this is due to components in severe asthma BAL. These 'components' could include extracellular vesicles secreted by cells into BAL fluid. Of interest, of the 4 severe asthma BAL samples which were associated with reducing TLR7 expression in healthy AM, baseline miRNA expression levels in 3 of the corresponding AM samples were amongst the highest of all the severe asthma subjects (Figure 4-3). It can be hypothesised that miR-150, miR-152 and mi375 may have been released from the AM in extracellular vesicles into BAL fluid. It has been shown that extracellular vesicles, or exosomes, can serve as intercellular transporters of

miRNAs for cell-to-cell communication (Fujita et al. 2014). Once this BAL fluid was applied onto healthy AM, the exosomes could transfer back into the recipient's AM and their contents affect cellular processes. These 'components' could also include bacterial products. Work from Southampton has shown that induced sputum from patients with severe asthma is more commonly colonised by potentially pathogenic bacteria: *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (personal communication with Professor Peter Howarth). These bacteria themselves may cause changes in miRNA and/or TLR7 expression or the inflammatory changes they induce within the airways may influence miRNA/TLR7 expression.

We were a little surprised to find that the typical Th1 and Th2 cytokines did not affect miR-150, miR-152 and miR-375 expression. However, in order to replicate the cytokine environment within the asthmatic airway, longer incubation periods may have been more appropriate. Treatment of cultured bronchial epithelial cells with IL-13 for 14 days has been shown to replicate some of the miRNA changes observed in asthmatic bronchial epithelium (Solberg et al. 2012). Contrary to the long incubation time used by Solberg *et al*, other studies have used shorter incubation times but higher concentrations of cytokines. TNF$\alpha$ (50-200 ng/ml) has been shown to increase miR-150 expression in monocyte derived macrophages in as little as 8 hours (Ghorpade et al. 2013) while treatment with IL-13 (100ng/ml) for 24 hours has been shown to reduce miR-375 expression in a human bronchial epithelial cell line (Lu et al. 2012). Therefore, it may have been more appropriate for us to use higher concentrations of cytokines and also explore different incubation times. However, numbers of AM recovered from BAL limit the use of multiple concentrations of cytokines and the concentrations employed were based on previous work done in our group.

Regarding cytokine stimulation and TLR7 expression, our results show that IL-4 reduced the expression of TLR7 in healthy AM ($p=0.045$ using paired t-test). Without corresponding changes in miRNA expression is it very unlikely that this is via a miRNA mediated mechanism. TLR7 activation has been shown to prevent Th2-mediated airway inflammation in a variety of animal models of acute and chronic asthma (Drake et al. 2012) and specifically reduce IL-4 and IL-13 levels in ovalubumin sensitised and challenged animals (Kaufman et al. 2012). This ability of TLR7 agonists to skew the immune system away from a

Th2 response has led to exploring their use in the treatment of asthma and allergic rhinitis (Greiff et al. 2012). Therefore, it is not entirely unexpected that Th2 stimulation of cells with IL-4 skews away from Th1 responses and reduces TLR7 expression.

As steroids can influence the transcription of a range of genes we wished to explore if the treatment that the patients with severe asthma were receiving i.e. glucocorticoids could affect the expression of miR-150, miR-152, miR-375 and TLR7. We evaluated the expression of miR-150, miR-152 and miR-375 in AM from well controlled MO subjects. We specifically recruited patients who had no history of disease exacerbation in the previous year and who had an ACQ score ≤1 (mean ACQ=0.7). Like patients with severe asthma, this group of patients were also on ICS therapy, but unlike the severe asthma group (mean ACQ=2.8), their disease was well controlled. Therefore, differences in miRNA expression between these two groups could theoretically be attributed to the underlying disease rather than ICS treatment.

Our results show that the expression of miR-150, miR-152 and miR-375 in MO was not significantly raised compared to healthy subjects suggesting that ICS were not increasing the expression of miRNA. But the expression of these miRNA was not significantly lower in MO compared to severe asthma except for miR-375. The lack of statistical significance could be due to the smaller number of subjects in the MO group.

Although both MO and severe asthma patients were on ICS treatment, severe asthma patients were receiving significantly higher doses (mean BDP equivalent dose 2253 mcg) compared to MO patients (mean BDP equivalent dose 725 mcg). As can be seen in Figure 6-8 the expression of all 3 miRNAs shows a trend of increasing expression from healthy to severe asthma and therefore, from our results we cannot fully exclude a steroid effect. Indeed, studies examining the effect of ICS on the expression of proinflammatory and anti-viral cytokines tend to show that their effects are dose dependent (Davies et al. 2011; Skevaki et al. 2009). However, our *ex vivo* model used concentrations of dexamethasone that are likely to be far greater than the serum corticosteroid levels in MO, and found no significant increase in miRNA levels. Ideally, we should have examined the expression of these miRNAs in subject on Step 4 and 5 of BTS asthma treatment who had well-controlled disease (ACQ≤1). This

would be quite difficult as almost by definition these patients have poorly controlled disease and an extremely small number would have an ACQ≤1. The interim analysis from the Wessex Severe Asthma Cohort, a large cohort of patients with severe asthma in the Wessex region, has shown that only 13 of the 200 patients (6.5%) analysed at the time had an ACQ≤1.

We propose that the increased expression of miRNAs in severe asthma is due to processes related to disease control and severity rather than the ICS treatment the patients were receiving. In support of our theory that ICS do not affect miRNA expression it been shown that the expression of miRNAs in bronchial biopsies from patients with asthma was no different before and after the use of ICS (Williams et al. 2009). Furthermore, microarrays done on bronchial epithelial brushes from steroid-naïve subjects showed that at baseline over 200 miRNAs were differentially expressed compared to non-asthmatic subjects and the expression of only 9 of these changed after a period of ICS use (Solberg et al. 2012), suggesting ICS have very minimal effects on the expression of pulmonary miRNAs.

Additionally, there is evidence from studies done in other disease areas that steroids can negatively influence miRNA biogenesis. MiRNA biogenesis is completely reliant on adequate cellular levels of certain enzymes- nuclear Drosha and cytoplasmic Dicer (Bartel 2004). Drosha processes the pri-miRNA into stem-loop intermediates which are then cleaved by Dicer and matured to their active ~22nt forms (Lee et al. 2003). A reduction in these enzymes would lead to a build-up of primary miRNA transcripts and a lack of functional miRNAs. Microarrays done on cells cultured with dexamethasone showed changes in the expression of a number of miRNAs with about 80% of these miRNAs being repressed. It was shown that this repression was due to steroid induced depletion of both Drosha and Dicer (Smith et al. 2010). If these actions of steroids were more global and occurring in AM as well then it makes it much less likely that increased expression of miR-150, miR-152 and miR-375 is due to the effects of ICS treatment.

While TLR7 expression in MO-AM was similar to healthy AM and significantly higher than SA-AM, *in vitro* experiments with dexamethasone clearly showed that at all the 3 concentrations used, steroids reduced the expression of TLR7 by ~50%. A number of discussion points arise from this result:

1. The magnitude of reduction in TLR7 expression induced by dexamethasone (~50%) is similar to the reduction in TLR7 seen in severe asthma AM compared to healthy.
2. This would suggest that standard asthma treatment i.e. ICS, albeit at higher doses, are impairing virus sensing mechanisms within AM by reducing expression of TLR7. Patients with severe asthma are on high doses of ICS and the results with the MO group clearly show that AM from patients on lower doses of ICS have higher TLR7 expression than severe asthma patients (Figure 6-10); this would support a dose dependent effect of steroids.
3. Higher expression of TLR7 in the MO group compared to severe asthma patients could also (or instead) be due to an effect of asthma control- the MO group had well-controlled disease while severe asthma patients had uncontrolled disease.
4. TLR7 expression in SA-AM did not correlate with the dose of ICS treatment the patient was receiving at the time of bronchoscopy (Figure 5-4). The effects of steroids on the suppression of inflammatory cytokines tend to dose dependent and if they were indeed reducing TLR7 expression then patients receiving higher doses of ICS should have had reduced TLR7 expression.
5. The literature suggests that steroids increase the expression of TLRs. TLR4 expression in PBMCs has been shown to increase after a 7 day course of oral prednisolone (Chun et al. 2010) while TLR2 expression was shown to be enhanced in BECs co-stimulated with budesonide and a TLR2 agonist (von Scheele et al. 2010). The latter study showed that the TLR2 enhancing effect occurred in addition to steroid induced inhibition of pro-inflammatory cytokines.
6. *In vitro* work with BECs has shown that budesonide does not inhibit the production of IFNβ after RV infection (Bochkov et al. 2013) suggesting that ICS do not have inhibitory effects on innate responses to RV.

Our results also show that TLR7 expression is significantly reduced in COPD AM, albeit in a small number of samples. We decided to investigate TLR7 expression in COPD AM because it has been shown that they have reduced IFN responses to RV (Mallia et al. 2011). COPD is characterised by recurrent lower respiratory tract bacterial infections and chronic bacterial colonisation of

airways. Patients with severe asthma frequently develop similar features and it can be postulated that bacterial presence and the associated cytokine milieu within the lower airways is impacting on TLR7 and miRNA expression. These results are extremely preliminary and confirmation in a larger sample and miRNA measurements are needed, but they would appear to support this theory.

In summary, our results indicate that steroid treatment does not affect the expression of miRNAs in SA-AM but steroids may reduce TLR7 expression. In reality, clinicians are unlikely to stop ICS treatment in patients with SA, even in those with recurrent viral exacerbations. However, our *in vitro* experiments clearly show that dexamethasone reduces TLR7 expression in AM and this finding warrants further confirmation with protein data and investigation using *in vivo* studies. Our results also suggest that changes in miRNA and TLR7 expression are more likely to be related to processes and/or components within the lower airways of SA patients.

# Chapter 7 Inhibition of miR-150, miR-152 and miR-375 improves RV-induced IFN production

## 7.1 Introduction

The results so far have shown that the expression of miR-150, miR-152 and miR-375 is increased in severe asthma and that the expression of TLR7 is reduced in severe asthma. TLR7 is one of the PRRs involved in the innate immune recognition of RV when it enters the airways and along with reduced expression, we have also shown that the function of this receptor is reduced in SA. The mechanistic molecular studies using cloned sequences of miRNAs and 3'UTR of TLR7 have provided direct evidence that these three miRNAs do indeed target the 3'UTR of TLR7, leading to reduced translation of the protein.

At present patients who develop an exacerbation of their asthma are treated with oral steroids and occasionally also nebulised bronchodilator therapy and antibiotics. There is no specific treatment that targets the underlying disease process and regardless of the cause of the exacerbation all patients receive similar treatment. Therefore there is a great need for novel therapeutic developments in this field.

In a cellular system, miRNA function can be inhibited with the use of anti-miRs. These are antisense oligonucleotides that are perfectly complementary to the miRNA and base pair with it to cause a reduction in the level of functional miRNA able to participate in gene suppression (Lennox et al. 2011). There is much interest in the use of these molecules as therapeutic agents for a number of disease processes but investigation into delivery mechanisms is still in early stages. We sought to investigate if inhibition of miR-150, miR-152 and mir-375 could lead to increased expression and function of TLR7 in AM. Asthmatic AM have been shown to produce far less type I and III IFNs when exposed to RV compared to healthy AM. If the expression of TLR7 could be improved and thus its function restored to normal levels it would ameliorate the defect in IFN production in asthmatic airways and consequently have great potential in the future therapeutic market for asthma exacerbations.

## 7.2 Hypothesis and Aims

### 7.2.1 Hypothesis

Inhibition of miR-150, miR-152 and miR-375 can augment the expression and function of TLR7 in AM thereby ameliorating the defective IFN response of these cells to RV infection

### 7.2.2 Aims

1. Successfully transfect AM with anti-miRs
2. Transfect AM with anti-miR-150, anti-miR-152 and anti-miR-375 and demonstrate improved function of TLR7

## 7.3 Methods and Results

### 7.3.1 AM can be successfully transfected with anti-miRs resulting in reduced expression of the desired miRNA

In the first instance, AM recovered from bronchoscopy were treated with anti-miR-150, anti-miR-152 and anti-miR-375. Previous work within our group has shown that dendritic cells and peripheral blood monocytes actively phagocytose oligonucleotides obviating the need to use a transfection reagent. This is beneficial because it is possible that the transfection reagent itself can influence the outcome of the experiment. It was hoped that AM would also phagocytose the oligonucleotide, and so a transfection reagent was not employed. As a control, scrambled RNA was used. In order to check the success of transfection, expression of two miRNAs was assessed in the transfected cells using qPCR. Figure 7-1 shows that AM can be successfully transfected by anti-miRs. Compared to mock transfected control, the expression of miR-150 is reduced by 90%, miR-152 by 50% and miR-375 by over 50% when AM are transfected with anti-miR-150, anti-miR-152 and anti-miR-375 respectively.

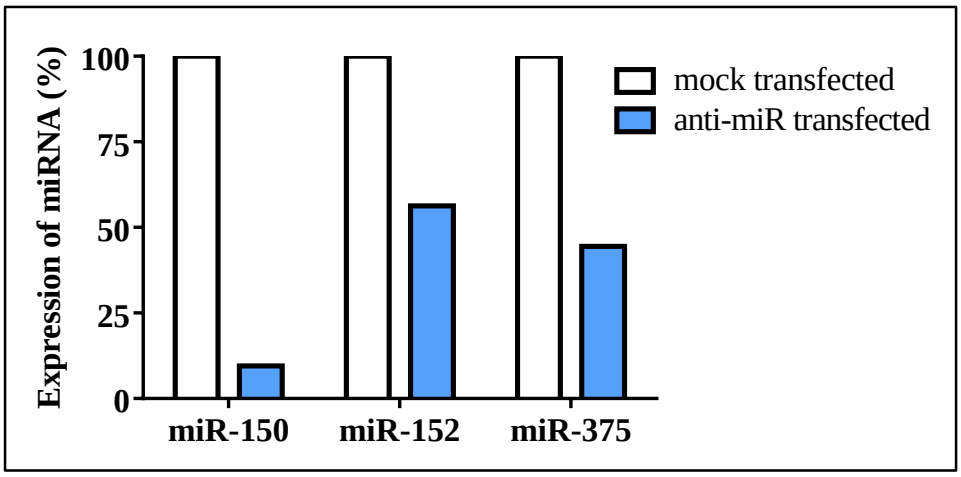


**Figure 7-1 Transfection of AM with antimiR-150, anti-miR-152 and antimiR-375 leads to reduction in the expression of the corresponding miRNAs**
AM were transfected with anti-150, anti-miR-152 and anti-375 and the expression of the corresponding miRNAs was assessed using qPCR. Expression is relative to the housekeeping gene RNU44.

### 7.3.2 Blocking miR-150, miR-152 and miR-375 in AM leads to significantly increased imiquimod-induced IFN production

AM were then transfected with all three anti-miRs (anti-miR-150, anti-miR-152 and anti-miR-375) so that the expression and function of all three miRNA could be inhibited. After 48 hours they were treated with imiquimod (5µg/ml) or medium alone as a control for 24 hours following which the expression of IFNα and IFNβ was measured using qPCR and ELISA.

Figure 7-2 shows that when AM are transfected with the three miRNAs and then challenged with the TLR7 agonist imiquimod, they produce significantly more IFNα and IFNβ mRNA compared to the mock transfected AM.

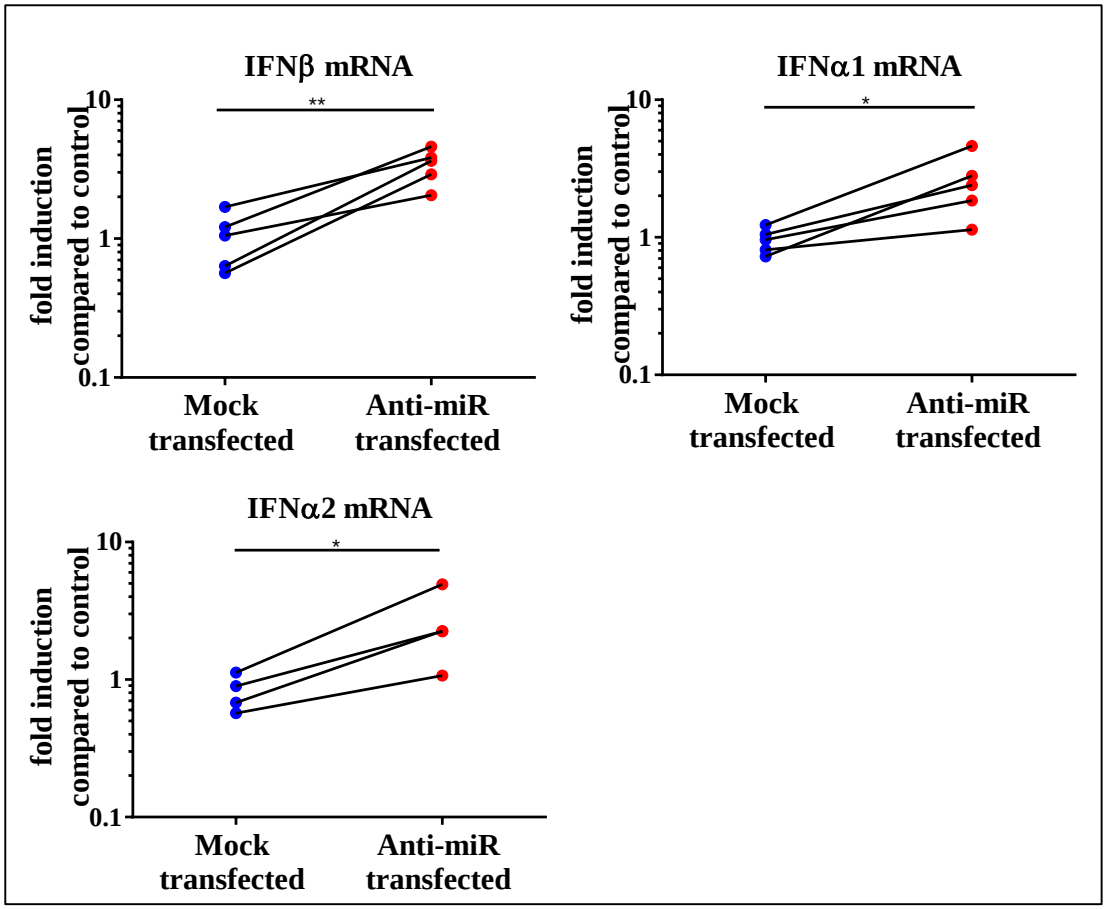


**Figure 7-2 Knock-down of miR-150, miR-152 and miR-375 improves TLR7 dependent IFN mRNA responses by imiquimod**
AM were transfected with a mix of anti-miR-150, anti-miR-152 and anti-miR-375 (Anti-miR transfected) or scrambled control oligonucleotides (mock transfected). 48 hours later cells were treated with imiquimod or medium alone and cells collected 24 hours later to determine levels of IFN. Expression is relative to treatment with medium alone and normalised against GAPDH. n=5 for the above experiments except IFNα2 where n=4. *p<0.05, **p<0.01 using paired t-test.

The expression of IFNβ protein was measured in the supernatant of these cells using ELISA and is shown in Figure 7-3. This assessment revealed that when transfected with the 3 anti-miRs, AM produced significantly more IFNβ protein compared to mock transfected AM. Figure 7-3 also shows that both the mock transfected and anti-miR transfected AM mounted an IFNβ response to imiquimod, although the response was only statistically significant in the anti-miR transfected group.

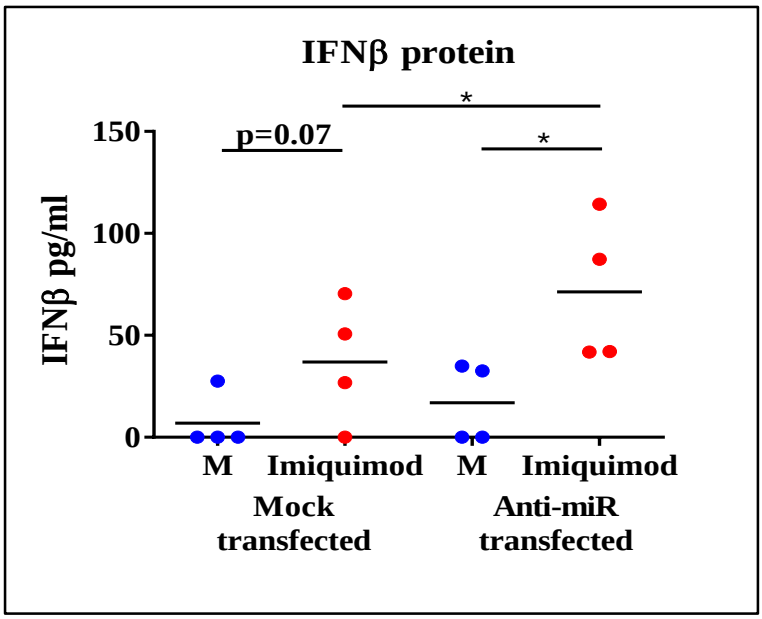


**Figure 7-3 Imiquimod-induced production of IFNβ protein by AM is increased when miR-150, miR-152 and miR-375 are inhibited by anti-miRs**
AM were transfected with the 3 anti-miRs (Anti-miR transfected) or scrambled control (Mock transfected) and 48 hours later treated with imiquimod (5μg/ml) or medium alone (M). IFNβ protein expression in the supernatant was measured 24 hours later by ELISA. AM from 4 subjects were used and the mean is displayed. *p<0.05 using repeated-measures ANOVA with Sidak's multiple comparison test and between group testing with paired t-test if results significant.

