The pathophysiology of rhinovirus induced exacerbations in mild
and moderate asthma

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Abstract

Rhinovirus infection is the most common cause of asthma exacerbations, however mechanisms underlying this remain poorly understood. A human model of experimental rhinovirus induced asthma exacerbation has been developed, however to date the exclusion of moderately-severe and poorly-controlled asthmatics has meant that the role of asthma severity and control on the outcome of rhinovirus infection is unknown. In addition, conventional sampling techniques such as bronchoalveolar lavage dilute many cytokines below limits of detection and consequently it has not been possible to measure key mediators of type 1 and 2 inflammation *in-vivo*.

Thirty-two mild-to-moderate asthmatics and 14 healthy subjects were inoculated nasally with rhinovirus-16. Bronchoscopies were performed 2 weeks prior to inoculation and on day 4 post-inoculation. A novel technique to sample undiluted mucosal airway lining fluid called ‘bronchosorption’ was developed and performed via bronchoscopy to enable more accurate measurement of cytokines. A similar technique termed ‘nasosorption’ was performed in the nose. Levels of a range of type 1 and 2 mediators were measured simultaneously in both the upper and lower airway throughout the infection.

Twenty-eight asthmatic and 11 healthy subjects developed objective evidence of infection. Asthmatics with moderately-severe disease and poor baseline control developed significantly greater lower respiratory symptoms and falls in lung function than milder and well-controlled asthmatics. The techniques of nasosorption and bronchosorption were able to identify significantly augmented type 2 immunity during infection *in-vivo* in asthmatic but not healthy subjects with levels of key mediators including IL-4, -5, -13, -33, TARC/CCL17, MDC/CCL22 all relating to exacerbation severity. Induction of type 1 mediators was comparable in the asthmatic and healthy nose but was increased in the asthmatic lung in keeping with the lower airway involvement by rhinovirus in asthma.
This is the first study to have demonstrated that baseline asthma severity and control influences the outcome of rhinovirus infection highlighting the importance of maintaining good asthma control. It is also the first to have shown the significant induction of a range of type 2 mediators by rhinovirus in asthma \textit{in-vivo}. The novel sampling techniques that have been developed will greatly advance our understanding of a range of respiratory conditions through the ability to measure previously undetectable inflammatory mediators.
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Asthma has been recognised since antiquity with reference to it in Egyptian papyrus records dating back to the second millennium BC. The term Asthma comes from the Greek verb *aazein*, meaning to pant, to exhale with the open mouth, sharp breath. In The *Iliad*, a Greek epic poem describing the siege of Troy, the expression *asthma* appeared for the first time. Galen, a Greek who practised as a physician in the second century BC clearly had some notion of the airflow obstruction that characterises asthma when he stated:

'...those who suffer from this disease have a feeling of constriction, and in consequence they breathe frequently and fast, raising the chest violently, without, however, breathing in much air.'

The 12th century rabbi and court physician to the Sultan Saladin of Arabia, Moses Maimonides wrote a classic *Treatise on Asthma* for Prince Al-Afdal, a patient of his. Maimonides revealed that his patient’s symptoms often started as a common cold during the wet months. Eventually the patient gasped for air and coughed until phlegm was expelled. He noted that the dry months of Egypt helped asthma sufferers.¹

Maimonides remarkable observation linking the common cold with the onset of acute asthma wasn’t discussed in any great deal again until the 1960’s.² Since then almost 1,500 articles have been published on the subject, yet the mechanisms underlying the relationship between viruses and asthma exacerbations remains elusive. Advancing our understanding of these mechanisms has been the overall aim of this thesis.
Statement of personal contribution to this study

This study was not possible without the assistance of several scientific and clinical research staff within Imperial College at various stages of this PhD. However the work presented in this thesis is my own unless stated otherwise. I personally assessed suitability for volunteers for this study at screening visits, was present at all of the 460 individual study visits for eligible volunteers, performed each of the 92 bronchoscopies and processed the vast majority of the upper and lower airway samples generated from these study visits. I personally carried out the validation studies for the nasosorption strips using the MSD platform, however the subsequent analysis of cytokine levels in nasosorption and bronchosorption samples using the MSD platform was carried out by Novartis (Horsham, UK). I carried out all the staining and counting of BAL cytospin slides other than immunohistochemistry which was carried out by Dr Jie Zhu at the Royal Brompton Hospital. Measurement of virus load by Taqman was initially carried out by myself and subsequently completed by research assistants Julia Aniscenko, Leila Gogsadze and Eteri Bakhsoliani. I carried out measurement of serum neutralising antibodies to rhinovirus-16 with the assistance of Dr Aurica Telcian, Imperial College.

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## Contents

**Chapter 1. Introduction**

1.1 Asthma 12  
1.2 Defining asthma exacerbations 12  
1.3 Epidemiology of Asthma Exacerbations 14  
1.4 Asthma control 16  
1.5 Categorical versus continuous measures of asthma control 17  
1.6 Asthma severity and the relationship with asthma control 18  
1.7 The role of viruses in asthma exacerbations 18  
1.8 Rhinoviruses 19  
1.9 Other viruses associated with asthma exacerbations 20  
1.10 Seasonal patterns of exacerbations 21  
1.11 Virus-allergen interactions 22  
1.12 Human model of experimental rhinovirus infection in asthma 23  
1.13 Effects of virus infection on airway hyperresponsiveness in asthma 25  
1.14 Symptoms during experimental rhinovirus infection 25  
1.15 The effects of experimental rhinovirus infection on FEV₁ and peak flow 27  
1.16 The immune response to rhinovirus infection 27  
1.17 Rhinovirus-induced signalling in epithelial cells 29  
1.18 Evidence for impaired anti-viral responses in asthma 30  
1.19 Relationship between exacerbations, airway remodelling, and lung function decline 31  
1.20 Type 2 inflammation in asthma 33  
1.21 Type 2 inflammation in virus-induced asthma exacerbations 34  
1.22 The airway epithelium as a regulator of type 2 responses in asthma 35  
1.23 IL-33 35  
1.24 IL-33 and asthma 36  
1.25 IL-33 and virus infections 37  
1.26 IL-25 37  
1.27 Thymic stromal lymphopoietin 38
Chapter 2. Materials and Methods

2.1 Validation of synthetic absorptive matrices for sampling airway mucosal lining fluid

2.2 Development of bronchial mucosal sampling (bronchosorption) device

2.3 Outline of clinical study and procedures

2.4 Study subjects

2.4.1 Inclusion criteria for asthmatics

2.4.2 Inclusion criteria for healthy subjects

2.4.3 Exclusion criteria for asthmatics

2.4.4 Exclusion criteria for healthy subjects

2.4.5 Defining asthma severity status

2.4.6 Defining asthma control status

2.5 Subject screening

2.5.1 First screening visit

2.5.2 Second screening visit

2.6 Skin prick testing
2.7 Pulmonary function testing 59
2.8 RV-16 serology testing 59
2.9 Histamine challenge 61
2.10 Virus inoculation 61
2.11 Symptom scores 62
2.11.1 Upper respiratory symptoms 62
2.11.2 Lower respiratory symptoms 62
2.12 Nasal lavage 64
2.13 Sampling nasal mucosal lining fluid: ‘Nasosorption’ 64
2.14 Bronchoscopy 65
2.14.1 Bronchoalveolar lavage 65
2.14.2 Bronchial brushings 65
2.14.3 Bronchial biopsies 66
2.14.4 Sampling bronchial mucosal lining fluid: ‘Bronchosorption’ 66
2.15 Virologic confirmation of RV-16 infection 67
2.16 PCR for additional respiratory viruses 68
2.17 Differential cell counting 69
2.18 Cytokine and chemokine measurement 69
2.19 Statistical analysis and software 71