Unfortunately, at the time that this analysis was done we used a standard ELISA platform for measuring IFNβ levels and not an MSD assay platform. The advantage of the latter is that much smaller sample volumes can be used, but as we used standard ELISA all the supernatant we had stored (200μl) was used and measurements of IFNα protein expression could not be made.

To confirm that the augmentation in IFN responses was due to the effects of these miRNAs on TLR7 we also stimulated the transfected cells with Poly:IC, a synthetic dsRNA that activates TLR3, RIG-1 and MDA5. This showed no difference in the expression of IFNβ between AM transfected with all three anti-miRs and mock transfected AM (Figure 8-4). This confirms that blocking miR-150, miR-152 and miR-375 were not influencing responses to dsRNA and that the increase in IFN production was due to miRNA mediated increases in TLR7 expression and function.

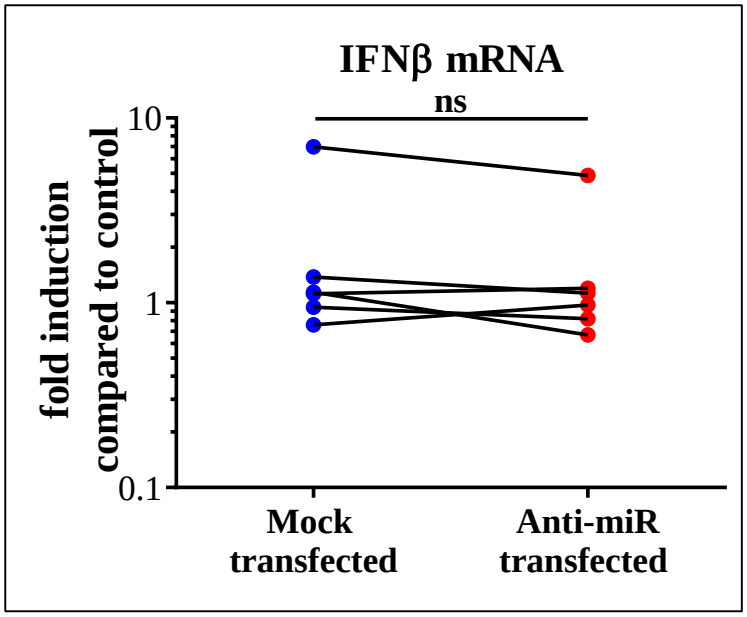


**Figure 7-4 Poly:IC induced IFNβ production by AM does not increase when cells are transfected with anti-miR-150, anti-miR-152 and anti-miR-375.** AM were transfected with the 3 anti-miRs (Anti-miR transfected) or scrambled control (Mock transfected) and 48 hours later treated with Poly:IC (10μg/ml) or medium alone. IFNβ mRNA expression was measured 24 hours later by qPCR and is shown relative to treatment with medium alone and standardised against GAPDH. AM from 6 subject were used. ns=not significant using paired t-test.

### 7.3.3 Transfection of AM with anti-miRs targeting miR-150, miR-152 and miR-375 leads to significantly increased IFN production when cells are challenged with rhinovirus

To show that the augmentation of TLR7-specific activity could be biologically relevant we also treated the transfected cells (both anti-miR transfected and mock-transfected) with RV (MOI 0.6) for 24 hours. AM transfected with anti-miR-150, anti-miR-152 and anti-miR-375 for 48 hours and exposed to RV showed significantly greater production of IFNβ and IFNα mRNA (Figure 7-5) and secreted protein (Figure 7-6) compared to mock transfected cells. These data suggest that blocking miR-150, miR-152 and miR-375 restores the anti-viral IFN responses in SA-AM.

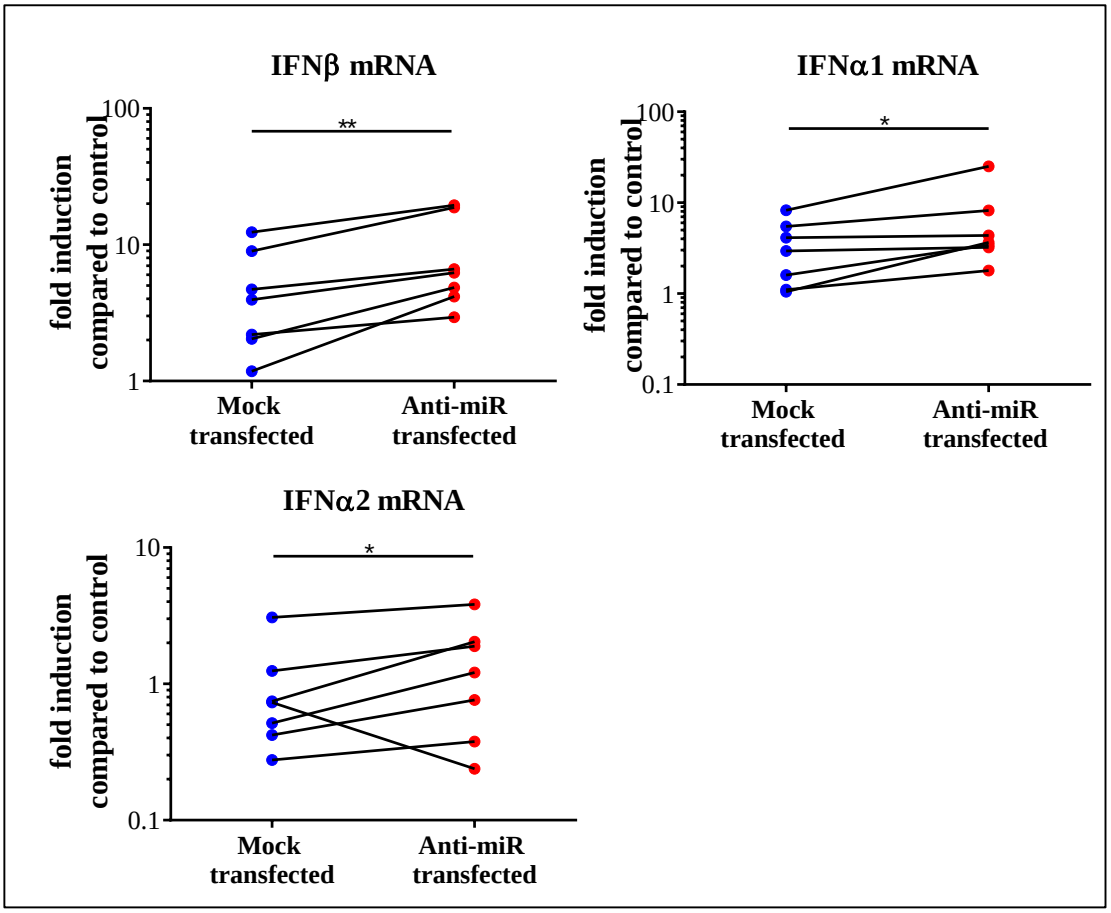


**Figure 7-5 Knock-down of miR-150, miR-152 and miR-375 improves anti-viral IFN responses in AM**
Alveolar macrophages were transfected with anti-miR-150, anti-miR-152 and anti-miR-375 (Anti-miR transfected) or scrambled control oligonucleotides (Mock transfected). 48 hours later cells were exposed to RV or UV-RV (UV-inactivated RV) and collected a further 24 hours later to determine levels of IFNβ and IFNα mRNA. AM from 7 subjects were used and expression is relative to treatment with medium alone and standardised against GAPDH. *p<0.05, **p<0.01 using repeated measured ANOVA with between group testing using paired t-test if results significant.

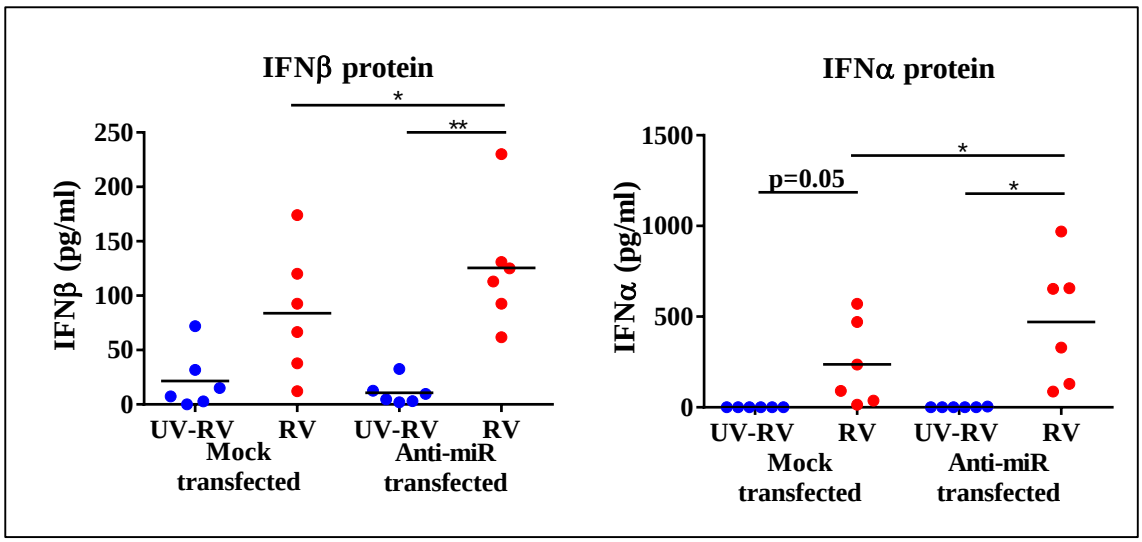


**Figure 7-6 Knock-down of miR-150, miR-152 and miR-375 improves anti-viral IFN protein responses in AM**
Alveolar macrophages were transfected with anti-miR-150, anti-miR-152 and anti-miR-375 (Anti-miR transfected) or scrambled control oligonucleotides (Mock transfected). 48 hours later cells were exposed to RV or UV-RV (UV-inactivated RV) and supernatants collected a further 24 hours later to determine levels of IFNβ and IFNα protein. AM from 6 subjects were used. *p<0.5, **p<0.01 using repeated measured ANOVA with between group testing using paired t-test if results significant.

As part of the cellular response to virus, IFNs induce the production of numerous ISGs that are vital to the execution of the cellular anti-viral innate response. Together these limit viral replication, inhibit viral budding from cells and induce apoptosis of infected cells, thereby limiting the magnitude and persistence of the viral infection (Schoggins et al. 2011; Swiecki et al. 2011). To assess whether blocking the 3 miRNAs could also have effects on these downstream genes we measured the expression of two of these ISGs- MxA and OAS.

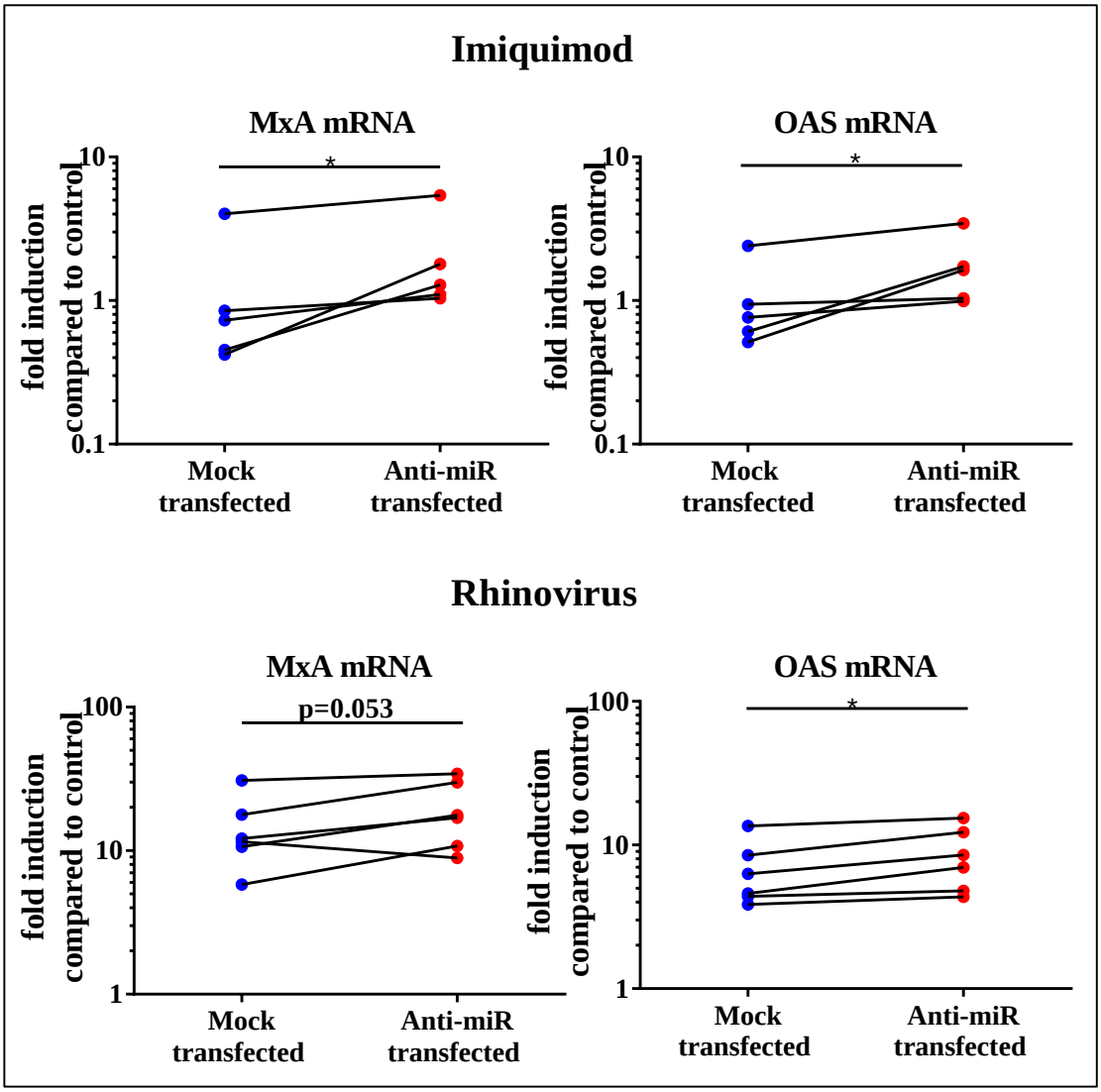


**Figure 7-7 Knock-down of miR-150, miR-152 and miR-375 augments TLR7 dependent and rhinovirus-induced production of ISGs**
Alveolar macrophages were transfected with anti-miR-150, anti-miR-152 and anti-miR-375 (Anti-miR transfected) or scrambled control oligonucleotides (Mock transfected). 48 hours later cells were exposed to imiquimod or RV or UV-RV (UV-inactivated RV) and collected a further 24 hours later to determine levels of MxA and OAS mRNA. AM from 5 subjects were used for the imiquimod experiments (top panel) and 6 subjects were used for the RV experiments (lower panel). Expression is relative to treatment with medium alone and standardised against GAPDH. *p<0.05 using paired t-test.

## 7.3.4 Effect of miRNA transfection on TLR7 expression

Finally in order to confirm that the increased IFN responses detected when the 3 miRNAs were blocked were indeed due to effects of the miRNAs on TLR7 expression we evaluated TLR7 expression and at the mRNA and protein level. TLR7 mRNA expression was evaluated 72 hours after AM had been transfected

with anti-miRs (or control). This was the same time point used for measurements of IFN mRNA levels. This showed a modest, but significant increase in the expression of TLR7 mRNA in AM transfected with anti-miRs compared to mock transfected samples.

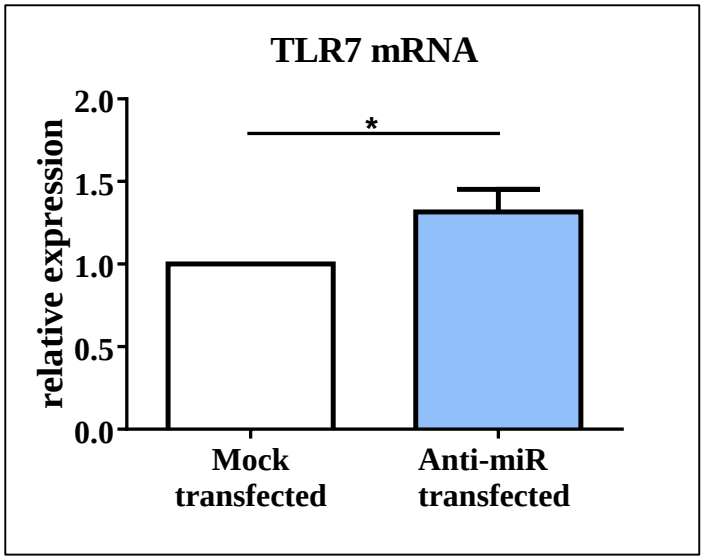


**Figure 7-8 Expression of TLR7 mRNA is increased in AM transfected with anti-miR-150, anti-miR-152 and anti-miR-375**
AM were transfected with anti-miR-150, anti-miR-152 and anti-miR-375 (Anti-miR transfected) or scrambled control (Mock transfected) and expression of TLR7 mRNA evaluated 72 hours later. Expression is standardised against GAPDH. *p<0.05 using paired t-test. Graph shows mean+SEM and results are from 11 experiments.

These results indicate that when miR-150, miR-152 and miR-375 are blocked, they are no longer able to exert any inhibitory effects on TLR7 mRNA and the expression of this mRNA increases in AM. Unfortunately, protein lysates of these cells had not been stored and so we could not evaluate TLR7 protein expression in these specific samples.

To investigate if the increase in TLR7 mRNA translates to an increase in TLR7 protein expression, healthy AM were transfected with pre-miR-150, pre-miR-152 and pre-miR-375 (500nM each) or scrambled control. Pre-miRNAs are small, chemically modified dsRNA molecules designed to mimic endogenous mature miRNAs. Therefore, transfecting a pre-miRNA into a cell essentially up-regulates the activity of that specific miRNA as the pre-miR, like endogenous miRNAs precursors, is processed by cellular enzymes into a miRNA and taken up into the RISC to regulate gene expression.

As before no transfection reagent was employed. 48 hours after transfections cells were lysed using the protein lysis buffer NP-40 and expression of TLR7 protein was assessed by Western blotting and densitometric analysis.

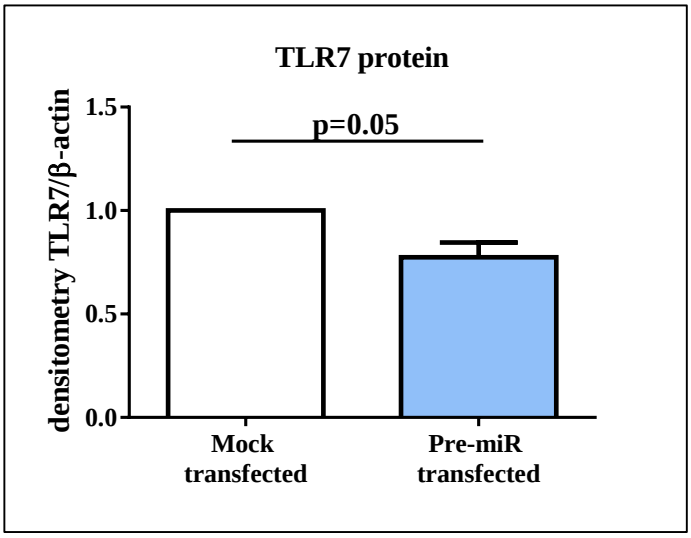


**Figure 7-9 Transfection of healthy AM with pre-miRs leads to reduction of TLR7 protein expression**
AM from 4 healthy subjects were transfected with pre-miR-150, pre-miR-152 and pre-miR-375 (pre-miR transfected) or scrambled control (mock transfected). Densitometric analysis of TLR7 protein expression was calculated related to β-actin. Graph displays mean+SEM; expression compared using paired t-test.

Figure 7-9 shows that although transfecting healthy AM with pre-miRs led to a robust reduction in TLR7 expression of about 25%, this was not statistically significant. Previous work done in our group has shown that AM more readily phagocytose anti-miRs compared to pre-miRs and one of the reasons that TLR7 protein levels were not suppressed further may be due to poor entry of the pre-miRs into the AM. Alternatives would have been to try and use a transfection reagent or perform this experiment using anti-miRs and SA-AM to show that transfecting anti-miR-150, anti-miR-152 and anti-miR-375 can increase TLR7 protein expression. However, practically it was easier to recruit healthy volunteers for these experiments than severe asthma subjects. Additionally, BAL cell recovery (and as a consequence the number of AM available for experimental work) tends to be lower in SA subjects which also makes performing western blots more challenging.

In summary, our results in this chapter clearly show that by blocking the expression of miR-150, miR-152 and miR-375 in SA-AM, RV-induced IFN production can be augmented and that this occurs via a TLR7 mediated mechanism.

## 7.4 Discussion

Asthmatic patients experience more severe and longer-lasting RV-induced exacerbations than healthy subjects (Corne et al. 2002; Message et al. 2008). Deficient RV induction of antiviral IFNs and ISGs by asthmatic BECs and AMs has been implicated in this increased susceptibility to LRT RV infection (Contoli et al. 2006; Sykes et al. 2012; Wark et al. 2005). Studies have shown that exogenous IFN, even in very small amounts, enables the asthmatic epithelium to elicit a normal anti-viral response and limit viral replication (Cakebread et al. 2011; Gaajetaan et al. 2013; Wark et al. 2005). Therefore strategies that could ameliorate the defective type I IFN response in asthmatic airway cells would certainly be useful therapeutic interventions to be used in the prevention and treatment of asthma exacerbations.

Within the airways, RV genetic material is recognised by a number of PRRs and their activation leads to a cascade of intracellular signalling steps, culminating in the expression of IFNs. These then stimulate the production of ISGs and together these antiviral agents work to reduce viral replication and increase viral clearance, thereby limiting the adverse effects of viral infection. Our results have shown that the expression of one of these PRRs, TLR7, is significantly reduced in AM from severe asthmatics and the expression of 3 miRNAs that target TLR7 is increased in severe asthma.

The next obvious step was to inhibit these three miRNAs in an attempt to increase the expression and thereby augment the function of TLR7. Our results demonstrate that AM transfected with the 3 antagomirs, in order to block miR-150, miR-152 and miR-375, and challenged with RV, showed an augmented IFN response compared to mock-transfected cells. More specifically, this effect was seen when the transfected cells were stimulated with imiquimod, which exclusively signals through TLR7, and is not seen when transfected AM are stimulated with Poly:IC. This suggests that the increase in IFN responses is due to miRNA-mediated changes in TLR7 expression and thereby function.

The changes we observed in TLR7 expression when miR-150, miR-152 and miR-375 were blocked were relatively modest, while we were able to detect significant increases in IFN expression. Other groups have also observed this phenomena where blocking miRNA function has significant effects on downstream genes yet changes in the putative target mRNA species cannot always be identified (Foster et al. 2013). One reason for this may be that miRNA circuits tend to be activated early in a cellular process and target transcripts are no longer present at the termination of the event, when measurements are made.

Some limitations of our results in this section need to be noted. Imiquimod and RV treatment of mock-transfected cells did mount an IFNβ protein response but this was not statistically significant. In contrast, the anti-miR transfected AM were able to mount a statistically significant IFNβ response to both imiquimod and RV. This could be because the underlying innate immune pathways in these cells are so deficient that unless the expression of TLR7 is boosted they are unable to mount a significant antiviral response. However, other groups have found that asthmatic AM do mount a significant type I and type III IFN response to RV, albeit far less in magnitude than that mounted by healthy AM (Contoli et al. 2006; Sykes et al. 2012). The number of samples used in the transfection experiments was quite small, due to the limited number of AM recoverable from BAL, and perhaps if we were able to increase the numbers in the groups statistically significant effects would be seen. Alternatively, as mentioned previously, our use of the 24 hour time point may not have been the most appropriate and in retrospect perhaps an additional time point should have been included. Finally, it may be that we employed too low an MOI for RV (0.6) and a higher MOI may have induced the production of significant amounts of IFN, even in the mock-transfected cells. Using IP-10 as a measure of IFN pathway activation, it has previously been shown that in AM, IP-10 release starts to increase at an MOI of 0.1 and peaks between an MOI of 1.0 to 10 (Korpi-Steiner et al. 2006), indicating that a higher MOI may have led to higher induction of IFN.

Recently, a phase II clinical trial has been completed that explored the use of inhaled IFNβ in patients with mild/moderate and severe asthma when they caught a cold (Djukanovic et al. 2014). It found that in patients on BTS Step 4 and 5 who developed symptoms of a cold, the use of inhaled IFNβ prevented

worsening of asthma symptoms and drop in peak flow compared to placebo. MiRNA based therapy would work similarly, but would have the advantage of utilising the cell's own machinery to produce more IFNβ in a spatiotemporal advantageous manner. Furthermore the lung is an attractive organ for delivery of therapeutic applications for modulating miRNA function because the drug can be directly applied to the lung via inhalation.

Unfortunately a single miRNA can also affect the expression of several other proteins and we have not tested for the specificity of our results in terms of any other effects inhibiting the 3 miRNAs may have in AM. It is difficult to predict the inhibitory effects of miRNAs and even though we have shown that miR-150, miR-152 and miR-375 are able to inhibit TLR7 expression, they may not have similar inhibitory effects on their other predicted targets due to reasons including accessibility of the target binding site, high target-to-miRNA ratio (Mullokandov et al. 2012) and the thermodynamic stability of the miRNA:mRNA duplex (Huang et al. 2010). Therefore, even though miR-150, miR-152 and miR-375 are predicted to target a number of other genes these may not all be present in macrophages and it is difficult to ascertain if these interactions will be biologically relevant.

It has been shown that miR-150 is able to regulate the expression of suppressor of cytokine signalling 1 (SOCS1) in PBMCs (Chen et al. 2014). OVA-treated SOCS1 null mice have been shown to develop an asthma phenotype characterised by increased serum IgE levels and elevation in IL-1, IL-5 and IL-13 expression in the lung (Lee et al. 2009) while SOCS1 induction by IL-13 in mice leads to reduced eotaxin expression and Th2 inflammatory responses (Fukuyama et al. 2009). It can be postulated that increased miR-150 in SA-AM could also reduce SOCS1 expression, increasing Th2 inflammation while blocking miR-150 with anti-miRs, as we have done, could increase SOC1 expression, putting a brake on the Th2 inflammatory responses. However, SOCS1 signalling pathways are extremely complex with reports showing SOCS1 suppresses RV-induced IFN expression in BECs (Edwards et al. 2012) and if similar effects occur in AM, then increasing SOCS1 with anti-miR-150 could reduce the beneficial effects of increasing TLR7 signalling.

The miR-152 family of miRNAs (miR-148 and miR-152) have also been implicated in asthma pathogenesis with increased expression of these miRNAs

in airway epithelial cells from asthmatic subjects with an asthmatic mother compared to those without an asthmatic mother (Nicodemus-Johnson et al. 2013). However asthma-relevant gene targets of miR-152 have not previously been identified.

MiR-375 has been shown to play a role in Th2 inflammation and altering the expression of this miRNA in asthmatic AM could have other disease relevant effects. In intestinal epithelial cells, miR-375 has been shown to be induced by IL-13 (Biton et al. 2011), a cytokine also associated with Th2 predominant asthma (Corren et al. 2011). Increased expression of miR-375 was shown to lead to increased goblet cell differentiation and Th2 responses while miR-375-knock out mice have reduced resistin-like molecule-β (RELMβ) expression. Work done by our group has shown that RELMβ expression in the airways increases with asthma severity and is specifically induced following bronchoconstriction of asthmatic airways (Grainge et al. 2012). In animal models, RELMβ induces collagen deposition and goblet cell hyperplasia (Mishra et al. 2007) and it can be postulated that anti-miR-375 induced reduction in miR-375 expression could also be beneficial by affecting these other pathways including reducing AM Th2 responses and RELMβ expression.

In summary we have shown that knock-down of miR-150, miR-152 and miR-375 in AM rescues the expression of TLR7 in these cells leading to augmented RV-induced expression of protective IFN. This provides a novel therapeutic target for virus-induced respiratory disease exacerbations. However, altering the expression of these miRNAs in AM may also affect other disease relevant pathways in AM which we have not explored and future work will need to address this, as well as effects on pathways not related to asthma, but which may be relevant in the lungs.

# Chapter 8 Discussion

## 8.1 General Discussion

Our results have demonstrated that alveolar macrophages from patients with severe asthma have a deficient IFN response to RV. We have shown that this is due to a miRNA mediated reduction in cellular TLR7 expression. The reduction in TLR7 expression correlates with clinical parameters of asthma control and can be ameliorated by blocking miR-150, miR-152 and miR-375. This leads to an augmented IFN response to RV by AM, implicating miRNA-based therapy as a novel therapeutic option for the prevention and treatment of virus-induced asthma exacerbations.

Asthma is a global health problem and with a mean prevalence of over 16% the UK has one of the highest prevalence rates for the disease worldwide (Bousquet et al. 2010). It is a chronic inflammatory disease characterised by variable and usually reversible airflow obstruction in association with airway hyperresponsiveness. A large proportion of the social, economic and personal burden of asthma is due to disease exacerbation (Johnston et al. 2006). Studies have shown that most exacerbations, both in children and adults, are associated with viral infection (Grissell et al. 2005; Johnston et al. 1995; Wark et al. 2002). Of the respiratory tract viruses detected, RV is most commonly found and detected in over two-thirds of cases (Nicholson et al. 1993). Natural and experimental infection of asthmatic patients with RV has been shown to lead to worsening of asthma control, deterioration in lung function and more prolonged hospital stay (Corne et al. 2002; Message et al. 2008).

It is thought that the reason asthmatic individuals are more severely affected and have more significant and long-lasting LRT symptoms when infected with RV compared to healthy individuals is because structural and immune cells within the asthmatic airway are deficient in their innate immune response to viral infection. IFNs are key antiviral cytokines that orchestrate the immune response to virus and together with other proteins whose production they stimulate (ISGs) work towards limiting viral replication and promoting apoptotic pathways within infected cells (Sadler et al. 2008; Samuel 2001). They also activate natural killer and T cells and thus play an important role in

the activation of the adaptive immune response (Le Bon et al. 2002). Therefore a deficiency in these cytokines would lead to increased viral replication, longer persistence of the virus within the airways and clinically lead to more severe and prolonged illness. However, there are inconsistencies in the literature regarding the IFN response to RV mounted by asthmatic airway cells. While a number of studies have shown that airway cells from individuals with asthma produce far less type I and III IFNs when exposed to RV *ex vivo* (Contoli et al. 2006; Sykes et al. 2012; Wark et al. 2005), recently it has been shown that bronchial epithelial cells from well-controlled mild-to-moderate asthmatics have an intact and satisfactory IFN response to RV *ex vivo* (Sykes et al. 2014). Furthermore, the IFN responses to RV have been shown to be intact in asthmatic peripheral blood monocytes (Sykes et al. 2012) and fibroblasts (Bedke et al. 2009). These latter studies highlight the uncertainty regarding how adequate the asthmatic anti-viral innate immune response is and given the burden of virus-induced disease exacerbation in asthma they indicate the importance of further investigation into this area.

Our results show that RV-induced IFN responses are significantly impaired in SA-AM compared to healthy AM (Figures 5-1 and 5-2). We decided to study AM because although they are the predominant immune cell within the lower airways, their role in the innate immune response to RV has not been extensively studied. Instead, most of the focus has been on the BEC probably because it is the primary site of RV replication within the airways. RV is a ssRNA virus and it forms a dsRNA intermediary during replication. Both the single stranded and double stranded forms of the viral genome can activate cellular PRRs and this initiates the host anti-viral response. Production of anti-viral (and pro-inflammatory) cytokines from BECs is replication dependent (Chen et al. 2006). There is much debate on whether RV can replicate in AM (Gern et al. 1996; Laza-Stanca et al. 2006) and this has formed the basis over the uncertainty regarding its contribution to the host anti-viral response. Studies have shown that only 5-10% of airway epithelial cells become infected with RV (Chen et al. 2006; Mosser et al. 2002). Nonetheless, during *in vivo* RV infection, BECs produce marked amounts of cytokines which suggests uninfected epithelial cells may also be producing these cytokines, most likely as a result of paracrine stimuli from adjacent cells. Since AM lie in extremely close proximity to BECs and are able internalise RV, it is most likely that these

stimuli are coming from infected AM. To support this it has been shown that epithelial cell cytokine release during RV infection is augmented by monocytic cell release of type I IFNs- monocytic cell-conditioned media added to BECs infected with RV led to a 40 fold increase in cytokine release compared to BECs cultured in isolation (Korpi-Steiner et al. 2010). Depletion of AM in mice leads to failure to clear virus from lungs of infected animals (Kumagai et al. 2007) and enhanced lung disease including alveolar damage, haemorrhage and excessive neutrophilic infiltrations (Narasaraju et al. 2011) suggesting that AM are essential to the initial anti-viral response. Therefore, AM are active participants in the host innate response to virus and rapidly internalise RV to release crucial anti-viral cytokines (Contoli et al. 2006; Korpi-Steiner et al. 2006) that have paracrine effects on nearby BECs alerting and activating them to participate in the overall anti-viral response.

We have shown that the expression of TLR7, a PRR that recognises the single stranded form of the RV genome, is significantly reduced in SA-AM (Figures 5-3 and 5-6). The reduction in TLR7 expression was shown to lead to a functional deficiency in the TLR7 signalling pathway resulting in reduced RV-induced IFN responses. As only minimal RV replication is thought to occur in AM most of the viral genetic material present within these cells would be ssRNA, making TLR7 is an extremely important PRR in AM. Reduced expression of TLR7 would reduce the ability of the AM to detect the presence of RV genome and this would ultimately lead to reduced IFN production. This is confirmed by our results showing that when AM from patients with severe asthma are stimulated with imiquimod, a specific TLR7 agonist, they produce significantly less IFN$\alpha$, IFN$\beta$ and ISGs compared to AM from healthy individuals. The expression and function of other PRRs (TLR3, RIG-1 and MDA5) involved in immune recognition of RV appear to be intact, albeit their contribution towards activating IFN signalling in AM, which do not fully support RV replication, is likely to be low.

However, the expression of TLR8, the other PRR activated by ssRNA did show reduced expression in severe asthma compared to healthy AM (although this did not reach statistical significance) (Figure 5-7). Reduced TLR8 expression would certainly contribute to the impact of reduced TLR7 expression on cellular IFN responses. It can be hypothesised that AM from subjects with reduced expression of both PRRs responsible for responses to ssRNA would be unable to mount an anti-viral response to RV infection and consequently

provide no protective IFN to nearby epithelium. This would greatly enhance the susceptibility to LRT virus infection and disease exacerbation.

In order to explore cellular mechanisms involved in the deficient IFN response mounted by SA-AM we decided to study miRNAs. MiRNAs are small non-coding RNA molecules that regulate gene expression at the post-transcriptional level by interacting with the 3'UTR of mRNA to reduce protein translation. We carried out miRNA microarrays on AM from healthy and asthmatic subjects and bioinformatics analyses followed by qPCR confirmation showed that the expression of miR-150, miR-152 and miR-375 is significantly increased in SA-AM and that they all can target TLR7 to reduce its expression.

In order to confirm that miR-150, miR-152 and miR-375 do indeed bind to the 3'UTR of TLR7, *in vitro* luciferase assays using cloned sequences of the 3 miRNAs and the 3'UTR of TLR7 were performed. These assays confirmed that all three miRNAs bind to the 3'UTR of TLR7 to reduce its expression and interestingly, when a vector containing all three miRNAs was used, much greater inhibition of translation was seen. This suggests that the miR-150, miR-152 and miR-375 are likely to work in a co-ordinated way to produce a clinically significant reduction in the expression of TLR7 and even small differences in the expression of the individual miRNA could have a significant effect when they collectively exert their effects on TLR7 expression. It is becoming apparent that miRNAs frequently operate in a coordinated fashion and so the combination of several miRNAs acting on multiple seed sequences in the same 3'UTR has considerably more biological effect than individual miRNAs alone (Peter 2010) and our results would support that. Interestingly, the reduction in TLR7 protein in severe asthma subjects was far greater (over 60%) than the reduction in TLR7 mRNA (50%) suggesting that the miRNAs are causing degradation (apparent by reduced TLR7 mRNA in SA-AM, Figure 5-3) and then inhibition of translation of remaining TLR7 mRNA transcripts (apparent by further reduction in the protein expression of TLR7, Figure 5-6).

We propose that TLR7 deficiency and impaired virus-induced IFN production is related to asthma severity and control rather than just the presence of asthma. We were unable to detect TLR7 deficiency in AM from subjects with mild (BTS treatment step 1, Figure 5-5) and moderate (BTS treatment step 3, Figure 6-10) asthma. Similarly, the literature shows that bronchial epithelial cells from

subjects with severe asthma have significantly reduced expression of TLR7 (Shikhagaie et al. 2014) while peripheral blood monocytes, CD4 and CD8 Tcells and dendritic cells (Lun et al. 2009; Roponen et al. 2010; Sykes et al. 2013), BAL cells (which are predominantly AM) (Sykes et al. 2012) and bronchial epithelial cells (Shikhagaie et al. 2014) from mild-to-moderate asthmatic subjects have normal levels of TLR7. Regarding IFN responses to virus, unlike the intact anti-viral responses observed in BECs from mild-to-moderate asthmatic subjects, those from children with severe asthma (Edwards et al. 2013) and adults with moderate-to-severe asthma (Parsons et al. 2014) have been shown to be significantly deficient. To further support our proposal, it was recently shown that nebulised IFNβ given to asthmatic patients who developed symptoms of a RV URT infection was only beneficial in patients with severe asthma (Djukanovic et al. 2014). It could be speculated that the reason for this is that patients with milder forms of the disease do not have deficiencies in IFN production and therefore do not gain any extra benefit after receiving extra exogenous IFN.

However, it could also be argued that reduced IFN responses by asthmatic airway cells are due to treatment with and dose of ICS. Sykes *et al* reported asthmatic BECs had an intact IFN response to virus and only 50% of their study patients were on ICS therapy, while in Parsons *et al*'s study, which did report asthmatic BECS had a deficient IFN response to virus, all patients were on ICS with a mean dose of 2267 mcg (BDP equivalent). All of our patients were on treatment with ICS, and their mean dose was 2300 mcg (BDP equivalent). However, *in vitro*, it has been shown that budesonide has beneficial effects in that it can reduce entry of RV into airway epithelial cells and pro-inflammatory cytokine release (IL-1β , IL-6 and IL-8) by RV infected cells (Yamaya et al. 2014), while induction of type I and III IFN genes is unaffected (Bochkov et al. 2013). This indicates that ICS therapy can moderate the pro-inflammatory responses without negatively impacting on anti-viral responses in the airways.

We also investigated if ICS treatment could affect TLR7, either directly or by altering the expression of miR-150, miR-152 and miR-375. Subjects with well-controlled ICS-treated asthma (MO group) had significantly higher TLR7 expression than severe asthma subjects suggesting that the ICS treatment was not reducing TLR7 expression (Figure 6-10). However, severe asthma subjects were receiving over 3 times the mean ICS dose of the MO patients (mean BDP

equivalent dose 2300mcg vs 720 mcg) and to fully exclude a steroid effect we would need to evaluate TLR7 expression in asthmatic patients matched for ICS dose i.e. on BTS treatment step 4 or 5, but who had well-controlled disease so that any differences in TLR7 expression could then be attributed to processes related to disease control rather than treatment. In reality this would be an extremely difficult group of patients to recruit and so we decided to evaluate changes in TLR7 in an *ex vivo* model where healthy AM were exposed to dexamethasone at varying concentrations.

This evaluation showed that *ex vivo*, steroids reduce TLR7 expression in AM. Treatment of PBMCs with budesonide *in vitro* has been shown to reduce RV and imiquimod induced IFN responses (Davies et al. 2011) and given our results, this may be due to a suppressive effect of steroids on TLR7 expression. Budesonide did not have an inhibitory effect on RV-induced IFN responses in BECs *in vitro* (Bochkov et al. 2013), but this may be because PRRs other than TLR7 are involved in viral responses in BECs. The effect of steroids on TLR7 mRNA expression needs further investigation firstly by exploring if it also translates to reduced protein expression and then investigating for confirmation in an *in vivo* model. If steroids do indeed reduce TLR7 expression in AM then a 'double-hit' scenario would arise in severe asthma whereby SA-AM have increased expression of miR-150, miR-152 and miR-375 leading to reduced TLR7 expression and are also exposed to high concentrations of steroids in the airways, further reducing TLR7 expression. This would ultimately result in a severely debilitated ability of these crucial immune cells to respond to virus presence. Clinically, this would certainly justify reducing ICS doses in patients with severe asthma who have evidence of low levels of airway inflammation.