**Chapter 3. Results 1: Development and Validation of Nasosorption and Bronchosorption**

3.1 Introduction 73
3.2 Assessment of Accuwick 74
3.3 Comparison of absorptive matrices and optimisation of protein recovery 76
3.4 Reproducibility of nasosorption 78
3.5 Comparison of nasosorption with nasal lavage 79
3.6 Development of bronchosorption 81
3.7 Comparison of bronchosorption with bronchoalveolar lavage 81
3.8 Discussion 83
Chapter 4. Results 2: Clinical and Virological Outcomes of Experimental Rhinovirus Infection in Asthma

4.1 Introduction 85
4.2 Subject demographics 86
4.3 Virologic confirmation of RV-16 infection 87
4.4 Adverse responses to rhinovirus inoculation 87
4.5 Upper respiratory symptom scores 91
4.6 Lower respiratory symptom scores 94
4.7 Changes in lung function with infection 98
4.8 Effects of virus infection on airway hyperresponsiveness in asthma 101
4.9 Assessment of virus load 102
4.10 Relationships between virus load and clinical outcome 105
4.11 Inflammatory cell measurements 105
4.12 Discussion 106

Chapter 5. Results 3: Type 1 Inflammation During Rhinovirus-Induced Exacerbations of Asthma

5.1 Introduction 112
5.2 Levels of IL-12 114
5.3 Relationships between IL-12 and clinical outcome 115
5.4 IFN-γ levels 115
5.5 Relationships between IFN-γ and clinical outcome in asthma 116
5.6 Levels of IL-15 117
5.7 Relationships between IL-15 and clinical outcome in asthma 118
5.8 Levels of IP-10/CXCL10 119
5.9 Relationships between IP-10/CXCL10 and clinical outcome in asthma 120
5.10 Levels of I-TAC/CXCL11 122
5.11 Relationships between I-TAC/CXCL11 and clinical outcome in asthma 123
5.12 Relationships between type 1 mediators and respiratory symptoms in healthy subjects 124
5.13 Relationships between type 1 mediators and virus load 126
5.14 Relationships between type 1 mediators and atopy 125
5.15 Relationships between baseline asthma severity and control and type 1 mediator levels 126
5.16 Relationships between nasal and bronchial levels of type 1 mediators 128
5.17 Relationships between baseline and virus-induced type 1 inflammation 128
5.18 Kinetics of type 1 mediator induction with rhinovirus in asthma 128
5.19 Relationships between the type 1 mediators 129
5.20 Discussion 131

Chapter 6. Results 4: Type 2 Inflammation During Rhinovirus-Induced Exacerbations of Asthma

6.1 Introduction 135
6.2 Levels of Th2 cytokines in airway mucosal lining fluid 136
6.3 Relationships between Th2 cytokines and virus-induced respiratory symptoms in asthma 138
6.4 Relationships between Th2 cytokines and virus-induced respiratory symptoms in healthy subjects 141
6.5 Relationships between Th2 cytokines and airway hyperresponsiveness 142
6.6 Relationships between Th2 cytokines in asthma 143
6.7 Levels of IL-33 in airway mucosal lining fluid 145
6.8 Relationship between IL-33 and respiratory symptom scores 145
6.9 Relationship between IL-33 and other mediators of type 2 inflammation 148
6.10 Levels of TARC/CCL17 in airway mucosal lining fluid 149
6.11 Levels of MDC/CCL22 in airway mucosal lining fluid 150
6.12 Relationships between Th2 chemokine levels in asthma 150
6.13 Relationships between Th2 chemokines and respiratory symptoms in asthma 152
6.14 Relationships between Th2 chemokines and respiratory symptoms in healthy subjects 154
6.15 Relationships between Th2 chemokines and Th2 cytokines in asthma 155
6.16 Relationships between type 2 mediators and virus load 157
6.17 Relationships between type 2 mediators and markers of atopy 158
6.18 Relationships between nasal and bronchial type 2 mediator levels in asthma 158
6.19 Relationships between baseline and virus-induced type 2 inflammation 161
6.20 Kinetics of nasal type 2 mediator induction in asthma 162
6.21 Discussion 164

Chapter 7. Discussion and future work 169

References 181
Appendix 193
Chapter 1

Introduction

1.1 Asthma

Asthma is the commonest chronic respiratory disease in the western world affecting up to 10% of adults and 30% of children. It is increasingly recognised as a heterogeneous condition with many factors including the underlying phenotype, trigger factor exposure, the degree of airflow obstruction and hyperresponsiveness, and the severity and type of airway inflammation all combining to create wide individual variability in disease manifestation. Its natural history includes acute episodic exacerbations against a background of chronic persistent inflammation and/or structural changes that are associated with symptoms and reduced lung function. These exacerbations constitute the greatest risk to patients, are a cause of significant anxiety to both patients and family members alike and generate the greatest financial burden for health care systems. Unsurprisingly therefore, prevention of exacerbations has consistently been described by international guidelines as one of the primary goals of asthma treatment. Indeed the GINA Asthma Challenge launched in May 2010 aims to reduce hospitalisations due to asthma by 50% over the next 5 years.

1.2 Defining Asthma Exacerbations

Airflow obstruction during exacerbations stems from a combination of concentric smooth muscle contraction, airway wall oedema, and luminal obstruction with mucus. Exacerbations vary greatly in speed of onset, intensity and in time to resolution both between and within individual patients. Several groups have put forward definitions of an asthma exacerbation (as applied to clinical trials) and attempted to stratify exacerbation severity. These include the Global Initiative for Asthma
The EPR-3 and GINA definition are broadly similar with GINA defining acute exacerbations as “episodes of progressive increase in shortness of breath, cough, wheezing, or chest tightness, or some combination of these symptoms, accompanied by decreases in expiratory airflow that can be quantified by measurement of lung function.” GINA adds that exacerbations are an acute and severe loss of control that requires urgent treatment. But although GINA refers to the severity of exacerbations they do not define exact criteria by which to distinguish severity levels in clinical studies.

The ATS/ERS statement published in 2009 defined exacerbations as “events characterized by a change from the patient’s previous status.” The task force stratified its definition by severity:

**Severe asthma exacerbations** are events that require urgent action on the part of the patient and physician to prevent a serious outcome, such as hospitalization or death from asthma. The occurrence of severe asthma exacerbations should be used as a marker of poor asthma control and should include either: use of systemic corticosteroids or an increase from a stable maintenance dose for at least 3 days; or a hospitalization or A&E visit because of asthma requiring systemic corticosteroids.