Both our *in vivo* study with MO subjects and the *ex vivo* model using dexamethasone on healthy AM showed that steroids do not increase the expression of miR-150, miR-152 and miR-375. This is consistent with other studies looking at the effects of steroids on miRNA expression. These have shown that by reducing the expression of miRNA processing enzymes (Smith et al. 2010) steroids tend to reduce the overall expression of miRNAs in a cell and would therefore be unlikely to increase their expression. What is apparent from our results it that if steroids are affecting the expression of TLR7 in AM, then it is extremely unlikely to be via miRNA mediated mechanisms.

Stimulation of TLR7 by the single stranded viral genome in AM is likely to occur before the formation of the double stranded intermediary and activation of relevant PRRs in BECs. Therefore, reduced TLR7 expression and function in SA-AM would delay the development of an 'anti-viral' state in nearby BECs, facilitating persistence of virus locally and associated immunopathology. Furthermore, RV infection of PBMCs has been shown to lead to increased expression of TLR7 (Pritchard et al. 2014), representing the cell's effort to increase virus surveillance mechanisms. Based on our results we propose that due to increased expression of miR-150, miR-152 and miR-375, this up-regulation of TLR7 expression would be hindered in SA-AM, further reducing IFN responses. Additionally, TLR8 expression has been shown to decrease with virus infection (Pritchard et al. 2014) and if it is already expressed at low levels in SA-AM (Figure 5-7) any further decrease would leave the SA-AM with markedly reduced TLR7 and TLR8 expression resulting is nearly no ability to recognise ssRNA and mount an IFN response.

We propose that reduced IFN responses from SA-AM drive the vulnerability of these subjects to not just lower respiratory tract viral infection but recurrent disease exacerbations in that patients with greater reductions in TLR7 expression would have more frequent disease exacerbations. We found a clear co-relation between the level of TLR7 expression in SA-AM and the number of exacerbations the patient had experienced in the previous year (Figure 5-13). These exacerbations may not necessarily all be associated with virus infection, but RV infection has been shown to predispose to bacterial and allergy driven asthma exacerbations. RV infection of AM has been shown to reduce the anti-bacterial activity of AM (Oliver et al. 2008), predisposing to bacterial LRT infection. Viral infection also increases the risk of allergen driven exacerbations in patients sensitised and exposed to sensitising allergen (Murray et al. 2004). Therefore, once infected with RV within the lower airways, asthmatic individuals are at an increased risk of bacterial and allergen driven exacerbations.

During and after an exacerbation, asthma symptom control deteriorates compared to baseline and this can be reflected by an increase in the ACQ score. Our results also showed that TLR7 expression in SA-AM inversely correlated with the patient's ACQ score (Figure 5-13). This was despite the bronchoscopy taking place at least 4-6 weeks after an exacerbation and could

indicate that reduced TLR7 expression leads to an exacerbation prone phenotype and that due to recurrent exacerbations, overall asthma control is poor. In fact, when TLR7 expression and ACQ score of MO subjects i.e. well controlled with no history of recent disease exacerbation, were included in this analysis, the strength of the correlation increased ($r= -0.7245$, $p<0.0001$ vs $r= -0.5608$, $p=0.007$).

The next question that arises is what is driving the deregulation of miRNA expression in SA-AM and thus leading to reduced TLR7 expression. Reduced IFN responses to RV have been reported to occur in airway cells in other chronic respiratory diseases such as COPD and CF (Mallia et al. 2011; Vareille et al. 2012). Asthma, COPD and CF have distinct pathogeneses in that COPD is most frequently due environmental factors (smoking), CF secondary to genetic mutations (in the CFTR gene) and asthma due to gene-environment interactions. We hypothesised that common mechanisms must underlie the defective IFN response to virus in these three conditions and to support this we also found reduced TLR7 in AM from COPD subjects (Figure 6-7). Recurrent LRT infections leading to chronic inflammation of the airways is a common phenomenon in severe asthma, COPD and CF, although the predominance of bacterial lower respiratory tract infections is better documented in COPD and CF. Therefore, we hypothesised that the expression of miRNAs in SA-AM were altered in response to inflammatory changes associated with bacterial presence in the lower airways. Our characterisation of the SA-AM showed that they tended to have a more M1/inflammatory phenotype with increased expression of IL-6, IL-8, TNF$\alpha$ and IL-23 (Figures 3-5 and 3-6) and it is probably that this is in response to the presence of bacterial particles and related pro-inflammatory cytokines in their microenvironment. In order to replicate the lower airways in severe asthma, we treated healthy AM with BAL fluid from patients with severe asthma but no significant changes in miRNA expression were detected at 24 hours. However, the expression of TLR7 was significantly reduced after exposure to 4 of the 10 BAL samples and showed a trend towards reduced expression after exposure to a further 2. This suggests that there are elements within the lower airways of patients with severe asthma that is driving the reduced expression of TLR7 within AM.

Although this experiment did not demonstrate that these elements were working via miRNAs, mediation by miRNAs cannot be excluded. Changes in

miRNA expression are often transient and may have returned to baseline at the time of our measurement. It is also possible that the BAL fluid contains extracellular vesicles containing miRNAs released from AM. *In vitro* work has shown that over-expressing a particular miRNA in a cell leads to its enrichment in extracellular vesicles (Guduric-Fuchs et al. 2012) and as we have shown increased expression of miR-150, miR-152 and miR-375 in AM, it is possible that they their expression is increased in extracellular vesicles in the BAL fluid. When this BAL fluid is applied onto healthy AM, the miRNAs could enter the cells and exert their inhibitory effects on gene translation. Furthermore, it appears that a common mechanism for selective miRNA export may exist as some miRNAs are enriched similarly in extracellular vesicles released by a range of cell types (Guduric-Fuchs et al. 2012). Of interest miR-150 is one of the preferentially exported miRNAs.

An important finding of our work is showing that manipulation of miR-150, miR-152 and miR-375 levels in AM can lead to functional improvements in IFN responses to RV (Figures 7-5 and 7-6). AM transfected with anti-miRs targeting all three miRNAs and subsequently challenged with RV produced significantly more IFN$\alpha$ and IFN$\beta$ than AM transfected with a scrambled control, which does not influence the expression of the miRNAs. This augmentation in IFN production was also seen when the transfected AM were stimulated with imiquimod (Figures 7-2 and 7-3), confirming that the increase in IFN responses is through miRNA mediated increase in TLR7 expression and function. This was confirmed by our results showing increased TLR7 mRNA in AM transfected with the anti-miRs compared to mock-transfected AM.

It has previously been shown that exogenous IFN$\beta$ added to asthmatic bronchial epithelial cells *in vitro* enables them to reduce viral replication (Wark et al. 2005) and RV induction of inflammatory cytokines IP-10, RANTES and IL-6 (Cakebread et al. 2011) to levels similar to those observed in non-asthmatic cells. Therefore, increasing the endogenous production of IFN by asthmatic AM could help restore them into behaving more like 'healthy' AM and when these cells are subsequently faced with a RV infection they secrete sufficient amounts of IFN to promote viral clearance and limit virus-induced pro-inflammatory changes. Furthermore, even extremely small amounts of exogenous IFN have been shown to induce a long-lasting anti-viral state in cells (Gaajetaan et al. 2013). Therefore, the increase in IFN induction provided by

increased signalling through TLR7 should be able to provide more robust IFN signalling to nearby epithelial cells to create an 'anti-viral' state. Clinically this could lead to more rapid resolution of symptoms and a reduction in the deleterious effects of virus induced exacerbation of asthma.

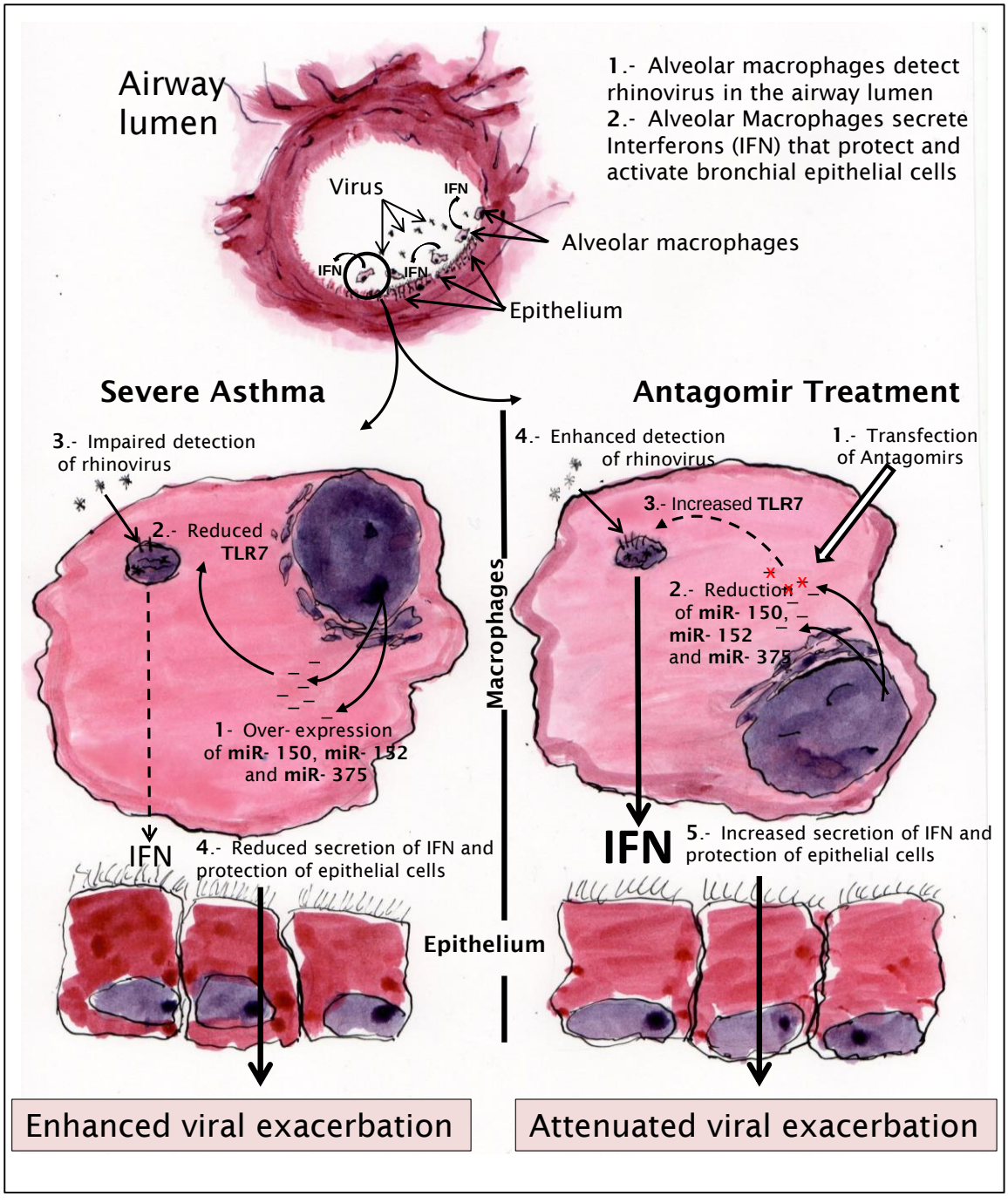


**Figure 8-1 Schematic model showing the interaction between alveolar macrophages and rhinovirus within the airways and the relevance of TLR7 and miRNAs.**

Reduced TLR7 in severe asthma could theoretically also impact on other pathways which we have not evaluated in this study. Animal studies suggest TLR7 may also play a role in the development of asthma. TLR7 null mice exposed to *Pneumovirus* in infancy develop an asthmatic phenotype characterised by BHR, elevated IgE levels and increased lung expression of IL-4, IL-5 and IL-13, when re-exposed to the virus or other aeroallergen in later life (Kaiko et al. 2013). Several prospective birth cohort studies have shown that wheezy LRT infections in infancy are a risk factor for the development of asthma in later childhood (Jackson et al. 2008; Kusel et al. 2007; Sigurs et al. 2000; Stein et al. 1999). It can be speculated that TLR7 deficiency in children, leading to lack of Th1 responses during critical periods of immune system maturation may allow Th2 adaptive immunity to predominate in the lung (Braun-Fahrlander et al. 2002), predisposing to the development of asthma in later life. The expression of TLR7 in AM from children has not been evaluated and it would extremely interesting to measure it in childhood cohorts that are being followed up and look for development of asthma.

The ability of TLR7 stimulation to skew the immune system away from a Th2 response has been clearly demonstrated in multiple animal models of asthma in which TLR7 agonists reduce acute and chronic inflammatory changes, BHR and airway remodelling (Biffen et al. 2012; Camateros et al. 2007; Jin et al. 2006; Meng et al. 2011; Moisan et al. 2006; Quarcoo et al. 2004; Xirakia et al. 2010). More recently, TLR7 stimulation has been shown to have a direct bronchodilatory effect via a TLR7-dependent-nitric oxide pathway in airway smooth muscle cells (Kaufman et al. 2011). It is possible that this bronchodilatory mechanism protects against airway obstruction during early respiratory virus infection and also suggests that TLR7 has relevance as rescue therapy in asthma for reversal of acute bronchoconstriction. Reduced expression of TLR7, as we have detected in SA-AM, could exaggerate Th2 allergic inflammation and impede this protective bronchodilation occurring during a viral infection. It can also be speculated that by blocking miR-150, miR-152 and miR-375 and increasing TLR7 expression in SA-AM, in addition to increases in virus-induced IFN production, these other pathways may also be impacted.

Obviously, the use of miRNA based therapy in clinical care is still in research stages but our results certainly have the potential to be translated from bench

to bedside. Drug delivery to the lung, especially to AM is practically attractive because the inhalation route can be employed and due to their phagocytic nature, AM readily take-up anti-miRs, obviating the need for a transfection reagent. The use of inhaled IFNβ during an asthma exacerbation has recently been reported, with benefits in asthma control in patients with severe asthma (Djukanovic et al. 2014). The use of inhaled IFNβ equates to providing the airway epithelium with exogenous IFNβ, while by manipulating miRNA expression in AM, endogenous IFN production can be increased, using the cells own machinery. The latter would appear to be more preferable because the effects of IFN would be in a spatiotemporally appropriate way. However, in this study we have not evaluated any detrimental effects the inhibition of miR-150, miR-152 and miR-375 may have on AM. Bioinformatics tools predict multiple gene targets for these miRNAs and future work would need to ascertain relevant pathways that may be negatively affected in our attempt to boost TLR7 signalling with anti-miR-150, anti-miR-152 and anti-miR-375. Reassuringly, reports suggest that the functional 'miRNome' of a cell is considerably smaller than previously inferred (Foster et al. 2013) and the actual biologically relevant targets within AM are almost certainly much fewer than that predicted by *in silico* tools.

Treating patients with severe asthma in a more personalised fashion is already underway with the introduction of mepolizumab (Pavord et al. 2012), an anti-IL-5 monoclonal antibody, and lebrikizumab (Corren et al. 2011), an anti-IL-13 antibody, for patients with eosinophilic inflammation and Th2-high disease respectively. Similarly we propose that any future translation of miRNA- based therapy be targeted to a specific group of severe asthma patients- those with an exacerbation prone phenotype. The BIOAIR trial identified that about a third of patients with severe asthma had frequent exacerbations, defined as ≥2 exacerbations a year (Kupczyk et al. 2014), while among 3151 patients presenting to U.S emergency departments with acute asthma, 21% reported six or more visits (Griswold et al. 2005). In our cohort of severe asthma patients the mean number of exacerbations a year was 5.9 (range 2-12) and it could be argued that similar TLR7 deficiency may not be present in patients with less frequent exacerbations. Therefore, detailed patient characterisation and careful selection will aid to identify those that would benefit most from boosting AM-driven IFN responses to virus.

We chose to recruit our group of severe asthmatics from the Difficult Airways Clinic held at University Hospital Southampton. Therefore our patient group includes some patients who are either current or past smokers. This group of patients is frequently excluded from clinical trials in asthma partly because of the confounding effects of smoking and it can be argued that our results may have been influenced by the influence of cigarette smoke on miRNAs. However, microRNA arrays done on AM from smokers with a mean 30 pack year smoking history show that the expression of miR-150 was significantly reduced in AM from smokers compared to never-smokers while smoking did not affect the expression of miR-152 or miR-375 significantly (Graff et al. 2012). Therefore it is extremely unlikely that increased expression of miR-150, miR-152 and miR-375 in SA-AM was due to the effect of smoking in some of the patients.

In our study we chose to focus on patients with severe asthma. As a group these patients constitute about 10% of asthma population yet account for 50% of health care costs (Holgate et al. 2008; Wenzel 2005). These patients remain symptomatic and experience recurrent exacerbations despite being on maximal therapy and so represent a huge unmet clinical need. A better understanding of the pathogenesis of virus induced exacerbation would be greatly beneficial to the whole asthma population but even more so for this group of patients simply because they seem to suffer from the most exacerbations. If a novel treatment could be created that prevented recurrent exacerbations this group could be spared considerable morbidity both in the present and in the future.

Based on our results it can be hypothesised that TLR7 deficiency can lead to a phenotype of asthma which is prone to recurrent prolonged exacerbations due to reduced ability to sense the presence of the virus. Virus infection of the lower airways also predisposes to bacterial and allergen driven disease exacerbations and recurrent exacerbations can lead to worsening airway inflammation, further disruption of the epithelial barrier and worsening lung function. As this cycle in repeated, in time chronic epithelial injury followed by aberrant repair would lead to thickening of the basement membrane and airway remodelling, all features frequently observed in severe asthma. Our current results show that TLR7 deficiency correlates inversely with number of disease exacerbations and asthma control and it could be speculated that

measures that increase TLR7 expression in SA-AM would also reduce the incidence of disease exacerbation and improve asthma control.

In conclusion we have shown that miRNA mediated deficiency in the expression of TLR7 by AM in severe asthma leads to impaired innate anti-viral response of these cells, making the individual more prone to longer lasting LRT viral exacerbations. More importantly, we also conclude that by manipulating the expression of miRNAs in these cells, the defective IFN response to virus can be ameliorated making it an extremely promising and novel treatment for asthma exacerbations in the future. However, patient selection would be vitally important as it is most likely that patients with severe asthma who are prone to recurrent exacerbations would benefit the most.

# 8.2 Future work

Future work leading on from this study can be divided into three broad themes:

1. Further detailed investigation into the effects of blocking miR-150, miR-152 and miR-375 in AM
2. Further investigation into airway processes that play a role in altering the expression of miR-150, miR-152 and miR-375 and TLR7.
3. Further investigation into other innate pathways related to IFN responses to RV

## 8.2.1 Further detailed investigation in the effects of blocking miR-150, miR-152 and miR-375 in AM

### 8.2.1.1 Expression of inflammatory cytokines and chemokines

In our study we have explored RV induction of IFN by AM. However, RV infection also leads to cellular expression of a range of other pro-inflammatory cytokines and chemokines. It is thought that the influx of inflammatory cells and their products in the airways contribute to the deleterious effects of virus infection. Signalling via TLR7 also leads to activation of the transcription factor NF-κ B and subsequent expression of genes included TNFα , IL-1, IL-6, IL-8 and IL-12 (Thompson et al. 2007). Therefore the effect of anti-miR-150, anti-miR-152 and anti-miR-375 on the downstream expression of these cytokines and chemokines need to be evaluated as they would impact on the overall immunopathology associated with RV infection.

### 8.2.1.2 Off-target effects of anti-miR therapy

MiRNAs have the ability to affect multiple gene pathways and other cellular effects of blocking miR-150, miR-152 and miR-375 in AM need to be explored. miR-152 knock-out mice do not exist, but reassuringly studies using miR-150 and miR-375 knock out mice did not report any significant pulmonary side effects (Mi et al. 2012; Poy et al. 2009). One potential benefit of blocking miR-150 is that signalling via MyD88, the intracellular signalling protein required for a number TLRs, would increase as miR-150 also targets MyD88 (Ghorpade et al. 2013).

#### 8.2.1.3 Animal models

Having shown the benefit of blocking miR-150, miR-152 and miR-375 in human AM *ex vivo*, future work should aim to test this model in an *in vivo*, animal model. Unfortunately, the binding sites for these three miRNAs in the 3'UTR of TLR7 is not conserved across species, preventing the use of a mouse model. Therefore, other options would have to be explored.

### 8.2.2 Further investigation into airway processes that play a role in altering the expression of miR-150, miR-152 and miR-375 and TLR7

#### 8.2.2.1 Exploring composition of severe asthma BAL fluid

Work from Southampton has shown that induced sputum from severe asthma subjects show predominant colonisation with potentially pathogenic bacterial species: *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*. Having shown that exposure to severe asthma BAL fluid can alter the expression of TLR7 in healthy AM, future work should investigate the components of severe asthma BAL fluid in more detail, in particular look for presence of pathogenic bacteria. It will also be useful to evaluate the expression of cytokines and chemokines in the BAL fluid as these too may be influencing TLR7 and miRNA expression.