**Moderate asthma exacerbations** are events that should result in a temporary change of treatment in an effort to prevent the exacerbation from becoming severe. A moderate exacerbation should include 1 or more of the following: deterioration in symptoms, deterioration in lung function, and increased use of short-acting β-agonist (SABA) bronchodilator. These features should last for 2 days or more but not be severe enough to warrant systemic corticosteroid use and/or hospitalization or ED visits for asthma.
Most recently, the 'Asthma Outcomes' workshop organised by a consortium of six U.S governmental and nongovernmental organisations, including the National Heart, Lung, and Blood Institute published their report. On review of the literature they noted that almost no 2 studies define asthma exacerbation in the same way and proposed the following definition, primarily based on the ATS/ERS statement:

“An exacerbation is a worsening of asthma requiring the use of systemic corticosteroids (or for patients on a stable maintenance dose, an increase in the use of systemic corticosteroids) to prevent a serious outcome.”

In contrast to the ATS/ERS statement however, the subcommittee did not endorse severity stratification in the definition as 'there is not a validated way to define the lower threshold of moderate exacerbations and to distinguish a moderate exacerbation from loss of chronic asthma control'. In addition, possible confusion between disease severity as opposed to the severity of exacerbations can arise. Lastly, they noted that severity of an exacerbation can refer to two distinct phenomena: (1) the intensity of symptoms in general or (2) the magnitude of individual features, such as the severity of airway obstruction. If a gradation of exacerbations is to be utilized, the terminology would need to be unambiguous.

1.3 Epidemiology of Asthma Exacerbations

The frequency with which acute exacerbations of asthma are reported in the literature varies according to the definition of exacerbation used and the severity and/or level of disease control of the asthmatic population being studied. In the OPTIMA trial rates in mild asthmatics were 0.92 per patient per year in those on low dose inhaled corticosteroids (ICS), compared to 0.36 in patients taking high dose ICS and a long-acting β-agonist (LABA). In the FACET study, rates of severe exacerbations in moderate asthmatics were 0.91 per patient per year in those treated with low dose ICS and 0.34 in patients on high dose ICS and LABA. In patients with more severe asthma in a trial of
ant-IgE therapy exacerbation rates over a 48 week period were 0.88 in the placebo group and 0.66 in the treatment group. Taken together, these data suggest that asthmatics on optimum treatment should only experience on average 1 exacerbation every 3 years. However these studies fail to reflect the heterogeneity of exacerbations within the asthmatic population as some patients will rarely or never experience an exacerbation whereas others experience frequent exacerbations. In addition, as poorly-controlled patients and those with frequent exacerbations are often excluded from these studies the true rates may be greater than reported.

In this respect, surveys of ‘real life’ asthma patients indicate that the incidence of exacerbations is much higher than seen in patients recruited to clinical trials. In a survey of 1003 patients in the USA with uncontrolled asthma 70% had an unscheduled physician visit, 36% an Emergency Department visit and 14% a hospitalization in the last year. This is compared to patients with controlled asthma of which 43% experienced an unscheduled physician visit, 10% an Emergency Department visit and 3% a hospitalization in the last year.

In addition to a relationship with disease control, the frequency of severe exacerbations also relates to asthma severity. A study from the National Heart, Lung, and Blood Institute’s Severe Asthma Research Program (SARP) found that the percentage of asthmatics with three or more exacerbations per year was 5% in the mild group, 13% in the moderate group and 54% in the severe patients. Factors associated with frequent exacerbations appear to include female sex, obesity, psychopathology, chronic sinusitis, gastro-oesophageal reflux, respiratory infections and obstructive sleep apnoea.

Fortunately, deaths from asthma are relatively rare but still numbered almost 3,500 (3,262 adults and 185 children aged 0–17) in 2007 in the USA and an estimated 1400 deaths in 2002 in the UK. Currently the Royal College of Physicians are carrying out a National Review of Asthma Deaths (NRAD) in the UK which should provide a more accurate figure in the next year or so.
1.4 Asthma Control

Our understanding of asthma control has evolved in recent years to include both the idea of current control but also the link with future risk. In many ways this is similar to several other chronic conditions such as renal disease, hypertension and diabetes in which the attainment of good control lessens the risk of future adverse events. Asthma control has been defined as the extent to which the various manifestations of asthma have been reduced or removed by treatment, and includes the two domains mentioned above:

1. The level of current clinical asthma control, which is gauged from features such as symptoms and the extent to which the patient can carry out activities of daily living, and

2. The risk of future adverse events including loss of control, exacerbations, accelerated decline in lung function, and side-effects of treatment.

Assessment of asthma control therefore falls into two broad categories relating to these components. In recent years 17 questionnaire instruments measuring asthma control have been developed. These composite score instruments for the most part contain comparable content. The four most commonly used are: the Asthma Control Questionnaire (ACQ), the Asthma Control Test (ACT), the Asthma Therapy Assessment Questionnaire (ATAQ), and the Asthma Control Scoring System (ACSS). The ACQ and ACT are considered the best validated measures and have been designated as the two asthma control composite score instruments for NIH-initiated clinical research in adults. To date, the majority of clinical trials have used the ACQ whilst the ACT has the most published validation data.

It is important to bear in mind that these tools reflect chronic disease activity and specifically the status of the individual within the recall window of the instrument in question and have not been validated to assess exacerbations.
The ACQ\textsuperscript{16} was developed by Juniper for assessing control in both clinical trials and clinical practise. Questions rely on recall of the previous 7 days (as oppose to 4 weeks for the ACT) and comprise breathlessness, nocturnal waking, symptoms on waking, wheeze, activity limitation, frequency of SABA use, and pre-bronchodilator FEV1\% predicted. All seven items are scored on a 7-point scale without weighting (0= good control, 6 = poor control) and the overall score is the mean of the responses. Asthma control groups are defined by well-accepted ACQ cut-off points of $\leq 0.75$ for well-controlled and $\geq 1.50$ for not well-controlled (or poorly-controlled) asthma respectively.\textsuperscript{17}

In addition to the contrasting recall period, the ACQ also differs from the ACT by including pulmonary function parameters. The ACT should therefore be used in conjunction with PFTs to better assess the asthma control impairment. However despite these differences, studies comparing the two have demonstrated strong correlations between the measures ($r = -0.82$ to -0.89) and showed similar reproducibility and sensitivity/specificity for detecting poorly-controlled asthma.\textsuperscript{18-20}

Poor asthma control is frequently due to poor compliance, under-prescribing, poor inhaler technique or environmental factors such as allergen exposure, respiratory viral infection and smoking. Less commonly is it due to intrinsically severe disease and resistance to therapy.