#### 8.2.2.2 Exploring the expression of TLR7 in other diseases

We have shown that the expression of TLR7 is also reduced in AM from patients with COPD and this warrants further investigation. In the first instance the number of patients in the COPD group would need to be increased and reduction in TLR7 expression also confirmed at the protein level. However, this preliminary finding is in keeping with our proposal that deficiencies in the IFN response to virus in asthma are due to chronic inflammatory processes occurring within the lower airways, and not necessarily secondary to asthma-specific factors. As epithelial cells from patients with CF, another chronic inflammatory respiratory disease, have also been found to have reduced IFN responses to RV, it would be interesting to evaluate TLR7 expression in airway cells from these patients.

#### 8.2.2.3 Exploring the expression of TLR7 in children

A significant proportion of children with virus-induced wheezing go on to develop asthma, especially children hospitalised with RV-induced wheezing (Gern 2009). Given the animal data suggesting TLR7 deficient mice exposed to respiratory viruses in infancy develop an asthma-like phenotype on re-exposure to virus in adulthood (Kaiko et al. 2013), it would be interesting and useful to evaluate the expression of TLR7 in AM from children being followed up for recurrent virus-induced wheeze. This would provide insight into the role of TLR7 in both viral respiratory illness but also pathogenesis of asthma.

#### 8.2.2.4 Exploring the effect of steroids on TLR7 expression

ICS therapy is the mainstay of asthma treatment and is used in patients with mild to severe asthma. Preliminary *ex vivo* experiments that we carried out showed that dexamethasone reduces the expression of TLR7 in AM (at all the three concentrations tested). This warrants further investigation as potential this could imply that treatment offered by clinicians to patients with asthma is suppressing immune responses to virus. In the first instance, evaluation of the expression of TLR7 protein in the samples already collected would be useful and if they confirm steroid induced reductions in expression then further *in vivo* studies would be justified. A potential study protocol could involve TLR7 evaluation in AM (isolated from BAL) before and 2-4 weeks after receiving high dose ICS therapy.

### 8.2.3 Further investigation into other IFN relevant cellular pathways

We have identified that there is a trend towards reduced TLR8 expression in SA-AM compared to healthy and this warrants further investigation and confirmation with protein level data. Because RV undergoes minimal or no replication in AM, if indeed TLR8 was also deficient in SA-AM this would hugely impact RV-induced IFN responses by these cells as it would mean both PRRs responsible for detecting ssRNA are deficient. Recent reports also suggest that TLR8 is activated by bacterial RNA (Cervantes et al. 2012), which may be also relevant to patients with severe asthma.

# Chapter 9 Appendices

## 9.1 Appendix 1: Study Exclusion Criteria

1. History of bronchopulmonary aspergillosis or uncontrolled infection
2. Clinically significant cardiopulmonary abnormalities unrelated to asthma
3. Current or past history of TB, SLE or multiple sclerosis
4. Clinically significant neurological, renal, endocrine, gastrointestinal, hepatic or haematological abnormalities uncontrolled with standard treatment
5. Alcohol or recreational drug abuse
6. Co-morbidity that precludes participation in study
7. Diagnosis of immunodeficiency requiring treatment
8. Treatment with immunomodulators (except theophylines or LTRAs)
9. Currently undergoing allergen desensitisation therapy
10. Regularly using sedatives, hypnotics or tranquillisers
11. Positive hepatitis viral antigens or antibodies
12. Blood donation within 3 months either end of the study
13. Live immunisation within 4 weeks prior to the study
14. On oxygen therapy
15. Participating in another study currently or within past 3 months
16. Unable to adequately understand instructions

# 9.2 Appendix 2: Asthma Control Questionnaire©

*Circle the number of the response that best describes how you have been during the past week. Please answer questions 1-6.*

1. On average, during the past week, how often were you woken by your asthma during the night?
    0) Never
    1) Hardly ever
    2) A few times
    3) Several times
    4) Many times
    5) A great many times
    6) Unable to sleep because of asthma
2. On average, during the past week, how bad were your asthma symptoms when you woke up in the morning?
    0) No symptoms
    1) Very mild symptoms
    2) Mild symptoms
    3) Moderate symptoms
    4) Quite severe symptoms
    5) Severe symptoms
    6) Very severe symptoms
3. In general, during the past week, how limited were you in your activities because of your asthma?
    0) Not limited at all
    1) Very slightly limited
    2) Slightly limited
    3) Moderately limited
    4) Very limited
    5) Extremely limited
    6) Totally limited
4. In general, during the past week, how much shortness of breath did you experience because of your asthma?
    0) None
    1) A very little
    2) A little
    3) A moderate amount
    4) Quite a lot
    5) A great deal
    6) A very great deal

5. In general, during the past week, how much of the time did you wheeze?
    0) Not at all
    1) Hardly any of the time
    2) A little of the time
    3) A moderate amount of the time
    4) A lot of the time
    5) Most of the time
    6) All the time
6. On average, during the past week, how many puffs of short-acting bronchodilator (e.g. Ventolin) have you used each day?
    0) None
    1) 1-2 puffs most days
    2) 3-4 puffs most days
    3) 5-8 puffs most days
    4) 9-12 puffs most days
    5) 13-16 puffs most days
    6) More than 16 puffs most days
7. To be completed by a member of the clinic staff/ study investigator

    $FEV_1$ pre bronchodilator: …….

    $FEV_1$ predicted: …….

    $FEV_1$ % predicted: …….

    0) >95% predicted
    1) 95-90% predicted
    2) 89-80% predicted
    3) 79-70% predicted
    4) 69-60% predicted
    5) 59-50% predicted
    6) <50% predicted

# 9.3 Appendix 3 Patient characteristics

| Healthy Subjects | | | | | |
|---|---|---|---|---|---|
| | Sex | Age | BMI | % predicted $FEV_1$ | % predicted FVC |
| 1 | Male | 19 | 23.72 | 110 | 108 |
| 2 | Male | 19 | 23.55 | 95 | 98 |
| 3 | Male | 19 | 19 | 96 | 89 |
| 4 | Female | 21 | 25.38 | 99 | 107 |
| 5 | Female | 20 | 31.96 | 108 | 103 |
| 6 | Male | 18 | 22.99 | 100 | 99 |
| 7 | Female | 21 | 19.28 | 104 | 95 |
| 8 | Female | 54 | 24.17 | 141 | 145 |
| 9 | Male | 19 | 24.97 | 104 | 95 |
| 10 | Female | 22 | 22.79 | 83 | 83 |
| 11 | Female | 20 | 21.63 | 101 | 94 |
| 12 | Female | 20 | 21.57 | 93 | 104 |
| 13 | Female | 19 | 21.26 | 77 | 79 |
| 14 | Female | 20 | 22.25 | 97 | |
| 15 | Female | 20 | 25.80 | 109 | 111 |
| 16 | Male | 22 | 24.22 | 98 | 110 |
| 17 | Female | 54 | 24.17 | 141 | 145 |
| 18 | Male | 19 | 24.97 | 104 | 95 |
| 19 | Female | 19 | 21.51 | 77 | 77 |
| 20 | Female | 23 | 25.88 | 120 | 113 |
| 21 | Male | 19 | 25.98 | 105 | 98 |
| 22 | Male | 19 | 25.57 | 103 | 111 |
| 23 | Male | 21 | 26.52 | 103 | 110 |
| 24 | Male | 23 | 24.30 | 98 | 106 |
| 25 | Female | 21 | 19.60 | 97 | 98 |
| 26 | Male | 23 | 32.37 | 109 | 112 |
| 27 | Male | 25 | 23.04 | 106 | 110 |
| 28 | Male | 29 | 25.98 | 85 | 79 |
| 29 | Male | 20 | 30.07 | 84 | 85 |
| 30 | Female | 49 | 32.05 | 105 | 112 |
| 31 | Male | 24 | 25.75 | 113 | 114 |
| 32 | Male | 21 | 22.86 | 103 | 113 |
| 33 | Female | 38 | 23.26 | 97 | 106 |
| 34 | Male | 25 | 24.90 | 114 | 130 |
| 35 | Female | 25 | 29.33 | 100 | 96 |
| 36 | Female | 51 | 22.48 | 124 | 140 |
| 37 | Female | 23 | 23.67 | 107 | 109 |
| 38 | Male | 23 | 20.60 | 109 | 102 |
| 39 | Female | 21 | 21.72 | 102 | 102 |
| 40 | Female | 21 | 22.72 | 117 | 109 |
| 41 | Male | 48 | 32.28 | 139 | 141 |
| 42 | Male | 24 | 27.34 | 116 | 112 |
| 43 | Male | 21 | 25.69 | 115 | 122 |

| | | | | | |
|---|---|---|---|---|---|
| 44 | Male | 21 | 22.10 | 98 | 110 |
| 45 | Female | 49 | 22.81 | 110 | 136 |
| 46 | Male | 40 | 24.60 | 91 | 95 |
| 47 | Male | 46 | 27.81 | 120 | 118 |
| 48 | Male | 47 | 25.46 | 100 | 87 |
| 49 | Male | 50 | 29.08 | 113 | 122 |
| 50 | Female | 47 | 26.25 | 123 | 129 |
| 51 | Male | 20 | 27.78 | 101 | 94 |

**Mild Asthma Subjects**

| | Sex | Age | BMI | % predicted $FEV_1$ | % predicted FVC | Atopy |
|---|---|---|---|---|---|---|
| 1 | Female | 18 | 21.33 | 96 | 100 | Yes |
| 2 | Male | 18 | 26.30 | 134 | 133 | Yes |
| 3 | Male | 20 | 23.10 | 105 | 120 | Yes |
| 4 | Female | 20 | 17.26 | 108 | 133 | Yes |
| 5 | Male | 24 | 23.20 | 107 | 113 | Yes |
| 6 | Male | 30 | 19.05 | 94 | 95 | Yes |
| 7 | Female | 30 | 22.03 | 97 | 102 | Yes |
| 8 | Female | 19 | 22.59 | 103 | 105 | Yes |
| 9 | Female | 20 | 20.94 | 108 | 104 | Yes |
| 10 | Female | 22 | 25.76 | 93 | 114 | Yes |
| 11 | Male | 18 | 26.30 | 134 | 133 | Yes |
| 12 | Female | 26 | 22.83 | 99 | 107 | Yes |
| 13 | Female | 19 | 22.86 | 103 | 118 | Yes |
| 14 | Male | 19 | 21.55 | 79 | 91 | Yes |
| 15 | Female | 20 | 19.05 | 94 | 95 | Yes |
| 16 | Female | 20 | 21.67 | 92 | 110 | Yes |
| 17 | Female | 22 | 25.76 | 93 | 114 | Yes |
| 18 | Female | 23 | 20.58 | 87 | 105 | Yes |
| 19 | Female | 22 | 20.10 | 92 | 106 | Yes |

**Moderate Asthma subjects**

| | Sex | Age | BMI | % predicted $FEV_1$ | % predicted FVC | Atopy | ACQ | ICS dose (mcg) |
|---|---|---|---|---|---|---|---|---|
| 1 | Female | 23 | 27.41 | 89 | 105 | No | 1.00 | 400 |
| 2 | Male | 54 | 28.56 | 80 | 96 | No | 0.29 | 800 |
| 3 | Female | 35 | 23.60 | 106 | 123 | Yes | 0.71 | 400 |
| 4 | Female | 49 | 29.67 | 92 | 115 | Yes | 0.57 | 800 |
| 5 | Male | 52 | 28.25 | 64 | 89 | Yes | 0.57 | 800 |
| 6 | Female | 25 | 24.61 | 99 | 113 | No | 0.57 | 800 |
| 7 | Female | 46 | 36.77 | 80 | 110 | Yes | 1.00 | 1000 |
| 8 | Male | 34 | 30.99 | 84 | 101 | Yes | 0.86 | 800 |

| Severe Asthma Subjects | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Sex | Age | BMI | % predicted $FEV_1$ | % predicted FVC | Atopy | ACQ | ICS dose (mcg) |
| 1 | Female | 51 | 34.67 | 48 | 60 | Yes | 4.86 | 3000 |
| 2 | Female | 28 | 23.28 | 51 | 76 | Yes | 3.86 | 3000 |
| 3 | Female | 41 | 25.54 | 76 | 99 | No | 3.86 | 2000 |
| 4 | Female | 22 | 27.22 | 85 | 116 | Yes | 3.43 | 2400 |
| 5 | Male | 28 | 35.43 | 83 | 97 | Yes | 3.71 | 2800 |
| 6 | Female | 45 | 38.28 | 64 | 80 | No | 2.71 | 1600 |
| 7 | Male | 61 | 22.66 | 88 | 102 | Yes | 3.30 | 1000 |
| 8 | Female | 51 | 43.12 | 76 | 76 | Yes | 2.80 | 2400 |
| 9 | Female | 58 | 33.91 | 56 | 71 | | 3.43 | 3000 |
| 10 | Female | 37 | 37.88 | 77 | 99 | Yes | 2.14 | 4000 |
| 11 | Female | 45 | 28.77 | 94 | 100 | Yes | 2.14 | 3000 |
| 12 | Female | 35 | 48.67 | 41 | 67 | Yes | 4.86 | 2000 |
| 13 | Male | 63 | 35.03 | 38 | 58 | Yes | 3.30 | 2000 |
| 14 | Female | 27 | 42.92 | 68 | 102 | Yes | 3.00 | 2000 |
| 15 | Male | 62 | 34.11 | 34 | 65 | No | 2.71 | 1000 |
| 16 | Female | 33 | 24.98 | 123 | 124 | Yes | 2.71 | 3000 |
| 17 | Male | 63 | 33.02 | 82 | 100 | No | 3.57 | 2400 |
| 18 | Female | 23 | 36.33 | 103 | 64 | No | 2.71 | 1600 |
| 19 | Male | 40 | 25.68 | 44 | 71 | Yes | 1.28 | 2400 |
| 20 | Female | 39 | 38.74 | 42 | 58 | No | 3.86 | 2000 |
| 21 | Male | 59 | 33.06 | 48 | 66 | Yes | 4.29 | 1600 |
| 22 | Female | 37 | 34.88 | 77 | 108 | Yes | 4.42 | 3000 |
| 23 | Male | 62 | 46.09 | 65 | 59 | Yes | 3.43 | 3600 |
| 24 | Female | 57 | 34.34 | 50 | 78 | No | 0.80 | 3600 |
| 25 | Female | 50 | 20.90 | 26.5 | 62 | No | 2.86 | 1000 |
| 26 | Male | 33 | 23.45 | 81 | 99 | Yes | 1.71 | 2000 |
| 27 | Female | 36 | 21.23 | 62 | 66 | Yes | 3.71 | 2000 |
| 28 | Female | 51 | 22.03 | 77 | 84 | Yes | 2.89 | 2900 |
| 29 | Female | 21 | 44.12 | 109 | 113 | Yes | 3.40 | 3300 |
| 30 | Male | 41 | 20.24 | 60 | 82 | Yes | 2.86 | 3200 |
| 31 | Female | 31 | 27.18 | 50 | 59 | No | 3.43 | 1200 |
| 32 | Female | 68 | 25.68 | 74 | 87 | No | 2.28 | 1280 |
| 33 | Male | 68 | 28.41 | 72 | 98 | Yes | 2.86 | 3200 |
| 34 | Female | 53 | 36.18 | 113 | 114 | Yes | 1.70 | 2000 |
| 35 | Female | 56 | 29.92 | 104 | 113 | No | 1.14 | 2400 |

**Table 9-1 Characteristics of patients used in the study**
ACQ, Asthma control questionnaire score; BMI, Body mass index; $FEV_1$, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroid; ICS dose is shown as the equivalent dose in micrograms of beclometasone.

# Chapter 10 References

Akira, S. (2003). "Toll-like receptor signaling." J Biol Chem 278(40): 38105-38108.

Alm, J. S., J. Swartz, et al. (1999). "Atopy in children of families with an anthroposophic lifestyle." Lancet 353(9163): 1485-1488.

Ancochea, J., A. Gonzalez, et al. (1993). "Expression of lymphocyte activation surface antigens in bronchoalveolar lavage and peripheral blood cells from young healthy subjects." Chest 104(1): 32-37.

Antonicelli, L., C. Bucca, et al. (2004). "Asthma severity and medical resource utilisation." Eur Respir J 23(5): 723-729.

Arruda, E., T. R. Boyle, et al. (1995). "Localization of human rhinovirus replication in the upper respiratory tract by in situ hybridization." J Infect Dis 171(5): 1329-1333.

Bai, T. R., J. M. Vonk, et al. (2007). "Severe exacerbations predict excess lung function decline in asthma." Eur Respir J 30(3): 452-456.

Balachandran, S., C. N. Kim, et al. (1998). "Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling." EMBO J 17(23): 6888-6902.

Barbalat, R., L. Lau, et al. (2009). "Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands." Nat Immunol 10(11): 1200-1207.

Barnes, P. J. (2006). "Corticosteroids: the drugs to beat." Eur J Pharmacol 533(1-3): 2-14.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell 116(2): 281-297.

Barton, G. M. (2007). "Viral recognition by Toll-like receptors." Semin Immunol 19(1): 33-40.

Barton, G. M. and J. C. Kagan (2009). "A cell biological view of Toll-like receptor function: regulation through compartmentalization." Nat Rev Immunol 9(8): 535-542.

Bateman, E. D., H. A. Boushey, et al. (2004). "Can guideline-defined asthma control be achieved? The Gaining Optimal Asthma ControL study." Am J Respir Crit Care Med 170(8): 836-844.

Bedke, N., H. M. Haitchi, et al. (2009). "Contribution of bronchial fibroblasts to the antiviral response in asthma." J Immunol 182(6): 3660-3667.

Bendelja, K., V. Vojvoda, et al. (2010). "Decreased Toll-like receptor 8 expression and lower TNF-alpha synthesis in infants with acute RSV infection." Respir Res 11: 143.

Benoit, M., B. Desnues, et al. (2008). "Macrophage polarization in bacterial infections." J Immunol 181(6): 3733-3739.

Bentley, A. M., S. R. Durham, et al. (1993). "Expression of endothelial and leukocyte adhesion molecules interacellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1 in the bronchial mucosa in steady-state and allergen-induced asthma." J Allergy Clin Immunol 92(6): 857-868.

Bentley, J. K., U. S. Sajjan, et al. (2013). "Rhinovirus colocalizes with CD68- and CD11b-positive macrophages following experimental infection in humans." J Allergy Clin Immunol 132(3): 758-761 e753.

Bentwich, I., A. Avniel, et al. (2005). "Identification of hundreds of conserved and nonconserved human microRNAs." Nat Genet 37(7): 766-770.

Beran, O., R. Potmesil, et al. (2011). "Differences in Toll-like receptor expression and cytokine production after stimulation with heat-killed gram-positive and gram-negative bacteria." Folia Microbiol (Praha) 56(2): 138-142.

Bieback, K., E. Lien, et al. (2002). "Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling." J Virol 76(17): 8729-8736.

Biffen, M., H. Matsui, et al. (2012). "Biological characterization of a novel class of toll-like receptor 7 agonists designed to have reduced systemic activity." Br J Pharmacol 166(2): 573-586.

Biswas, S. K. and A. Mantovani (2010). "Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm." Nat Immunol 11(10): 889-896.

Biton, M., A. Levin, et al. (2011). "Epithelial microRNAs regulate gut mucosal immunity via epithelium-T cell crosstalk." Nat Immunol 12(3): 239-246.

Bochkov, Y. A., W. W. Busse, et al. (2013). "Budesonide and formoterol effects on rhinovirus replication and epithelial cell cytokine responses." Respir Res 14: 98.

Bourdin, A., D. Neveu, et al. (2007). "Specificity of basement membrane thickening in severe asthma." J Allergy Clin Immunol 119(6): 1367-1374.

Bousquet, J., P. Chanez, et al. (1990). "Eosinophilic inflammation in asthma." N Engl J Med 323(15): 1033-1039.

Bousquet, J., E. Mantzouranis, et al. (2010). "Uniform definition of asthma severity, control, and exacerbations: document presented for the World Health Organization Consultation on Severe Asthma." J Allergy Clin Immunol 126(5): 926-938.

Braun-Fahrlander, C., J. Riedler, et al. (2002). "Environmental exposure to endotoxin and its relation to asthma in school-age children." N Engl J Med 347(12): 869-877.

Breving, K. and A. Esquela-Kerscher (2010). "The complexities of microRNA regulation: mirandering around the rules." Int J Biochem Cell Biol 42(8): 1316-1329.

Brodersen, P. and O. Voinnet (2009). "Revisiting the principles of microRNA target recognition and mode of action." Nat Rev Mol Cell Biol 10(2): 141-148.

Bronevetsky, Y. and K. M. Ansel (2013). "Regulation of miRNA biogenesis and turnover in the immune system." Immunol Rev 253(1): 304-316.

Brown, B. D., B. Gentner, et al. (2007). "Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state." Nat Biotechnol 25(12): 1457-1467.

Bucchieri, F., S. M. Puddicombe, et al. (2002). "Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis." Am J Respir Cell Mol Biol 27(2): 179-185.

Busacker, A., J. D. Newell, Jr., et al. (2009). "A multivariate analysis of risk factors for the air-trapping asthmatic phenotype as measured by quantitative CT analysis." Chest 135(1): 48-56.

Busse, W. W., S. Banks-Schlegel, et al. (2000). "Pathophysiology of severe asthma." J Allergy Clin Immunol 106(6): 1033-1042.

Busse, W. W., W. J. Morgan, et al. (2011). "Randomized trial of omalizumab (anti-IgE) for asthma in inner-city children." N Engl J Med 364(11): 1005-1015.

Cakebread, J. A., Y. Xu, et al. (2011). "Exogenous IFN-beta has antiviral and anti-inflammatory properties in primary bronchial epithelial cells from asthmatic subjects exposed to rhinovirus." J Allergy Clin Immunol 127(5): 1148-1154 e1149.

Calhoun, W. J., E. C. Dick, et al. (1994). "A common cold virus, rhinovirus 16, potentiates airway inflammation after segmental antigen bronchoprovocation in allergic subjects." J Clin Invest 94(6): 2200-2208.

Camateros, P., M. Tamaoka, et al. (2007). "Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463." Am J Respir Crit Care Med 175(12): 1241-1249.

Campbell, M. J., G. R. Cogman, et al. (1997). "Age specific trends in asthma mortality in England and Wales, 1983-95: results of an observational study." BMJ 314(7092): 1439-1441.

Care, A., D. Catalucci, et al. (2007). "MicroRNA-133 controls cardiac hypertrophy." Nat Med 13(5): 613-618.

Careau, E. and E. Y. Bissonnette (2004). "Adoptive transfer of alveolar macrophages abrogates bronchial hyperresponsiveness." Am J Respir Cell Mol Biol 31(1): 22-27.

Cervantes, J. L., B. Weinerman, et al. (2012). "TLR8: the forgotten relative revindicated." Cell Mol Immunol 9(6): 434-438.

Chang, T. C., E. A. Wentzel, et al. (2007). "Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis." Mol.Cell 26(5): 745-752.

Chauhan, A. J., H. M. Inskip, et al. (2003). "Personal exposure to nitrogen dioxide (NO2) and the severity of virus-induced asthma in children." Lancet 361(9373): 1939-1944.

Chen, R. F., K. D. Yang, et al. (2014). "Augmented miR-150 expression associated with depressed SOCS1 expression involved in dengue haemorrhagic fever." J Infect.

Chen, Y., E. Hamati, et al. (2006). "Rhinovirus induces airway epithelial gene expression through double-stranded RNA and IFN-dependent pathways." Am J Respir Cell Mol Biol 34(2): 192-203.

Cherfils-Vicini, J., S. Platonova, et al. (2010). "Triggering of TLR7 and TLR8 expressed by human lung cancer cells induces cell survival and chemoresistance." J Clin Invest 120(4): 1285-1297.

Cheuk, D. K., I. W. Tang, et al. (2007). "Rhinovirus infection in hospitalized children in Hong Kong: a prospective study." Pediatr Infect Dis J 26(11): 995-1000.

Chiba, Y. and M. Misawa (2010). "MicroRNAs and Their Therapeutic Potential for Human Diseases: MiR-133a and Bronchial Smooth Muscle Hyperresponsiveness in Asthma." J.Pharmacol.Sci.

Chiba, Y., M. Tanabe, et al. (2009). "Down-regulation of miR-133a contributes to up-regulation of Rhoa in bronchial smooth muscle cells." Am J Respir Crit Care Med 180(8): 713-719.

Cho, S. H., L. A. Stanciu, et al. (2005). "Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma." Am J Respir Crit Care Med 171(3): 224-230.

Choy, D. F., B. Modrek, et al. (2011). "Gene expression patterns of Th2 inflammation and intercellular communication in asthmatic airways." J Immunol 186(3): 1861-1869.

Chun, E., S. H. Lee, et al. (2010). "Toll-like receptor expression on peripheral blood mononuclear cells in asthmatics; implications for asthma management." J Clin Immunol 30(3): 459-464.

Chung, K. F. (2006). "Intrinsic differences of the airway epithelium in childhood allergic asthma." Am J Respir Crit Care Med 174(10): 1066-1067.

Chung, K. F., S. E. Wenzel, et al. (2014). "International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma." Eur Respir J 43(2): 343-373.

Chung, Y., J. Y. Hong, et al. (2014). "Rhinovirus Infection Induces IL-13 Production from CD11b-positive, Exudative M2-polarized Exudative Macrophages." Am J Respir Cell Mol Biol.

Cockcroft, D. W., K. Y. Murdock, et al. (1992). "Sensitivity and specificity of histamine PC20 determination in a random selection of young college students." J Allergy Clin Immunol 89(1 Pt 1): 23-30.

Collison, A., C. Herbert, et al. (2011). "Altered expression of microRNA in the airway wall in chronic asthma: miR-126 as a potential therapeutic target." BMC Pulm Med 11: 29.

Collison, A., J. Mattes, et al. (2011). "Inhibition of house dust mite-induced allergic airways disease by antagonism of microRNA-145 is comparable to glucocorticoid treatment." J Allergy Clin Immunol 128(1): 160-167 e164.

Comhair, S. A., P. R. Bhathena, et al. (2001). "Extracellular glutathione peroxidase induction in asthmatic lungs: evidence for redox regulation of expression in human airway epithelial cells." FASEB J 15(1): 70-78.

Contoli, M., G. Caramori, et al. (2005). "Mechanisms of respiratory virus-induced asthma exacerbations." Clin Exp Allergy 35(2): 137-145.

Contoli, M., S. D. Message, et al. (2006). "Role of deficient type III interferon-lambda production in asthma exacerbations." Nat Med 12(9): 1023-1026.

Corne, J. M. and S. T. Holgate (1997). "Mechanisms of virus induced exacerbations of asthma." Thorax 52(4): 380-389.

Corne, J. M., C. Marshall, et al. (2002). "Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study." Lancet 359(9309): 831-834.

Corren, J., R. F. Lemanske, et al. (2011). "Lebrikizumab treatment in adults with asthma." N Engl J Med 365(12): 1088-1098.

Crapo, R. O., R. Casaburi, et al. (2000). "Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999." Am J Respir Crit Care Med 161(1): 309-329.

Custovic, A., S. L. Johnston, et al. (2013). "EAACI position statement on asthma exacerbations and severe asthma." Allergy 68(12): 1520-1531.

Custovic, A., S. C. Taggart, et al. (1996). "Exposure to house dust mite allergens and the clinical activity of asthma." J Allergy Clin Immunol 98(1): 64-72.

Davies, D. E. (2009). "The role of the epithelium in airway remodeling in asthma." Proc Am Thorac Soc 6(8): 678-682.

Davies, J. M., M. L. Carroll, et al. (2011). "Budesonide and formoterol reduce early innate anti-viral immune responses in vitro." PLoS ONE 6(11): e27898.

Davis, M. J., T. M. Tsang, et al. (2013). "Macrophage M1/M2 polarization dynamically adapts to changes in cytokine microenvironments in Cryptococcus neoformans infection." MBio 4(3): e00264-00213.

de Blic, J. and P. Scheinmann (2007). "The use of imaging techniques for assessing severe childhood asthma." J Allergy Clin Immunol 119(4): 808-810.

Der, S. D., A. Zhou, et al. (1998). "Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays." Proc Natl Acad Sci U S A 95(26): 15623-15628.

Didierlaurent, A., J. Goulding, et al. (2008). "Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection." J Exp Med 205(2): 323-329.

Diebold, S. S., T. Kaisho, et al. (2004). "Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA." Science 303(5663): 1529-1531.

Dijkstra, A., J. M. Vonk, et al. (2006). "Lung function decline in asthma: association with inhaled corticosteroids, smoking and sex." Thorax 61(2): 105-110.

Djukanovic, R., T. Harrison, et al. (2014). "The effect of inhaled IFN-beta on worsening of asthma symptoms caused by viral infections. A randomized trial." Am J Respir Crit Care Med 190(2): 145-154.

Donnelly, R. P. and S. V. Kotenko (2010). "Interferon-lambda: a new addition to an old family." J Interferon Cytokine Res 30(8): 555-564.

Dougherty, R. H. and J. V. Fahy (2009). "Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype." Clin Exp Allergy 39(2): 193-202.

Doull, I. J., F. C. Lampe, et al. (1997). "Effect of inhaled corticosteroids on episodes of wheezing associated with viral infection in school age children: randomised double blind placebo controlled trial." BMJ 315(7112): 858-862.

Doyle, S., S. Vaidya, et al. (2002). "IRF3 mediates a TLR3/TLR4-specific antiviral gene program." Immunity 17(3): 251-263.

Drake, M. G., E. H. Kaufman, et al. (2012). "The therapeutic potential of Toll-like receptor 7 stimulation in asthma." Inflamm Allergy Drug Targets 11(6): 484-491.

Du, Q., L. F. Zhou, et al. (2009). "Imiquimod, a toll-like receptor 7 ligand, inhibits airway remodelling in a murine model of chronic asthma." Clin Exp Pharmacol Physiol 36(1): 43-48.

Edwards, M., G. Vera, et al. (2012). Nuclear Suppressor Of Cytokine Signalling-1 Suppresses Rhinovirus Induced Interferon Expression And Is Associated With Impaired Interferon Expression In Asthma. C13. MECHANISMS INVOLVED IN EXACERBATION OF ASTHMA AND COPD, American Thoracic Society**:** A3877-A3877.

Edwards, M. R., N. Regamey, et al. (2013). "Impaired innate interferon induction in severe therapy resistant atopic asthmatic children." Mucosal Immunol 6(4): 797-806.

Ekman, A. K., M. Adner, et al. (2011). "Toll-like receptor 7 activation reduces the contractile response of airway smooth muscle." Eur J Pharmacol 652(1-3): 145-151.

ENFUMOSA (2003). "The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic severe asthma. European Network for Understanding Mechanisms of Severe Asthma." Eur Respir J 22(3): 470-477.

Faure, E., L. Thomas, et al. (2001). "Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation." J Immunol 166(3): 2018-2024.

Finberg, R. W., J. P. Wang, et al. (2007). "Toll like receptors and viruses." Rev Med Virol 17(1): 35-43.

Fiore, R., G. Siegel, et al. (2008). "MicroRNA function in neuronal development, plasticity and disease." Biochim Biophys Acta 1779(8): 471-478.

Fiorentino, D. F., A. Zlotnik, et al. (1991). "IL-10 inhibits cytokine production by activated macrophages." J Immunol 147(11): 3815-3822.

FitzGerald, J. M., A. Becker, et al. (2004). "Doubling the dose of budesonide versus maintenance treatment in asthma exacerbations." Thorax 59(7): 550-556.

Flood-Page, P., C. Swenson, et al. (2007). "A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma." Am J Respir Crit Care Med 176(11): 1062-1071.

Forbes, R. L., P. G. Gibson, et al. (2012). "Impaired type I and III interferon response to rhinovirus infection during pregnancy and asthma." Thorax 67(3): 209-214.

Foster, P. S., M. Plank, et al. (2013). "The emerging role of microRNAs in regulating immune and inflammatory responses in the lung." Immunol Rev 253(1): 198-215.

Friedman, J. M. and P. A. Jones (2009). "MicroRNAs: critical mediators of differentiation, development and disease." Swiss Med Wkly 139(33-34): 466-472.

Frotscher, B., K. Anton, et al. (2002). "Inhibition of IgE production by the imidazoquinoline resiquimod in nonallergic and allergic donors." J Invest Dermatol 119(5): 1059-1064.

Fry, R. C., J. E. Rager, et al. (2014). "Air toxics and epigenetic effects: ozone altered microRNAs in the sputum of human subjects." Am J Physiol Lung Cell Mol Physiol 306(12): L1129-1137.

Fuchs, R. and D. Blaas (2010). "Uncoating of human rhinoviruses." Rev Med Virol 20(5): 281-297.

Fujita, Y., Y. Yoshioka, et al. (2014). "Intercellular communication by extracellular vesicles and their microRNAs in asthma." Clin Ther 36(6): 873-881.

Fukuyama, S., T. Nakano, et al. (2009). "Pulmonary suppressor of cytokine signaling-1 induced by IL-13 regulates allergic asthma phenotype." Am J Respir Crit Care Med 179(11): 992-998.

Fuller, R. W. (1989). "The role of the alveolar macrophage in asthma." Respir Med 83(3): 177-178.

Gaajetaan, G. R., T. H. Geelen, et al. (2013). "Interferon-beta induces a long-lasting antiviral state in human respiratory epithelial cells." J Infect 66(2): 163-169.

Gauvreau, G. M., L. P. Boulet, et al. (2011). "Effects of interleukin-13 blockade on allergen-induced airway responses in mild atopic asthma." Am J Respir Crit Care Med 183(8): 1007-1014.

Gern, J. E. (2009). "Rhinovirus and the initiation of asthma." Curr Opin Allergy Clin Immunol 9(1): 73-78.

Gern, J. E. and W. W. Busse (2002). "Relationship of viral infections to wheezing illnesses and asthma." Nat Rev Immunol 2(2): 132-138.

Gern, J. E., E. C. Dick, et al. (1996). "Rhinovirus enters but does not replicate inside monocytes and airway macrophages." J Immunol 156(2): 621-627.

Gern, J. E., D. M. Galagan, et al. (1997). "Detection of rhinovirus RNA in lower airway cells during experimentally induced infection." Am J Respir Crit Care Med 155(3): 1159-1161.

Ghorpade, D. S., S. Holla, et al. (2013). "Sonic hedgehog-dependent induction of microRNA 31 and microRNA 150 regulates Mycobacterium bovis BCG-driven toll-like receptor 2 signaling." Mol Cell Biol 33(3): 543-556.

Gill, M. A., G. Bajwa, et al. (2010). "Counterregulation between the FcepsilonRI pathway and antiviral responses in human plasmacytoid dendritic cells." J Immunol 184(11): 5999-6006.

Giranda, V. L., B. A. Heinz, et al. (1992). "Acid-induced structural changes in human rhinovirus 14: possible role in uncoating." Proc Natl Acad Sci U S A 89(21): 10213-10217.

Girard, D. E. (1981). "The history of Osler's Principles and Practice of Medicine." Med Times 109(7): 1s-4s, 13s.

Goleva, E., P. J. Hauk, et al. (2008). "Corticosteroid-resistant asthma is associated with classical antimicrobial activation of airway macrophages." J Allergy Clin Immunol 122(3): 550-559 e553.

Gordon, S. and F. O. Martinez (2010). "Alternative activation of macrophages: mechanism and functions." Immunity 32(5): 593-604.

Gordon, S. B. and R. C. Read (2002). "Macrophage defences against respiratory tract infections." Br Med Bull 61: 45-61.

Graff, J. W., L. S. Powers, et al. (2012). "Cigarette smoking decreases global microRNA expression in human alveolar macrophages." PLoS ONE 7(8): e44066.

Grainge, C., V. Dulay, et al. (2012). "Resistin-like molecule-beta is induced following bronchoconstriction of asthmatic airways." Respirology 17(7): 1094-1100.

Grainge, C. L., L. C. Lau, et al. (2011). "Effect of bronchoconstriction on airway remodeling in asthma." N Engl J Med 364(21): 2006-2015.

Green, R. M., A. Custovic, et al. (2002). "Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study." BMJ 324(7340): 763.

Greiff, L., A. Cervin, et al. (2012). "Repeated intranasal TLR7 stimulation reduces allergen responsiveness in allergic rhinitis." Respir Res 13: 53.

Grimson, A., K. K. Farh, et al. (2007). "MicroRNA targeting specificity in mammals: determinants beyond seed pairing." Mol Cell 27(1): 91-105.

Grissell, T. V., H. Powell, et al. (2005). "Interleukin-10 gene expression in acute virus-induced asthma." Am J Respir Crit Care Med 172(4): 433-439.

Griswold, S. K., C. R. Nordstrom, et al. (2005). "Asthma exacerbations in North American adults: who are the "frequent fliers" in the emergency department?" Chest 127(5): 1579-1586.

Grunberg, K., R. F. Sharon, et al. (2000). "Experimental rhinovirus 16 infection increases intercellular adhesion molecule-1 expression in bronchial epithelium of asthmatics regardless of inhaled steroid treatment." Clin Exp Allergy 30(7): 1015-1023.

Guduric-Fuchs, J., A. O'Connor, et al. (2012). "Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types." BMC Genomics 13: 357.

Guo, H., N. T. Ingolia, et al. (2010). "Mammalian microRNAs predominantly act to decrease target mRNA levels." Nature 466(7308): 835-840.

Gupta, S., S. Siddiqui, et al. (2009). "Qualitative analysis of high-resolution CT scans in severe asthma." Chest 136(6): 1521-1528.

Hackett, T. L. and D. A. Knight (2007). "The role of epithelial injury and repair in the origins of asthma." Curr Opin Allergy Clin Immunol 7(1): 63-68.

Haines, C. J., Y. Chen, et al. (2013). "Autoimmune memory T helper 17 cell function and expansion are dependent on interleukin-23." Cell Rep 3(5): 1378-1388.

Haller, O. and G. Kochs (2002). "Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity." Traffic 3(10): 710-717.

Hammell, M. (2010). "Computational methods to identify miRNA targets." Semin Cell Dev Biol 21(7): 738-744.

Harrison, T. W., J. Oborne, et al. (2004). "Doubling the dose of inhaled corticosteroid to prevent asthma exacerbations: randomised controlled trial." Lancet 363(9405): 271-275.

Haselkorn, T., R. S. Zeiger, et al. (2009). "Recent asthma exacerbations predict future exacerbations in children with severe or difficult-to-treat asthma." J Allergy Clin Immunol 124(5): 921-927.

Hayashi, F., K. D. Smith, et al. (2001). "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5." Nature 410(6832): 1099-1103.

Heaney, L. G. and R. Horne (2012). "Non-adherence in difficult asthma: time to take it seriously." Thorax 67(3): 268-270.

Heil, F., H. Hemmi, et al. (2004). "Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8." Science 303(5663): 1526-1529.

Hewson, C. A., A. Jardine, et al. (2005). "Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells." J Virol 79(19): 12273-12279.

Hilty, M., C. Burke, et al. (2010). "Disordered microbial communities in asthmatic airways." PLoS ONE 5(1): e8578.

Holgate, S., H. Bisgaard, et al. (2008). "The Brussels Declaration: the need for change in asthma management." Eur Respir J 32(6): 1433-1442.

Holgate, S. T. (1999). "Genetic and environmental interaction in allergy and asthma." J Allergy Clin Immunol 104(6): 1139-1146.

Holgate, S. T. (2007). "Epithelium dysfunction in asthma." J Allergy Clin Immunol 120(6): 1233-1244; quiz 1245-1236.

Holgate, S. T. (2008). "Pathogenesis of asthma." Clin Exp Allergy 38(6): 872-897.

Holgate, S. T. (2011). "Asthma: a simple concept but in reality a complex disease." Eur J Clin Invest 41(12): 1339-1352.

Holgate, S. T., H. S. Arshad, et al. (2010). "A new look at the pathogenesis of asthma." Clin.Sci.(Lond) 118(7): 439-450.

Holgate, S. T. and D. E. Davies (2009). "Rethinking the pathogenesis of asthma." Immunity. 31(3): 362-367.

Hon, L. S. and Z. Zhang (2007). "The roles of binding site arrangement and combinatorial targeting in microRNA repression of gene expression." Genome Biol 8(8): R166.

Hoover-Litty, H. and J. M. Greve (1993). "Formation of rhinovirus-soluble ICAM-1 complexes and conformational changes in the virion." J Virol 67(1): 390-397.

Horn, M. E., S. E. Reed, et al. (1979). "Role of viruses and bacteria in acute wheezy bronchitis in childhood: a study of sputum." Arch Dis Child 54(8): 587-592.

Howarth, P. H., K. S. Babu, et al. (2005). "Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma." Thorax 60(12): 1012-1018.

Huang, Y., Q. Zou, et al. (2010). "A study of miRNAs targets prediction and experimental validation." Protein Cell 1(11): 979-986.

Huang, Y. J. (2013). "Asthma microbiome studies and the potential for new therapeutic strategies." Curr Allergy Asthma Rep 13(5): 453-461.

Hume, D. A. (2008). "Macrophages as APC and the dendritic cell myth." J Immunol 181(9): 5829-5835.

Humphreys, D. T., B. J. Westman, et al. (2005). "MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function." Proc Natl Acad Sci U S A 102(47): 16961-16966.

Iikura, K., T. Katsunuma, et al. (2011). "Peripheral blood mononuclear cells from patients with bronchial asthma show impaired innate immune responses to rhinovirus in vitro." Int Arch Allergy Immunol 155 Suppl 1: 27-33.

Ishii, K. J. and S. Akira (2005). "TLR ignores methylated RNA?" Immunity 23(2): 111-113.

Jackson, D. J., R. E. Gangnon, et al. (2008). "Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children." Am J Respir Crit Care Med 178(7): 667-672.