1.5 Categorical Versus Continuous Measures of Asthma Control

The transformation of several continuous variables such as symptoms and reliever use into individual categorical variables to which scores are assigned and cut-points applied to allow categorisation of patients into well-controlled and poorly-controlled groups is potentially problematic and inevitably creates an indeterminate zone – a ‘no-man’s land’ where patients are neither poorly- nor well-controlled. Many different terms have been used to describe this middle category including ‘partially controlled’, ‘not well controlled’ ‘acceptable control’, and ‘sub-optimal control’.\textsuperscript{3} Added to this complexity is the lack of complete agreement regarding threshold criteria of
the different categories. The current study uses the term ‘partially-controlled’ to describe asthmatics that are neither well-controlled nor poorly-controlled.

1.6 Asthma Severity and the Relationship with Asthma Control

The concept of asthma severity has evolved substantially in recent years and is currently used to refer to the intensity of treatment required to control the patient’s asthma. Asthma severity may be influenced by genetic and environmental factors, the underlying disease activity, and the dominant inflammatory pathways and processes that vary according to differing patient phenotypes.

In 2004, the GINA executive committee recommended that their report be revised to emphasise asthma management based on clinical control rather than the classification of patients by severity. Up until the publication of their revised document in 2006 the classification of asthma was according to the severity of clinical features (see section 2.4.5 for the full classification).

A number of authors have reviewed the relationship between control and severity with differing conclusions reflecting the evolution of how these terms have been defined and assessed. The terms are not synonymous, despite them being used interchangeably all too frequently. Patients with severe asthma may be well-controlled on very high doses of multiple asthma medications whereas a patient with mild asthma may be poorly controlled in the absence of any regular inhaled therapy. Yet despite this they do have a close, dynamic relationship with each other and are clearly linked. Asthmatic subjects in this study are defined both in terms of their severity and their asthma control status.

1.7 The Role of Viruses in Asthma Exacerbations

In healthy people the common cold is usually a self-limiting upper airway disease with a short duration. However since the early 1960s, viral respiratory tract infections have been consistently reported as triggers for exacerbations of asthma in both adults and children. The development of
highly sensitive and specific molecular diagnostic and detection techniques using polymerase chain reaction (PCR) technology in the 1990s led to greatly improved detection of respiratory tract viruses and allowed clear demonstration of the important link between viral infections and asthma exacerbations. When PCR is used to supplement or instead of conventional techniques, viruses have been found in approximately 80% of wheezing episodes in school-aged children and in approximately one half to three quarters of acute wheezing episodes in adults. With the exception of respiratory syncytial virus (RSV) in infants hospitalised with bronchiolitis, rhinoviruses are by far the most frequently detected virus type.

1.8 Rhinoviruses

Human rhinoviruses (HRV) belong to the family Picornaviridae and are classified within the genus of enteroviruses. Rhinoviruses are single-stranded, non-enveloped RNA viruses with a genome that consists of approximately 7,200 nucleotides. They were first cultured in 1953 and over 100 serotypes have been identified by culture. These serotypes have been divided into type A and B genotypes originally based on their sensitivities to anti-viral drugs, but more recently based on their genetic sequences. Indeed genomic sequencing has permitted the identification of a further new group of noncultururable rhinoviruses known as HRV type-C.

The most common way to catch a cold is thought to be by introduction of the fingertip into the nose or eye. Rhinoviruses in the nose are thought to be deposited on the skin surface in the nares and not directly onto the nasal mucosa. How RV is then introduced to the mucosa remains unclear, as does the question of whether RV is able to penetrate the mucous blanket casing the epithelium or whether mucociliary clearance carries the virus back to the nasopharynx.

All respiratory tract viruses enter and replicate within airway epithelial cells and can damage both ciliated and nonciliated respiratory epithelial cells, leading to necrosis of the airway epithelium, ciliostasis, loss of cilia, and impairment of mucociliary clearance. The majority of RV serotypes
(including RV-16) attach to epithelial cells through intercellular adhesion molecule 1 (ICAM-1). A smaller number of other serotypes use the low-density lipoprotein (LDL) receptor whilst the receptors for HRV type C have not been identified as yet. Indeed, rhinovirus infection itself upregulates expression of ICAM-1 both in vivo and in vitro to further the availability of receptors to bind to and infect epithelial cells.\textsuperscript{35,36} Following attachment to one of these receptors on respiratory epithelial cells, the viral genome is released from the endosome into the cytoplasm, viral replication takes place and virus is subsequently released on cell lysis.

Epidemiological studies using RT-PCR for HRV detection suggest that children are the main reservoir for RV and that families or schools / day-care centres are the most common site for spread of infection. Pre-school age children have about six RV infections per year falling to 2-3 infections in adults. Infections can occur year-round but seasonal peaks are seen which relate to social behaviour rather than being a direct result of climate-related virus viability alone (discussed further in section 1.10).

1.9 Other Viruses Associated with Asthma Exacerbations

Influenza is a common infection during the winter months frequently reaching local or national epidemic proportions. Following the 2009 H1N1 influenza A pandemic, a number of studies highlighted asthma as an important co-morbid condition associated with markers of increased illness severity such as hospitalisation, admission to ICU, and mortality.\textsuperscript{37–39} Indeed, during the peak 2009 H1N1 flu season, children with asthma were infected almost twice as often with H1N1 compared with other respiratory viruses and experienced increased severity of cold symptoms with H1N1 compared with viruses.\textsuperscript{40} Vaccination against pandemic H1N1 influenza is therefore specifically recommended in asthmatics. It should however be noted that whilst several studies do demonstrate a reduction in the number of exacerbations following vaccination, other studies have not shown a clear benefit.\textsuperscript{41–44}
RSV is the main pathogen causing severe bronchiolitis in infants with most infections occurring between December and February each year. In infants and young children, differentiating between acute wheeze, bronchiolitis, post-bronchiolitis wheeze, and acute exacerbations of asthma is frequently difficult. Subsequently the interpretation of paediatric studies is complex and the prevalence of RSV can vary widely from study to study. In an Australian birth cohort study, RSV accounted for 16.8% (second behind RV) of cases of wheezy respiratory infections in the first year of life\textsuperscript{45} whereas a detection frequency of 27% was seen in a similar British study.\textsuperscript{46} RSV in older children and adults is much less frequent however it is seen in older adults where it is frequently an under recognised trigger in acute asthma. A study by Falsey et al demonstrated that 7.2% of hospitalisations for asthma in those over the age of 65 were associated with RSV infection.\textsuperscript{47}

Other respiratory tract viruses such as coronaviruses, human metapneumoviruses, parainfluenza viruses, adenoviruses, and bocaviruses, have all been detected in subjects with asthma exacerbations. However, in a recent epidemiologic study performed after the discovery of several new respiratory viruses such as bocavirus, the only virus type significantly associated with asthma exacerbations in children aged 2 to 17 years were rhinoviruses.\textsuperscript{48}

1.10 Seasonal Patterns of Exacerbations

Exacerbations of asthma are seasonal and the period of study should always be borne in mind when interpreting exacerbation aetiology. For example a study in infants carried out in September found no cases of influenza at all,\textsuperscript{49} whereas a proportion of 20% was seen in another study during the flu season.\textsuperscript{50}

RSV, metapneumoviruses, and influenza viruses (with the exception of 2009 H1N1) are usually limited to the winter and early spring. RV infections can occur throughout the year but are most common in the spring and autumn. In children, seasonal peaks in asthma exacerbations occur frequently in autumn corresponding to the weeks following the start of the new school term.\textsuperscript{49} This
phenomenon has been termed the ‘September epidemic’. Among older adolescents and young adults a similar albeit more blunted picture is seen with a peak occurring a week after the school age children. In older adults a peak is seen in December to January.