Jackson, D. J., A. Sykes, et al. (2011). "Asthma exacerbations: origin, effect, and prevention." J Allergy Clin Immunol 128(6): 1165-1174.

Jardim, M. J., L. Dailey, et al. (2012). "Distinct microRNA expression in human airway cells of asthmatic donors identifies a novel asthma-associated gene." Am J Respir Cell Mol Biol 47(4): 536-542.

Jin, S. X., K. S. Yin, et al. (2006). "Imiquimod attenuates airway inflammation and decreases the expression of thymus and activation regulated chemokine in allergic asthmatic mice." Chin Med J (Engl) 119(5): 412-416.

Johnston, N. W., S. L. Johnston, et al. (2005). "The September epidemic of asthma exacerbations in children: a search for etiology." J Allergy Clin Immunol 115(1): 132-138.

Johnston, N. W. and M. R. Sears (2006). "Asthma exacerbations . 1: epidemiology." Thorax 61(8): 722-728.

Johnston, S. L. and R. J. Martin (2005). "Chlamydophila pneumoniae and Mycoplasma pneumoniae: a role in asthma pathogenesis?" Am J Respir Crit Care Med 172(9): 1078-1089.

Johnston, S. L., P. K. Pattemore, et al. (1995). "Community study of role of viral infections in exacerbations of asthma in 9-11 year old children." BMJ 310(6989): 1225-1229.

Juarez, E., C. Nunez, et al. (2010). "Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes." Respir Res 11: 2.

Jude, J. A., M. Dileepan, et al. (2012). "miR-140-3p regulation of TNF-alpha-induced CD38 expression in human airway smooth muscle cells." Am J Physiol Lung Cell Mol Physiol 303(5): L460-468.

Juniper, E. F., P. M. O'Byrne, et al. (1999). "Development and validation of a questionnaire to measure asthma control." Eur Respir J 14(4): 902-907.

Kaiko, G. E., Z. Loh, et al. (2013). "Toll-like receptor 7 gene deficiency and early-life Pneumovirus infection interact to predispose toward the development of asthma-like pathology in mice." J Allergy Clin Immunol 131(5): 1331-1339 e1310.

Kariko, K., M. Buckstein, et al. (2005). "Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA." Immunity 23(2): 165-175.

Karta, M. R., M. L. Gavala, et al. (2014). "LPS modulates rhinovirus-induced chemokine secretion in monocytes and macrophages." Am J Respir Cell Mol Biol 51(1): 125-134.

Kato, A., S. Favoreto, Jr., et al. (2007). "TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells." J Immunol 179(2): 1080-1087.

Kato, H., O. Takeuchi, et al. (2006). "Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses." Nature 441(7089): 101-105.

Kaufman, E. H., A. D. Fryer, et al. (2011). "Toll-like receptor 7 agonists are potent and rapid bronchodilators in guinea pigs." J Allergy Clin Immunol 127(2): 462-469.

Kaufman, E. H. and D. B. Jacoby (2012). "Upping the antedrug: is a novel anti-inflammatory Toll-like receptor 7 agonist also a bronchodilator?" Br J Pharmacol 166(2): 569-572.

Kawai, T. and S. Akira (2011). "Toll-like receptors and their crosstalk with other innate receptors in infection and immunity." Immunity 34(5): 637-650.

Kedde, M., M. J. Strasser, et al. (2007). "RNA-binding protein Dnd1 inhibits microRNA access to target mRNA." Cell 131(7): 1273-1286.

Kelly, M. M., E. M. King, et al. (2012). "Corticosteroid-induced gene expression in allergen-challenged asthmatic subjects taking inhaled budesonide." Br J Pharmacol 165(6): 1737-1747.

Khaitov, M. R., V. Laza-Stanca, et al. (2009). "Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells." Allergy 64(3): 375-386.

Kim, E. Y., J. T. Battaile, et al. (2008). "Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease." Nat Med 14(6): 633-640.

Kim, Y. K., S. Y. Oh, et al. (2007). "Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma." J Immunol 178(8): 5375-5382.

Kiriakidou, M., G. S. Tan, et al. (2007). "An mRNA m7G cap binding-like motif within human Ago2 represses translation." Cell 129(6): 1141-1151.

Koga, T., Y. Oshita, et al. (2006). "Characterisation of patients with frequent exacerbation of asthma." Respir Med 100(2): 273-278.

Korpi-Steiner, N. L., M. E. Bates, et al. (2006). "Human rhinovirus induces robust IP-10 release by monocytic cells, which is independent of viral replication but linked to type I interferon receptor ligation and STAT1 activation." J Leukoc Biol 80(6): 1364-1374.

Korpi-Steiner, N. L., S. M. Valkenaar, et al. (2010). "Human monocytic cells direct the robust release of CXCL10 by bronchial epithelial cells during rhinovirus infection." Clin Exp Allergy 40(8): 1203-1213.

Kotenko, S. V., G. Gallagher, et al. (2003). "IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex." Nat Immunol 4(1): 69-77.

Krek, A., D. Grun, et al. (2005). "Combinatorial microRNA target predictions." Nat Genet 37(5): 495-500.

Krieg, A. M. (2002). "CpG motifs in bacterial DNA and their immune effects." Annu Rev Immunol 20: 709-760.

Krol, J., I. Loedige, et al. (2010). "The widespread regulation of microRNA biogenesis, function and decay." Nat Rev Genet 11(9): 597-610.

Krutzfeldt, J. and M. Stoffel (2006). "MicroRNAs: a new class of regulatory genes affecting metabolism." Cell Metab 4(1): 9-12.

Kulshreshtha, R., M. Ferracin, et al. (2007). "A microRNA signature of hypoxia." Mol Cell Biol 27(5): 1859-1867.

Kumagai, Y., O. Takeuchi, et al. (2007). "Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses." Immunity 27(2): 240-252.

Kumar, M., T. Ahmad, et al. (2011). "Let-7 microRNA-mediated regulation of IL-13 and allergic airway inflammation." J Allergy Clin Immunol 128(5): 1077-1085 e1071-1010.

Kupczyk, M., A. ten Brinke, et al. (2014). "Frequent exacerbators--a distinct phenotype of severe asthma." Clin Exp Allergy 44(2): 212-221.

Kurt-Jones, E. A., M. Chan, et al. (2004). "Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis." Proc Natl Acad Sci U S A 101(5): 1315-1320.

Kurt-Jones, E. A., L. Popova, et al. (2000). "Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus." Nat Immunol 1(5): 398-401.

Kusel, M. M., N. H. de Klerk, et al. (2007). "Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma." J Allergy Clin Immunol 119(5): 1105-1110.

Lagos-Quintana, M., R. Rauhut, et al. (2001). "Identification of novel genes coding for small expressed RNAs." Science 294(5543): 853-858.

Lahti, E., V. Peltola, et al. (2009). "Induced sputum in the diagnosis of childhood community-acquired pneumonia." Thorax 64(3): 252-257.

Lambert, H. P. and H. Stern (1972). "Infective factors in exacerbations of bronchitis and asthma." Br Med J 3(5822): 323-327.

Lambrecht, B. N. and H. Hammad (2012). "The airway epithelium in asthma." Nat Med 18(5): 684-692.

Langley, S. J., S. Goldthorpe, et al. (2003). "Exposure and sensitization to indoor allergens: association with lung function, bronchial reactivity, and exhaled nitric oxide measures in asthma." J Allergy Clin Immunol 112(2): 362-368.

Laskin, D. L., B. Weinberger, et al. (2001). "Functional heterogeneity in liver and lung macrophages." J Leukoc Biol 70(2): 163-170.

Laza-Stanca, V., L. A. Stanciu, et al. (2006). "Rhinovirus replication in human macrophages induces NF-kappaB-dependent tumor necrosis factor alpha production." J Virol 80(16): 8248-8258.

Le Bon, A. and D. F. Tough (2002). "Links between innate and adaptive immunity via type I interferon." Curr Opin Immunol 14(4): 432-436.

Leckie, M. J., A. ten Brinke, et al. (2000). "Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response." Lancet 356(9248): 2144-2148.

Lee, C., T. B. Kolesnik, et al. (2009). "Suppressor of cytokine signalling 1 (SOCS1) is a physiological regulator of the asthma response." Clin Exp Allergy 39(6): 897-907.

Lee, I., S. S. Ajay, et al. (2009). "New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites." Genome Res 19(7): 1175-1183.

Lee, J., T. H. Chuang, et al. (2003). "Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7." Proc Natl Acad Sci U S A 100(11): 6646-6651.

Lee, R. C., R. L. Feinbaum, et al. (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell 75(5): 843-854.

Lee, Y., C. Ahn, et al. (2003). "The nuclear RNase III Drosha initiates microRNA processing." Nature 425(6956): 415-419.

Lemaitre, B., E. Nicolas, et al. (1996). "The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults." Cell 86(6): 973-983.

Lemanske, R. F., Jr., E. C. Dick, et al. (1989). "Rhinovirus upper respiratory infection increases airway hyperreactivity and late asthmatic reactions." J Clin Invest 83(1): 1-10.

Lennox, K. A. and M. A. Behlke (2011). "Chemical modification and design of anti-miRNA oligonucleotides." Gene Ther 18(12): 1111-1120.

Lewis, B. P., C. B. Burge, et al. (2005). "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets." Cell 120(1): 15-20.

Li, Y. and S. Hua (2014). "Mechanisms of pathogenesis in allergic asthma: role of interleukin-23." Respirology 19(5): 663-669.

Lieberman, D., S. Printz, et al. (2003). "Atypical pathogen infection in adults with acute exacerbation of bronchial asthma." Am J Respir Crit Care Med 167(3): 406-410.

Lien, E., T. J. Sellati, et al. (1999). "Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products." J Biol Chem 274(47): 33419-33425.

Liu, S. Y., D. J. Sanchez, et al. (2011). "New developments in the induction and antiviral effectors of type I interferon." Curr Opin Immunol 23(1): 57-64.

Lloyd, C. M. and E. M. Hessel (2010). "Functions of T cells in asthma: more than just T(H)2 cells." Nat Rev Immunol 10(12): 838-848.

Lopez-Souza, N., G. Dolganov, et al. (2004). "Resistance of differentiated human airway epithelium to infection by rhinovirus." Am J Physiol Lung Cell Mol Physiol 286(2): L373-381.

Louie, J. K., A. Roy-Burman, et al. (2009). "Rhinovirus associated with severe lower respiratory tract infections in children." Pediatr Infect Dis J 28(4): 337-339.

Lu, J., G. Getz, et al. (2005). "MicroRNA expression profiles classify human cancers." Nature 435(7043): 834-838.

Lu, L. F. and A. Liston (2009). "MicroRNA in the immune system, microRNA as an immune system." Immunology 127(3): 291-298.

Lu, T. X., J. Hartner, et al. (2011). "MicroRNA-21 limits in vivo immune response-mediated activation of the IL-12/IFN-gamma pathway, Th1 polarization, and the severity of delayed-type hypersensitivity." J Immunol 187(6): 3362-3373.

Lu, T. X., E. J. Lim, et al. (2012). "MiR-375 is downregulated in epithelial cells after IL-13 stimulation and regulates an IL-13-induced epithelial transcriptome." Mucosal Immunol 5(4): 388-396.

Lu, T. X., A. Munitz, et al. (2009). "MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression." J Immunol 182(8): 4994-5002.

Lun, S. W., C. K. Wong, et al. (2009). "Expression and functional analysis of toll-like receptors of peripheral blood cells in asthmatic patients: implication for immunopathological mechanism in asthma." J Clin Immunol 29(3): 330-342.

Lund, J. M., L. Alexopoulou, et al. (2004). "Recognition of single-stranded RNA viruses by Toll-like receptor 7." Proc Natl Acad Sci U S A 101(15): 5598-5603.

Mack, G. S. (2007). "MicroRNA gets down to business." Nat Biotechnol 25(6): 631-638.

Malathi, K., B. Dong, et al. (2007). "Small self-RNA generated by RNase L amplifies antiviral innate immunity." Nature 448(7155): 816-819.

Malathi, K., J. M. Paranjape, et al. (2005). "A transcriptional signaling pathway in the IFN system mediated by 2'-5'-oligoadenylate activation of RNase L." Proc Natl Acad Sci U S A 102(41): 14533-14538.

Malley, R., P. Henneke, et al. (2003). "Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection." Proc Natl Acad Sci U S A 100(4): 1966-1971.

Mallia, P., S. D. Message, et al. (2011). "Experimental rhinovirus infection as a human model of chronic obstructive pulmonary disease exacerbation." Am J Respir Crit Care Med 183(6): 734-742.

Mann, J. K., J. R. Balmes, et al. (2010). "Short-term effects of air pollution on wheeze in asthmatic children in Fresno, California." Environ Health Perspect 118(10): 1497-1502.

Mantovani, A., A. Sica, et al. (2004). "The chemokine system in diverse forms of macrophage activation and polarization." Trends Immunol 25(12): 677-686.

Martinez, F. O., S. Gordon, et al. (2006). "Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression." J Immunol 177(10): 7303-7311.

Martinez, F. O., L. Helming, et al. (2009). "Alternative activation of macrophages: an immunologic functional perspective." Annu Rev Immunol 27: 451-483.

Masoli, M., D. Fabian, et al. (2004). "The global burden of asthma: executive summary of the GINA Dissemination Committee report." Allergy 59(5): 469-478.

Mathonnet, G., M. R. Fabian, et al. (2007). "MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F." Science 317(5845): 1764-1767.

Mattes, J., A. Collison, et al. (2009). "Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease." Proc Natl Acad Sci U S A 106(44): 18704-18709.

Melgert, B. N., N. H. ten Hacken, et al. (2011). "More alternative activation of macrophages in lungs of asthmatic patients." J Allergy Clin Immunol 127(3): 831-833.

Meng, L., X. He, et al. (2011). "TLR3 and TLR7 modulate IgE production in antigen induced pulmonary inflammation via influencing IL-4 expression in immune organs." PLoS ONE 6(2): e17252.

Message, S. D., V. Laza-Stanca, et al. (2008). "Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production." Proc Natl Acad Sci U S A 105(36): 13562-13567.

Metias, S. M., E. Lianidou, et al. (2009). "MicroRNAs in clinical oncology: at the crossroads between promises and problems." J Clin Pathol 62(9): 771-776.

Mi, Q. S., Y. P. Xu, et al. (2012). "Lack of microRNA miR-150 reduces the capacity of epidermal Langerhans cell cross-presentation." Exp Dermatol 21(11): 876-877.

Miller, E. K., K. M. Edwards, et al. (2009). "A novel group of rhinoviruses is associated with asthma hospitalizations." J Allergy Clin Immunol 123(1): 98-104 e101.

Miller, E. K., X. Lu, et al. (2007). "Rhinovirus-associated hospitalizations in young children." J Infect Dis 195(6): 773-781.

Miller, M. K., J. H. Lee, et al. (2007). "Recent asthma exacerbations: a key predictor of future exacerbations." Respir Med 101(3): 481-489.

Minerbi-Codish, I., D. Fraser, et al. (1998). "Influence of asthma in pregnancy on labor and the newborn." Respiration 65(2): 130-135.

Minor, T. E., E. C. Dick, et al. (1974). "Viruses as precipitants of asthmatic attacks in children." JAMA 227(3): 292-298.

Mishra, A., M. Wang, et al. (2007). "Resistin-like molecule-beta is an allergen-induced cytokine with inflammatory and remodeling activity in the murine lung." Am J Physiol Lung Cell Mol Physiol 293(2): L305-313.

Mohamed, J. S., M. A. Lopez, et al. (2010). "Mechanical stretch up-regulates microRNA-26a and induces human airway smooth muscle hypertrophy by suppressing glycogen synthase kinase-3beta." J Biol Chem 285(38): 29336-29347.

Moisan, J., P. Camateros, et al. (2006). "TLR7 ligand prevents allergen-induced airway hyperresponsiveness and eosinophilia in allergic asthma by a MYD88-dependent and MK2-independent pathway." Am J Physiol Lung Cell Mol Physiol 290(5): L987-995.

Molitoris, J. K., K. S. McColl, et al. (2011). "Glucocorticoid-mediated repression of the oncogenic microRNA cluster miR-17~92 contributes to the induction of Bim and initiation of apoptosis." Mol Endocrinol 25(3): 409-420.

Moller-Larsen, S., M. Nyegaard, et al. (2008). "Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders." Thorax 63(12): 1064-1069.

Moore, W. C., E. R. Bleecker, et al. (2007). "Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program." J Allergy Clin Immunol 119(2): 405-413.

Moore, W. C., D. A. Meyers, et al. (2010). "Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program." Am J Respir Crit Care Med 181(4): 315-323.

Moreira, A. P., K. A. Cavassani, et al. (2010). "Serum amyloid P attenuates M2 macrophage activation and protects against fungal spore-induced allergic airway disease." J Allergy Clin Immunol 126(4): 712-721 e717.

Mosser, A. G., R. Brockman-Schneider, et al. (2002). "Similar frequency of rhinovirus-infectible cells in upper and lower airway epithelium." J Infect Dis 185(6): 734-743.

Mosser, A. G., R. Vrtis, et al. (2005). "Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues." Am J Respir Crit Care Med 171(6): 645-651.

Mueller, T., T. Terada, et al. (2006). "Th2 cytokines down-regulate TLR expression and function in human intestinal epithelial cells." J Immunol 176(10): 5805-5814.

Mullokandov, G., A. Baccarini, et al. (2012). "High-throughput assessment of microRNA activity and function using microRNA sensor and decoy libraries." Nat Methods 9(8): 840-846.

Murray, C. S., G. Poletti, et al. (2006). "Study of modifiable risk factors for asthma exacerbations: virus infection and allergen exposure increase the risk of asthma hospital admissions in children." Thorax 61(5): 376-382.

Murray, C. S., A. Simpson, et al. (2004). "Allergens, viruses, and asthma exacerbations." Proc Am Thorac Soc 1(2): 99-104.

Murray, P. J. and T. A. Wynn (2011). "Obstacles and opportunities for understanding macrophage polarization." J Leukoc Biol 89(4): 557-563.

Nakajima, H. and K. Hirose (2010). "Role of IL-23 and Th17 Cells in Airway Inflammation in Asthma." Immune Netw 10(1): 1-4.

Narasaraju, T., E. Yang, et al. (2011). "Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis." Am J Pathol 179(1): 199-210.

Nichols, T., A. Hansell, et al. (1999). "The contribution of 'holiday deaths' to seasonal variations in asthma mortality in England and Wales." Clin Exp Allergy 29(10): 1415-1417.

Nicholson, K. G., J. Kent, et al. (1993). "Respiratory viruses and exacerbations of asthma in adults." BMJ 307(6910): 982-986.

Nicodemus-Johnson, J., B. Laxman, et al. (2013). "Maternal asthma and microRNA regulation of soluble HLA-G in the airway." J Allergy Clin Immunol 131(6): 1496-1503.

Nicolae, D., N. J. Cox, et al. (2005). "Fine mapping and positional candidate studies identify HLA-G as an asthma susceptibility gene on chromosome 6p21." Am.J.Hum.Genet. 76(2): 349-357.

Nottrott, S., M. J. Simard, et al. (2006). "Human let-7a miRNA blocks protein production on actively translating polyribosomes." Nat Struct Mol Biol 13(12): 1108-1114.

Nuanualsuwan, S. and D. O. Cliver (2003). "Infectivity of RNA from inactivated poliovirus." Appl Environ Microbiol 69(3): 1629-1632.

O'Byrne, P. M., P. J. Barnes, et al. (2001). "Low dose inhaled budesonide and formoterol in mild persistent asthma: the OPTIMA randomized trial." Am J Respir Crit Care Med 164(8 Pt 1): 1392-1397.

O'Byrne, P. M., S. Pedersen, et al. (2009). "Severe exacerbations and decline in lung function in asthma." Am J Respir Crit Care Med 179(1): 19-24.

O'Connell, R. M., K. D. Taganov, et al. (2007). "MicroRNA-155 is induced during the macrophage inflammatory response." Proc Natl Acad Sci U S A 104(5): 1604-1609.

O'Neill, S., J. Sweeney, et al. (2014). "The cost of treating severe refractory asthma in the UK: an economic analysis from the British Thoracic Society Difficult Asthma Registry." Thorax.

Ober, C. (2005). "Perspectives on the past decade of asthma genetics." J Allergy Clin Immunol 116(2): 274-278.

Oliver, B. G., S. Lim, et al. (2008). "Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages." Thorax 63(6): 519-525.

Olsen, P. H. and V. Ambros (1999). "The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation." Dev Biol 216(2): 671-680.

Orom, U. A., F. C. Nielsen, et al. (2008). "MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation." Mol Cell 30(4): 460-471.

Ortega, H. G., M. C. Liu, et al. (2014). "Mepolizumab Treatment in Patients with Severe Eosinophilic Asthma." N Engl J Med.

Oswald, H., P. D. Phelan, et al. (1997). "Childhood asthma and lung function in mid-adult life." Pediatr Pulmonol 23(1): 14-20.