1.11 Virus-allergen interactions

All asthmatic children are infected with 1 or more strains of RV each year, yet only a proportion of infections are associated with severe exacerbations. This fact has led to the speculation that RV acts as a cofactor along with environmental allergens to initiate an exacerbation to an extent that neither alone can achieve. In 2002 Green reported that allergen sensitization, exposure to sensitising allergens, and respiratory virus infection acted in a synergistic manner to significantly increase the risk of hospitalisation in adults with acute asthma. Four years later, Murray observed even greater synergistic interaction in children. Individually, these factors did not increase the risk for hospital admission in asthmatic children. Simpson observed that most children classified as ‘atopic’ using conventional definitions were clustered into four distinct classes. Only one of these classes termed ‘multiple early sensitisation’ which comprised approximately a quarter of the ‘atopic’ children was significantly associated with risk of hospitalisation with asthma. This supports the idea that atopic status is on a spectrum of severity rather than simply a ‘yes’ or ‘no’ diagnosis. Olenec and co-workers sampled nasal secretions weekly from asthmatic children and noted that the relation between infection and subsequent illness varied with the presence of sensitisation. Specifically, greater upper and lower respiratory tract symptoms were seen in children with atopic asthma as opposed those with non-atopic asthma. Taken together these studies suggest atopic asthma is associated with more severe illness following virus infection than asthma in the absence of allergic sensitisation.

In contrast to the above findings and using the human experimental RV infection model, de Kluijver and co-workers recruited 36 house dust mite (HDM) allergic, asthmatic patients in a three-arm,
parallel, placebo-controlled, double-blind study. Patients inhaled a low dose of HDM allergen for 10 consecutive working days and/or were subsequently infected with RV16. Whilst both allergen exposure and RV infection individually resulted in a significant fall in FEV$_1$, no synergistic or additive effect on any of the clinical or inflammatory outcomes were seen.$^{55}$ These findings followed an earlier study by Avila and colleagues who were also unsuccessful in demonstrating allergen-virus synergism when using three high-dose nasal allergen challenges in the week preceding rhinovirus challenge. $^{56}$ It is possible that the two controlled studies failed to see evidence of synergism as neither continued allergen exposure during the viral infection - both studies having an interval of 3 days between the final allergen challenge and viral infection. Moreover, given the heterogeneous nature of asthma it is highly plausible that only a subgroup of highly atopic individuals demonstrate obvious allergen-virus synergy – a subgroup corresponding to the ‘multiple early sensitisation’ children described by Simpson.$^{53}$

1.12 Human Model of Experimental Rhinovirus Infection in Asthma

Experimental rhinovirus infection was introduced in humans prior to animal models due to the specificity of rhinovirus to the human species. It has only been since the development of a mouse model of RV infection by Bartlett and Walton $^{57}$ that this avenue has been open to researchers. Initially healthy subjects were inoculated, followed by atopic individuals and lastly by mild asthmatic volunteers. This model permits the study of a rhinovirus infection under controlled conditions, allows comparison of symptoms, airway physiology, lung function, airway inflammation, and use of asthma medication to name but a few. Its evolution to include the study of asthma has led to a significant increase in our understanding of the processes that underlie virus-induced exacerbations and offers a model with which new medications can be tested. Table 1 summarises the previous studies to have used the human model of experimental rhinovirus inoculation in asthma. The clinical outcome measures are then discussed more fully in the following sections.
Table 1. Studies using the human model of experimental rhinovirus inoculation in asthma

<table>
<thead>
<tr>
<th>Publication</th>
<th>Sample size</th>
<th>Endpoints</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemanske, JCI 1989 58</td>
<td>10 subjects with allergic rhinitis (non-asthmatic)</td>
<td>Airway hyperresponsiveness and late asthmatic reactions (LARs) to inhaled antigen</td>
<td>RV16 increases airway hyperresponsiveness and predisposes the allergic patient to develop LARs</td>
</tr>
<tr>
<td>Calhoun, JCI 1994 59</td>
<td>5 healthy, 7 subjects with allergic rhinitis (non-asthmatic)</td>
<td>Airway histamine release and eosinophil influx</td>
<td>RV-induced airway histamine release and eosinophilia in allergic but not healthy subjects</td>
</tr>
<tr>
<td>Cheung, AJRCCM 1995 50</td>
<td>14 mild asthmatic subjects</td>
<td>Airway hyperresponsiveness</td>
<td>RV16 increases airway hyperresponsiveness in asthma</td>
</tr>
<tr>
<td>Grunberg, AJRCCM 1997 61</td>
<td>13 mild asthmatic subjects</td>
<td>Airway hyperresponsiveness</td>
<td>RV16 increases airway hyperresponsiveness in asthma</td>
</tr>
<tr>
<td>Fleming, AJRCCM 1999 62</td>
<td>10 healthy, 11 mild asthmatic subjects</td>
<td>Cold score, spirometry, airway inflammatory cell counts</td>
<td>Similar clinical and inflammatory responses between asthmatic and healthy subjects</td>
</tr>
<tr>
<td>Gern, AJRCCM 2010 63</td>
<td>22 subjects with allergic rhinitis or mild asthma</td>
<td>Cytokine levels in sputum and nasal lavage; no clinical endpoints assessed</td>
<td>Increased RV16 induced IL-8 and G-CSF in atopic subjects</td>
</tr>
<tr>
<td>Grunberg, CEA 2000 35; AJRCCM 2001 64</td>
<td>25 mild asthmatic subjects</td>
<td>ICAM-1 expression; airway inflammation</td>
<td>Increased ICAM-1 expression with RV16; no effect of pre-treatment with steroids; no effect of steroids on RV-induced airway inflammation</td>
</tr>
<tr>
<td>Bardin, ERJ 2000 65</td>
<td>11 healthy, 5 atopic, 6 mild asthmatic subjects</td>
<td>Peak flow, Cold score</td>
<td>Greater falls in peak flow in asthmatics; no group difference in cold score</td>
</tr>
<tr>
<td>Jarjour, JACI 2000 66</td>
<td>8 mild asthmatic subjects</td>
<td>Airway inflammation</td>
<td>Increased IL-8, G-CSF and neutrophilia with RV16</td>
</tr>
<tr>
<td>Zambrano, JACI 2003 67</td>
<td>9 healthy, 16 mild asthmatic subjects</td>
<td>Symptom scores, Lung function, airway inflammation, IgE</td>
<td>Increased symptoms and reduced lung function in 'IgE high' asthmatics</td>
</tr>
<tr>
<td>Message, PNAS 2008 68</td>
<td>15 healthy, 10 mild asthmatic subjects</td>
<td>Symptom scores, lung function, airway inflammation</td>
<td>Greater symptom scores, falls in lung function and eosinophilia in asthma</td>
</tr>
<tr>
<td>DeMore, JACI 2009 69</td>
<td>18 healthy, 20 mild asthmatic subjects</td>
<td>Symptom scores, airway inflammation</td>
<td>Similar symptom scores and inflammatory responses; eosinophilia associated with worse colds</td>
</tr>
</tbody>
</table>
1.13 Effects of Virus Infection on Airway Hyperresponsiveness in Asthma