Papadopoulos, N. G., P. J. Bates, et al. (2000). "Rhinoviruses infect the lower airways." J Infect Dis 181(6): 1875-1884.

Papadopoulos, N. G., G. Sanderson, et al. (1999). "Rhinoviruses replicate effectively at lower airway temperatures." J Med Virol 58(1): 100-104.

Papi, A. and S. L. Johnston (1999). "Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-kappaB-mediated transcription." J Biol Chem 274(14): 9707-9720.

Parsons, K. S., A. C. Hsu, et al. (2014). "TLR3 and MDA5 signalling, although not expression, is impaired in asthmatic epithelial cells in response to rhinovirus infection." Clin Exp Allergy 44(1): 91-101.

Pauwels, R. A., C. G. Lofdahl, et al. (1997). "Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group." N Engl J Med 337(20): 1405-1411.

Pavord, I. D., S. Korn, et al. (2012). "Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial." Lancet 380(9842): 651-659.

Perry, M. M., J. E. Baker, et al. (2014). "Airway smooth muscle hyperproliferation is regulated by microRNA-221 in severe asthma." Am J Respir Cell Mol Biol 50(1): 7-17.

Peter, M. E. (2010). "Targeting of mRNAs by multiple miRNAs: the next step." Oncogene 29(15): 2161-2164.

Peters-Golden, M. (2004). "The alveolar macrophage: the forgotten cell in asthma." Am J Respir Cell Mol Biol 31(1): 3-7.

Petersen, C. P., M. E. Bordeleau, et al. (2006). "Short RNAs repress translation after initiation in mammalian cells." Mol Cell 21(4): 533-542.

Pichlmair, A., O. Schulz, et al. (2006). "RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates." Science 314(5801): 997-1001.

Platanias, L. C. (2005). "Mechanisms of type-I- and type-II-interferon-mediated signalling." Nat Rev Immunol 5(5): 375-386.

Pockros, P. J., D. Guyader, et al. (2007). "Oral resiquimod in chronic HCV infection: safety and efficacy in 2 placebo-controlled, double-blind phase IIa studies." J Hepatol 47(2): 174-182.

Pope, S. M., P. C. Fulkerson, et al. (2005). "Identification of a cooperative mechanism involving interleukin-13 and eotaxin-2 in experimental allergic lung inflammation." J Biol Chem 280(14): 13952-13961.

Popp, W., O. Braun, et al. (1989). "Increased expression of HLA-DR antigen on alveolar macrophages in pulmonary diseases." Virchows Arch A Pathol Anat Histopathol 414(5): 393-397.

Poy, M. N., L. Eliasson, et al. (2004). "A pancreatic islet-specific microRNA regulates insulin secretion." Nature 432(7014): 226-230.

Poy, M. N., J. Hausser, et al. (2009). "miR-375 maintains normal pancreatic alpha- and beta-cell mass." Proc Natl Acad Sci U S A 106(14): 5813-5818.

Prasse, A., M. Germann, et al. (2007). "IL-10-producing monocytes differentiate to alternatively activated macrophages and are increased in atopic patients." J Allergy Clin Immunol 119(2): 464-471.

Pritchard, A. L., O. J. White, et al. (2014). "Asthma is associated with multiple alterations in anti-viral innate signalling pathways." PLoS ONE 9(9): e106501.

Qin, H. B., B. Xu, et al. (2012). "Inhibition of miRNA-221 suppresses the airway inflammation in asthma." Inflammation 35(4): 1595-1599.

Quarcoo, D., S. Weixler, et al. (2004). "Resiquimod, a new immune response modifier from the family of imidazoquinolinamines, inhibits allergen-induced Th2 responses, airway inflammation and airway hyper-reactivity in mice." Clin Exp Allergy 34(8): 1314-1320.

Rabe, K. F., P. A. Vermeire, et al. (2000). "Clinical management of asthma in 1999: the Asthma Insights and Reality in Europe (AIRE) study." Eur Respir J 16(5): 802-807.

Rajewsky, N. (2006). "microRNA target predictions in animals." Nat Genet 38 Suppl: S8-13.

Rassa, J. C., J. L. Meyers, et al. (2002). "Murine retroviruses activate B cells via interaction with toll-like receptor 4." Proc Natl Acad Sci U S A 99(4): 2281-2286.

Raver-Shapira, N., E. Marciano, et al. (2007). "Transcriptional activation of miR-34a contributes to p53-mediated apoptosis." Mol.Cell 26(5): 731-743.

Re, F. and J. L. Strominger (2004). "IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells." J Immunol 173(12): 7548-7555.

Rebouillat, D. and A. G. Hovanessian (1999). "The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties." J Interferon Cytokine Res 19(4): 295-308.

Reddel, H. K., D. R. Taylor, et al. (2009). "An official American Thoracic Society/European Respiratory Society statement: asthma control and exacerbations: standardizing endpoints for clinical asthma trials and clinical practice." Am J Respir Crit Care Med 180(1): 59-99.

Reich, E., R. M. Franklin, et al. (1962). "Action of actinomycin D on animal cells and viruses." Proc Natl Acad Sci U S A 48: 1238-1245.

Roberts, W. K., A. Hovanessian, et al. (1976). "Interferon-mediated protein kinase and low-molecular-weight inhibitor of protein synthesis." Nature 264(5585): 477-480.

Robinson, D. S., Q. Hamid, et al. (1992). "Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma." N Engl J Med 326(5): 298-304.

Roche, W. R., S. Montefort, et al. (1993). "Cell adhesion molecules and the bronchial epithelium." Am Rev Respir Dis 148(6 Pt 2): S79-82.

Roponen, M., S. T. Yerkovich, et al. (2010). "Toll-like receptor 7 function is reduced in adolescents with asthma." Eur Respir J 35(1): 64-71.

Rosenstreich, D. L., P. Eggleston, et al. (1997). "The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma." N Engl J Med 336(19): 1356-1363.

Ruggiero, T., M. Trabucchi, et al. (2009). "LPS induces KH-type splicing regulatory protein-dependent processing of microRNA-155 precursors in macrophages." FASEB J 23(9): 2898-2908.

Saba, T. G., Y. Chung, et al. (2014). "Rhinovirus-induced macrophage cytokine expression does not require endocytosis or replication." Am J Respir Cell Mol Biol 50(5): 974-984.

Sadler, A. J. and B. R. Williams (2008). "Interferon-inducible antiviral effectors." Nat Rev Immunol 8(7): 559-568.

Sakamoto, M., S. Ida, et al. (1984). "Effect of influenza virus infection on allergic sensitization to aerosolized ovalbumin in mice." J Immunol 132(5): 2614-2617.

Samuel, C. E. (2001). "Antiviral actions of interferons." Clin Microbiol Rev 14(4): 778-809, table of contents.

Schatz, M., M. P. Dombrowski, et al. (2003). "Asthma morbidity during pregnancy can be predicted by severity classification." J Allergy Clin Immunol 112(2): 283-288.

Schatz, M., L. M. Meckley, et al. (2014). "Asthma exacerbation rates in adults are unchanged over a 5-year period despite high-intensity therapy." J Allergy Clin Immunol Pract 2(5): 570-574 e571.

Schembri, F., S. Sridhar, et al. (2009). "MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium." Proc.Natl.Acad.Sci.U.S.A 106(7): 2319-2324.

Schnare, M., G. M. Barton, et al. (2001). "Toll-like receptors control activation of adaptive immune responses." Nat Immunol 2(10): 947-950.

Schoggins, J. W. and C. M. Rice (2011). "Interferon-stimulated genes and their antiviral effector functions." Curr Opin Virol 1(6): 519-525.

Schoggins, J. W., S. J. Wilson, et al. (2011). "A diverse range of gene products are effectors of the type I interferon antiviral response." Nature 472(7344): 481-485.

Schroth, M. K., E. Grimm, et al. (1999). "Rhinovirus replication causes RANTES production in primary bronchial epithelial cells." Am J Respir Cell Mol Biol 20(6): 1220-1228.

Schulz, O., S. S. Diebold, et al. (2005). "Toll-like receptor 3 promotes cross-priming to virus-infected cells." Nature 433(7028): 887-892.

Schulze, J., M. Rosewich, et al. (2009). "Methacholine challenge--comparison of an ATS protocol to a new rapid single concentration technique." Respir Med 103(12): 1898-1903.

Scott, H. A., P. G. Gibson, et al. (2011). "Airway inflammation is augmented by obesity and fatty acids in asthma." Eur Respir J 38(3): 594-602.

Sears, M. R., J. M. Greene, et al. (2003). "A longitudinal, population-based, cohort study of childhood asthma followed to adulthood." N Engl J Med 349(15): 1414-1422.

See, H. and P. Wark (2008). "Innate immune response to viral infection of the lungs." Paediatr Respir Rev 9(4): 243-250.

Seggerson, K., L. Tang, et al. (2002). "Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation." Dev Biol 243(2): 215-225.

Shannon, J., P. Ernst, et al. (2008). "Differences in airway cytokine profile in severe asthma compared to moderate asthma." Chest 133(2): 420-426.

Sharma, A., M. Kumar, et al. (2012). "Antagonism of mmu-mir-106a attenuates asthma features in allergic murine model." J Appl Physiol (1985) 113(3): 459-464.

Shaw, D. E., M. A. Berry, et al. (2007). "Association between neutrophilic airway inflammation and airflow limitation in adults with asthma." Chest 132(6): 1871-1875.

Shaw, T. J., S. L. Wakely, et al. (2004). "Endobronchial ultrasound to assess airway wall thickening: validation in vitro and in vivo." Eur Respir J 23(6): 813-817.

Sheppard, P., W. Kindsvogel, et al. (2003). "IL-28, IL-29 and their class II cytokine receptor IL-28R." Nat Immunol 4(1): 63-68.

Shikhagaie, M. M., C. K. Andersson, et al. (2014). "Mapping of TLR5 and TLR7 in central and distal human airways and identification of reduced TLR expression in severe asthma." Clin Exp Allergy 44(2): 184-196.

Sigurs, N., R. Bjarnason, et al. (2000). "Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7." Am J Respir Crit Care Med 161(5): 1501-1507.

Skevaki, C. L., I. Christodoulou, et al. (2009). "Budesonide and formoterol inhibit inflammatory mediator production by bronchial epithelial cells infected with rhinovirus." Clin Exp Allergy 39(11): 1700-1710.

Smith, L. K., R. R. Shah, et al. (2010). "Glucocorticoids modulate microRNA expression and processing during lymphocyte apoptosis." J Biol Chem 285(47): 36698-36708.

Solberg, O. D., E. J. Ostrin, et al. (2012). "Airway epithelial miRNA expression is altered in asthma." Am J Respir Crit Care Med 186(10): 965-974.

Sporik, R., T. A. Platts-Mills, et al. (1993). "Exposure to house dust mite allergen of children admitted to hospital with asthma." Clin Exp Allergy 23(9): 740-746.

St-Laurent, J., V. Turmel, et al. (2009). "Alveolar macrophage subpopulations in bronchoalveolar lavage and induced sputum of asthmatic and control subjects." J Asthma 46(1): 1-8.

Staples, K. J., T. S. Hinks, et al. (2012). "Phenotypic characterization of lung macrophages in asthmatic patients: overexpression of CCL17." J Allergy Clin Immunol 130(6): 1404-1412 e1407.

Stefani, G. and F. J. Slack (2008). "Small non-coding RNAs in animal development." Nat Rev Mol Cell Biol 9(3): 219-230.

Stein, M., S. Keshav, et al. (1992). "Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation." J Exp Med 176(1): 287-292.

Stein, R. T., D. Sherrill, et al. (1999). "Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years." Lancet 354(9178): 541-545.

Stokes, J. R., R. L. Sorkness, et al. (1998). "Attenuation of virus-induced airway dysfunction in rats treated with imiquimod." Eur Respir J 11(2): 324-329.

Strachan, D. P. (1989). "Hay fever, hygiene, and household size." BMJ 299(6710): 1259-1260.

Subrata, L. S., J. Bizzintino, et al. (2009). "Interactions between innate antiviral and atopic immunoinflammatory pathways precipitate and sustain asthma exacerbations in children." J Immunol 183(4): 2793-2800.

Suzuki, T., M. Yamaya, et al. (2000). "Effects of dexamethasone on rhinovirus infection in cultured human tracheal epithelial cells." Am J Physiol Lung Cell Mol Physiol 278(3): L560-571.

Swiecki, M. and M. Colonna (2011). "Type I interferons: diversity of sources, production pathways and effects on immune responses." Curr Opin Virol 1(6): 463-475.

Sykes, A., M. R. Edwards, et al. (2012). "Rhinovirus 16-induced IFN-alpha and IFN-beta are deficient in bronchoalveolar lavage cells in asthmatic patients." J Allergy Clin Immunol 129(6): 1506-1514 e1506.

Sykes, A., M. R. Edwards, et al. (2013). "TLR3, TLR4 and TLRs7-9 Induced Interferons Are Not Impaired in Airway and Blood Cells in Well Controlled Asthma." PLoS ONE 8(6): e65921.

Sykes, A., J. Macintyre, et al. (2014). "Rhinovirus-induced interferon production is not deficient in well controlled asthma." Thorax 69(3): 240-246.

Taganov, K. D., M. P. Boldin, et al. (2006). "NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses." Proc Natl Acad Sci U S A 103(33): 12481-12486.

Takahasi, K., M. Yoneyama, et al. (2008). "Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses." Mol Cell 29(4): 428-440.

Takaoka, A., S. Hayakawa, et al. (2003). "Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence." Nature 424(6948): 516-523.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol 16(1): 3-9.

Takyar, S., H. Vasavada, et al. (2013). "VEGF controls lung Th2 inflammation via the miR-1-Mpl (myeloproliferative leukemia virus oncogene)-P-selectin axis." J Exp Med 210(10): 1993-2010.

Tan, Z., G. Randall, et al. (2007). "Allele-specific targeting of microRNAs to HLA-G and risk of asthma." Am J Hum Genet 81(4): 829-834.

Teichtahl, H., N. Buckmaster, et al. (1997). "The incidence of respiratory tract infection in adults requiring hospitalization for asthma." Chest 112(3): 591-596.

ten Brinke, A. (2008). "Risk factors associated with irreversible airflow limitation in asthma." Curr Opin Allergy Clin Immunol 8(1): 63-69.

ten Brinke, A., P. J. Sterk, et al. (2005). "Risk factors of frequent exacerbations in difficult-to-treat asthma." Eur Respir J 26(5): 812-818.

ten Brinke, A., J. T. van Dissel, et al. (2001). "Persistent airflow limitation in adult-onset nonatopic asthma is associated with serologic evidence of Chlamydia pneumoniae infection." J Allergy Clin Immunol 107(3): 449-454.

Thompson, A. J. and S. A. Locarnini (2007). "Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response." Immunol Cell Biol 85(6): 435-445.

Thomson, N. C. (2014). "Omalizumab decreases exacerbations and allows a step down in daily inhaled corticosteroid dose in adults and children with moderate-to-severe asthma." Evid Based Med 19(4): 135.

Thomson, N. C., R. Chaudhuri, et al. (2004). "Asthma and cigarette smoking." Eur Respir J 24(5): 822-833.

Toshchakov, V., B. W. Jones, et al. (2002). "TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages." Nat Immunol 3(4): 392-398.

Triantafilou, K., E. Vakakis, et al. (2011). "Human rhinovirus recognition in non-immune cells is mediated by Toll-like receptors and MDA-5, which trigger a synergetic pro-inflammatory immune response." Virulence 2(1): 22-29.

Truyen, E., L. Coteur, et al. (2006). "Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients." Thorax 61(3): 202-208.

Tsitsiou, E., A. E. Williams, et al. (2012). "Transcriptome analysis shows activation of circulating CD8+ T cells in patients with severe asthma." J Allergy Clin Immunol 129(1): 95-103.

Tversky, J. R., T. V. Le, et al. (2008). "Human blood dendritic cells from allergic subjects have impaired capacity to produce interferon-alpha via Toll-like receptor 9." Clin Exp Allergy 38(5): 781-788.

Tyner, J. W., O. Uchida, et al. (2005). "CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during viral infection." Nat Med 11(11): 1180-1187.

Ulrik, C. S. and P. Lange (1994). "Decline of lung function in adults with bronchial asthma." Am J Respir Crit Care Med 150(3): 629-634.

Unger, B. L., A. N. Faris, et al. (2012). "Rhinovirus attenuates non-typeable Hemophilus influenzae-stimulated IL-8 responses via TLR2-dependent degradation of IRAK-1." PLoS Pathog 8(10): e1002969.

van der Sluijs, K. F., L. J. van Elden, et al. (2004). "IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection." J Immunol 172(12): 7603-7609.

Vareille, M., E. Kieninger, et al. (2012). "Impaired type I and type III interferon induction and rhinovirus control in human cystic fibrosis airway epithelial cells." Thorax 67(6): 517-525.

von Scheele, I., K. Larsson, et al. (2010). "Budesonide enhances Toll-like receptor 2 expression in activated bronchial epithelial cells." Inhal Toxicol 22(6): 493-499.

Wang, X., E. R. Hinson, et al. (2007). "The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts." Cell Host Microbe 2(2): 96-105.

Wark, P. A., S. L. Johnston, et al. (2005). "Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus." J Exp Med 201(6): 937-947.

Wark, P. A., S. L. Johnston, et al. (2002). "Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma." Eur Respir J 19(1): 68-75.

Wark, P. A., S. L. Johnston, et al. (2002). "Chlamydia pneumoniae immunoglobulin A reactivation and airway inflammation in acute asthma." Eur Respir J 20(4): 834-840.

Wark, P. A., M. Tooze, et al. (2013). "Viral and bacterial infection in acute asthma and chronic obstructive pulmonary disease increases the risk of readmission." Respirology 18(6): 996-1002.

Weber, F., V. Wagner, et al. (2006). "Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses." J Virol 80(10): 5059-5064.

Wenzel, S. (2005). "Severe asthma in adults." Am J Respir Crit Care Med 172(2): 149-160.

Wenzel, S. E. (2012). "Asthma phenotypes: the evolution from clinical to molecular approaches." Nat Med 18(5): 716-725.

Williams, A. E. (2008). "Functional aspects of animal microRNAs." Cell Mol Life Sci 65(4): 545-562.

Williams, A. E., H. Larner-Svensson, et al. (2009). "MicroRNA expression profiling in mild asthmatic human airways and effect of corticosteroid therapy." PLoS ONE 4(6): e5889.

Williams, A. E., M. M. Perry, et al. (2007). "microRNA expression in the aging mouse lung." BMC Genomics 8: 172.

Witt, C. A., A. Sheshadri, et al. (2014). "Longitudinal changes in airway remodeling and air trapping in severe asthma." Acad Radiol 21(8): 986-993.

Woodruff, P. G., H. A. Boushey, et al. (2007). "Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids." Proc Natl Acad Sci U S A 104(40): 15858-15863.

Woodruff, P. G., B. Modrek, et al. (2009). "T-helper type 2-driven inflammation defines major subphenotypes of asthma." Am J Respir Crit Care Med 180(5): 388-395.

Wu, X. B., M. Y. Wang, et al. (2014). "Overexpression of microRNA-21 and microRNA-126 in the patients of bronchial asthma." Int J Clin Exp Med 7(5): 1307-1312.

Xiao, C., S. M. Puddicombe, et al. (2011). "Defective epithelial barrier function in asthma." J Allergy Clin Immunol 128(3): 549-556 e541-512.

Xirakia, C., O. Koltsida, et al. (2010). "Toll-like receptor 7-triggered immune response in the lung mediates acute and long-lasting suppression of experimental asthma." Am J Respir Crit Care Med 181(11): 1207-1216.

Yamaya, M., H. Nishimura, et al. (2014). "Formoterol and budesonide inhibit rhinovirus infection and cytokine production in primary cultures of human tracheal epithelial cells." Respir Investig 52(4): 251-260.

Yang, M., R. K. Kumar, et al. (2012). "Emerging roles of pulmonary macrophages in driving the development of severe asthma." J Leukoc Biol 91(4): 557-569.

Yazdanbakhsh, M., P. G. Kremsner, et al. (2002). "Allergy, parasites, and the hygiene hypothesis." Science 296(5567): 490-494.

Yuan, F., X. Fu, et al. (2014). "Induction of Murine Macrophage M2 Polarization by Cigarette Smoke Extract via the JAK2/STAT3 Pathway." PLoS ONE 9(9): e107063.

Zarember, K. A. and P. J. Godowski (2002). "Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines." J Immunol 168(2): 554-561.

Zhang, X., A. F. Clark, et al. (2008). "FK506-binding protein 51 regulates nuclear transport of the glucocorticoid receptor beta and glucocorticoid responsiveness." Invest Ophthalmol Vis Sci 49(3): 1037-1047.

Zhu, J., S. D. Message, et al. (2014). "Airway inflammation and illness severity in response to experimental rhinovirus infection in asthma." Chest.