Airway hyperresponsiveness (AHR) is one of the cardinal features of asthma and can be defined as increased sensitivity of the small airways to bronchoconstriction in response to inhaled substances, such as histamine or methacholine. In a prospective study of asthmatic children experiencing naturally occurring URTIs, Xepapadaki observed increased AHR following a virus infection (of which 82% were RV) that persisted for between 5 and 11 weeks after the onset of the virus infection.\textsuperscript{70}

Several experimental infection studies with RV have confirmed increased virus-induced AHR. Cheung inoculated 7 atopic asthmatic volunteers with RV16 highlighting an increased response to methacholine on days 2, 7 and 15 compared to the non-infected asthmatics.\textsuperscript{60} Grunberg inoculated 19 mild asthmatics with RV-16 observing a decrease in histamine PC\textsubscript{20} at day 4, which was most pronounced in those with a severe cold.\textsuperscript{61} Message noted similar findings when a histamine challenge was repeated on day 6 post-inoculation. Interestingly, in a study of atopic non-asthmatics with active allergic rhinitis, Gern observed similar results to those seen in asthma indicating that allergy in the absence of asthma was sufficient to influence lower airway responses to rhinovirus.\textsuperscript{71}

These studies are consistent with the findings observed in animal models of RV, paramyxovirus, RSV, and influenza virus infections.\textsuperscript{57,72–74} In particular, RV-infected ovalbumin-sensitised mice showed significantly enhanced airway hyper-responsiveness on day 1 compared with that observed in mice infected with virus alone or mice challenged with allergen alone.\textsuperscript{57}

1.14 Symptoms during Experimental Rhinovirus Infection

The severity of upper respiratory symptoms that accompany RV infections varies greatly. Indeed as many as 20-30% of healthy subjects may not experience any significant symptoms at all during PCR positive infections whilst others may experience severe and prolonged colds. A highly variable clinical response to the common cold is also observed in asthma - both in prospective longitudinal
Using a dose of between 5,000-30,000 TCID\textsubscript{50} RV-16 (tissue culture infective dose; that amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated), Grunberg\textsuperscript{80}, Cheung\textsuperscript{60} and Message\textsuperscript{68} all observed a peak in cold symptoms on day 2-4 post-inoculation with symptoms returning to baseline levels within one week in most subjects. The peak of asthma symptoms was less consistent with Grunberg seeing a peak between days 2-4, Cheung recorded an increase in asthma symptoms on day 4 with a peak on days 6-11 whilst Message observed a peak between days 4-6. A return to baseline levels by day 13 was observed in all studies, with many subjects returning to normal levels within a week of inoculation.

Studies using the human model of RV infection have on the whole reported similar increases in upper respiratory tract (URT) symptoms in both healthy subjects and asthmatics. However in a study by Zambrano, a subgroup of ‘high’ IgE asthmatics did experience significantly higher cold scores than healthy controls ($P < 0.02$). A strong trend ($P = 0.07$) was seen for the remaining ‘low’ IgE asthmatics.\textsuperscript{67} This is one of the few studies to suggest that asthmatics may experience more severe colds than healthy people and was consistent with findings of Corne, who in a prospective study of cold transmission, identified couples for whom one partner had asthma and the other did not.\textsuperscript{76} He then observed an extended duration of illness in the asthmatic partner of each couple in question, although this did not reach statistical significance for cold symptoms.

No group has observed a significant increase in lower respiratory symptoms in healthy subjects, however, De Kluijver demonstrated a significant increase in bronchial epithelial ICAM-1 expression (in the absence of any change in inflammatory cell counts) following the inoculation of 12 nonatopic healthy subjects with RV-16. This indicates that even in healthy subjects, limited sub-clinical changes to the lower airway are seen.\textsuperscript{81}
1.15 The Effect of Experimental Rhinovirus Infection on FEV$_1$ and Peak Flow

Following the inoculation of 7 mild asthmatics with RV-16, Cheung did not observe any significant change in FEV$_1$ although a strong trend was observed (p = 0.06). Fleming inoculated 11 mild asthmatics who similarly did not experience significant falls in FEV$_1$ or PEF, although they did record a significant increase in asthma symptoms.$^{62}$ In contrast, Grunberg observed a significant decrease in FEV$_1$ in asthmatics after RV16 infection, reaching a minimum level 2 days post-inoculation,$^{80}$ whilst Bardin observed a significant fall in PEF from baseline in asthmatics on days 4-9.$^{65}$ More recently, Message confirmed these latter findings with a maximal fall in PEF noted on day 5 post-inoculation in asthmatic but not healthy subjects.$^{68}$ It is interesting to note that the mean baseline FEV$_1$ (% pred) for the asthmatic subjects in the Cheung and Fleming studies were greater (95% and 92% predicted respectively) than for the asthmatics in the Grunberg study (86% predicted) indicating that perhaps the baseline severity of the asthmatic subjects recruited to these studies influenced outcome to some degree. Indeed the 7 asthmatics recruited by Cheung were so well-controlled that only 4 of them required sporadic SABA for relief of symptoms – the remaining subjects were so mild that even occasional SABA was unnecessary.$^{60}$

1.16 The Immune Response to Rhinovirus Infection

Infection with respiratory viruses poses a unique challenge to the immune system: not only must the virus be promptly eliminated, but tissue inflammation must be regulated to prevent unwanted consequences which could lead to eventual respiratory failure. In addition during the recovery phase of infection, the immune system must help coordinate tissue repair to restore normal lung architecture and function and prevent chronic defects resulting from remodelling.
Proud and colleagues carried out a study to define changes in gene expression using nasal scrapes of healthy subjects infected with RV-16. This study reveals the true complexity of the immune response to rhinovirus infection: at 48 hours post-inoculation almost 12,000 gene transcripts were significantly altered. The most abundantly induced genes encoded chemokines, signalling molecules, interferon-responsive genes and antivirals. Only a tiny proportion of these have been measured at the protein level in vitro and even fewer have been measured in vivo during a naturally-occurring or experimental RV infection in man.

Infection with RV induces a cascade of inflammatory responses and many are believed to be responsible for the majority of common cold symptoms experienced. Healthy volunteers challenged intranasally with mediators known to be secreted during RV infection experience a range of symptoms including sneezing and coughing (prostaglandins D2 and F2α), rhinorrhea and a sore throat (bradykinins), and increased nasal resistance (IL-8/CXCL8). Nasal sampling during naturally occurring colds reveals further increases in a large number of other mediators including IFN-γ, IL-1β, IL-6, IL-10, TNF-α, RANTES, MPO, and ECP. A similar group of cytokines were also found to be increased in nasal lavage following experimental infection as well as following infection of bronchial epithelial cells with rhinovirus. Many of these mediators are then able to induce neutrophilic, lymphocytic, and eosinophilic inflammation, as well as airway hyperresponsiveness and airway remodelling.

One such mediator is IP-10/CXCL10 (IFN-γ-inducible protein 10) - a ligand for the CXCR3 receptor found on activated type 1 T lymphocytes and natural killer cells. Spurrell demonstrated induction of IP-10/CXCL10 both in vitro and in vivo following human experimental infection with RV-16. In addition IP-10/CXCL10 levels in nasal lavages correlated with symptom severity, viral titre, and numbers of lymphocytes in airway secretions indicating a role for IP-10/CXCL10 in the pathogenesis of RV-induced colds and exacerbations of asthma. Generation of IP-10/CXCL10 was shown to require virus capable of replication but was not dependent on prior induction of type 1 interferons.
and Proud have since demonstrated that RV-16-induced IP-10/CXCL10 production is however dependent upon the transcription factor IFN-regulatory factor (IRF)-1.\textsuperscript{93}

CXCL8/IL-8, a major chemoattractant for neutrophils is increased following virus infection and correlates with cold symptoms both in asthmatic children with community acquired viral URTI’s as well as following experimental inoculation with RV-16.\textsuperscript{66,69,95} These results are supported by a naturally-occurring cold study in adult asthmatics which found an association between exacerbations and greater sputum neutrophil counts,\textsuperscript{96} as well as by an experimental inoculation study reporting significant increases in BAL neutrophil counts in asthmatic subjects.\textsuperscript{66}

In addition, DeMore observed that more asthmatics had detectable eosinophils in nasal lavage and sputum samples during infection than healthy volunteers,\textsuperscript{69} whilst Message observed significantly increased BAL eosinophil numbers during RV16 infection with strong trends for increases in BAL lymphocytes ($P = 0.06$) and neutrophils ($P = 0.07$).\textsuperscript{68}

1.17 Rhinovirus-induced signalling in epithelial cells

A number of transcription factors play a role in RV-mediated events in epithelial cells including members of the nuclear factor KB (NF-kB), signal transducer and activator of transcription (STAT), and interferon regulatory factor (IRF) families.\textsuperscript{97}

NF-kB has been shown by several groups to be upstream of epithelial cell mediator release and lead to the synthesis of cytokines, chemokines, adhesion molecules, respiratory mucins, and growth factors. Greater levels of NF-kB p65 and p50 activation are seen in bronchial biopsy specimens, sputum cells, and cultured bronchial epithelial cells (BECs) from asthmatic subjects.\textsuperscript{98,99} Viral infection causes upregulation of a plethora of pro-inflammatory molecules in an NF-kB–dependent manner including the chemokines: GRO-\textalpha/CXCL1, ENA-78/CXCL5, IL-8/CXCL8, IP-10/CXCL10, RANTES/CCL5, and eotaxin/CCL11;\textsuperscript{89,90,92,100–103} various cytokines and growth factors including IL-6, IL-
11, GM-CSF, fibroblast growth factor (FGF) -2, and vascular endothelial growth factor (VEGF);\textsuperscript{104–106} as well as respiratory mucins including MUC5AC and MUC5B.\textsuperscript{107–109}

Data from Chen et al supports a role for the Janus kinase (JAK)/STAT signalling pathway in RV-induced signalling, observing RV-16 induction of STAT-1 phosphorylation, and attenuation of RV-induced gene expression from human tracheobronchial epithelial cells following pre-incubation with a JAK1 inhibitor.\textsuperscript{110}

1.18 Evidence for Impaired Anti-Viral Responses in Asthma

The possibility that asthmatics have an abnormal antiviral response that predisposes them to greater virus-induced morbidity and lower airway involvement than healthy subjects has been investigated in a series of studies. The study by Corne et al\textsuperscript{76} was the first to suggest that there might be some inherent differences in the way that asthmatic subjects respond to respiratory tract viral infections. Wark et al\textsuperscript{111} subsequently provided a mechanistic insight into why this might be the case with studies involving cultured BECs from asthmatic and healthy subjects obtained at bronchoscopy, followed by infection with rhinovirus \textit{in vitro}. Although rhinovirus induction of the proinflammatory cytokine IL-6 and RANTES/CCL5 was not different between the 2 groups, asthmatic BECs produced lower levels of the type I IFN-β and also had higher levels of rhinovirus replication. Importantly, deficient IFN-β expression was observed in both steroid-treated and steroid-naive asthmatic subjects. The asthmatic BECs responded to exogenous treatment with IFN-β, exhibiting reduced rhinovirus release and demonstrating that the deficiency in asthmatic cells was associated with production of antiviral IFN-β rather than the actions of IFN-β.

Contoli et al\textsuperscript{112} provided further evidence of the importance of antiviral IFN’s using the human experimental infection model. Not only did bronchoalveolar lavage (BAL) cells from asthmatic subjects have lower levels of rhinovirus and LPS-induced type III IFN-λ, but deficient IFN-λ in asthmatic cells was related to the pathogenesis of asthma exacerbations \textit{in vivo}, with abundance of
IFN-λ being negatively correlated with airway symptom scores, changes in lung function, virus load, and markers of inflammation in vivo. Several other studies have also reported evidence of a deficient anti-viral response in asthma including a recent study by Sykes that demonstrated delayed and deficient RV induction of type I (α and β) IFNs in BAL cells of asthmatic subjects.  

However, not all investigators have been able to reproduce these findings with at least two groups being unable to confirm deficient epithelial cell responses in asthma. In addition, no study has as yet been able to demonstrate IFN delay or deficiency and/or increased virus shedding in vivo.  

At present, the mechanisms behind impaired interferon production in asthmatic subjects remain unknown and current data supporting a role for this in asthma is at the in-vitro level only. The measurement of IFN-α, -β, and -λ reported in this thesis represents the first attempt to define this impairment in asthma in-vivo. 

1.19 Relationship between exacerbations, airway remodelling and lung function decline  

Asthma is associated with accelerated loss of lung function compared to healthy subjects with patients developing varying degrees of irreversible airflow obstruction. This decline in lung function is believed to be secondary to airway remodelling – a feature of persistent asthma characterised by increased smooth muscle, subepithelial fibrosis and altered deposition of extracellular matrix (ECM). It is thought to involve repetitive epithelial injury on a background of chronic airway inflammation, a hypothesis supported by the results of a study by Bai et al highlighting that asthmatics who experienced at least one severe exacerbation had accelerated loss of lung function (30mL greater annual decline in FEV₁) compared with those who did not have any exacerbations.  

Bronchial biopsies from asthmatics have shown both RV protein and RNA in the submucosal layers, suggesting that limited infection of fibroblasts or other submucosal cells does occur and raising the possibility that viral infections contribute to remodelling processes in asthma. Kuo recently demonstrated increased deposition of the ECM protein, perlecan, and collagen V following RV
infection of human BECs, \(^{116}\) whilst Leigh demonstrated that RV infection of cultured epithelial cells led to upregulation of amphiregulin, activin A, and vascular endothelial growth factor (VEGF) protein levels.\(^{120}\) These three mediators have all been strongly implicated in the remodelling process: amphiregulin, a member of the epidermal growth factor (EGF) family, is released in response to airway epithelial injury and engages EGF receptors to drive the altered repair process that is characteristic of airway remodelling.\(^{121}\) Activin A is a member of the TGF (transforming growth factor)-\(\beta\) superfamily and has been implicated in the subepithelial fibrosis characteristic of airway remodelling.\(^{122,123}\) Expression of VEGF and its receptors is increased in asthma and VEGF is the major proangiogenic activity in asthmatic airways.\(^{124,125}\) Increases in the size and number of airway wall blood vessels are characteristic of airway remodelling even in mild asthma\(^{126}\) and overexpression of VEGF in the airways of mice has been shown to result in both vascular and airway remodelling.\(^{127}\) Moreover nasal lavage samples from subjects with confirmed natural RV infections showed significantly higher VEGF protein levels during peak cold symptoms compared with both baseline levels and with control levels.\(^{120}\)

It is possible that RV gains access to underlying fibroblasts through the epithelial disruption that occurs in asthmatic airways. This susceptibility may then allow RV replication in the lower airways to develop, especially in asthmatic subjects where there is reduced protection offered by epithelial derived IFNs. Bedke et al. infected normal and asthmatic airway fibroblasts \textit{in vitro} with RV1B illustrating both the ability of fibroblasts to support viral replication and their vigorous proinflammatory response with induction of IL-6 and IL-8.\(^{128}\) This latter response compounds the existing airway inflammation to contribute to an exacerbation. Furthermore, the study demonstrated that exogenous IFN-\(\beta\) protects fibroblasts against infection, providing further support of its potential use as a therapy against virus-induced asthma exacerbations.

TGF-\(\beta\) is a key regulator of wound repair and healing, and increased TGF-\(\beta\) and TGF-\(\beta\) mRNA in BAL and in endobronchial biopsies respectively have been reported in patients with
Thomas demonstrated that RV replication is enhanced in TGF-β1–treated fibroblasts and fibroblasts from patients with asthma, and this is linked to deficient type I IFN responses. TGF-β may therefore act as an endogenous immunosuppressant promoting virus replication during the evolution of virus-induced asthma exacerbations.131

Interestingly, reduced FEV₁ has been shown to be an important risk factor itself for multiple, severe asthma exacerbations.132,133 This may be because exacerbations cause the remodelling processes that result in this loss, or alternatively it may follow certain host factors that make both exacerbations and loss of lung function more likely.134

1.20 Type 2 Inflammation in Asthma

The role of type 2 inflammation during rhinovirus-induced exacerbations was one of the main focuses of this study. Measurement of a large number of mediators known to be important to this pathway was made in-vivo during infection including IL-4, IL-5, IL-13, IL-25, IL-33, TSLP, TARC/CCL17 and MDC/CCL22. This inflammatory pathway is therefore reviewed in detail below.

The importance of Th2 cytokines to asthma pathogenesis is well established and the notion that upregulation of Th2 cytokines is critical for the allergic inflammation associated with asthma is over 20 years old now.135,136 Bronchial biopsies from both atopic and non-atopic asthmatics are characterised by accumulation of CD4+ T lymphocytes producing Th2 cytokines as well as eosinophils and mast cells in the epithelium and lamina propria.136–138 IL-5 mediates the growth, differentiation, recruitment, activation, and survival of eosinophils, whereas IL-13 has multiple effects including smooth muscle changes relating to hyperresponsiveness, differentiation of mucus-secreting goblet cells, induction of chemokines, alternative macrophage activation, and promotion of a pro-fibrogenic stromal environment.139

The importance of the Th2 axis in driving both eosinophilic airway inflammation and bronchial hyperreactivity has been highlighted using animal models in which the individual Th2 cytokines have
been knocked out.\textsuperscript{140} In humans, the development and administration of humanised monoclonal antibodies (mAb) against the Th2 cytokines have provided further insights: Mepolizumab - a mAb against IL-5 was shown to be effective in two proof-of-concept studies reducing the frequency of severe exacerbations in patients with severe refractory eosinophilic asthma.\textsuperscript{141,142} In the much larger multi-centre DREAM trial, Pavord \textit{et al} also showed a significant reduction in exacerbations in patients with eosinophilic inflammation.\textsuperscript{143} Moreover, a recent trial of lebrikizumab (anti IL-13 mAb) demonstrated a 60\% reduction in the rate of exacerbations in a ‘high-Th2’ subgroup of asthmatics compared to the placebo group.\textsuperscript{144} The cause of the exacerbations in these studies is not known, however it is reasonable to assume that their aetiology reflects other studies showing a majority to be triggered by viruses.

What these studies make overly apparent is that targeted therapies need an equally targeted group of patients as only about 50\% of asthmatics appear to have measurable Th2 inflammation,\textsuperscript{145} and earlier trials of mepolizumab in a more heterogeneous group of subjects failed to show the expected improvements in outcome.\textsuperscript{146}

\textbf{1.21 Type 2 Inflammation in Virus-Induced Asthma Exacerbations}

In airway sputum cells, Gern observed that an increased Th1 response (defined by the ratio of IFN-\textgreek{y}:IL-5 mRNA) was associated with more mild colds and more rapid clearance of virus.\textsuperscript{63} This implied that an increased Th2 response would result in the reverse scenario, i.e. more severe virus-induced morbidity. In support of this, Message found that IL-4, IL-5, and IL-13 production by BAL T cells were all associated with more severe virus-induced asthma symptoms following subsequent infection (ex-vivo).\textsuperscript{68} In addition, Bartlett & Walton observed significant induction of IL-4 (day 1) and IL-13 (days 1 and 2) expression in rhinovirus infected, ova-sensitised mice compared with ova-sensitised controls using the mouse model of RV-induced exacerbations.\textsuperscript{57} Interestingly, of the 11 mild asthmatics inoculated with RV16 by Fleming, only 2 had measurable IL-5 in nasal lavage specimens - these two asthmatics experienced the most intense cold symptoms.\textsuperscript{62}
When these results are considered in the light of the recent mepolizumab and lebrikizumab trials, augmented type 2 responses appear likely to have a role in the pathogenesis of virus-induced exacerbations.

1.22 The Airway Epithelium as a Regulator of Type 2 Responses in Asthma

The bronchial epithelium provides a barrier between the microbe-rich outside world and the internal parenchyma. However, it is far more than simply an inert barrier and is immunologically active, playing a pivotal role in both innate and adaptive immunity.

Our understanding of the relationship between the airway epithelium and Th2 inflammation comes largely from the response to allergen and relatively little has been added to the literature from studies with viruses. Current concepts of epithelial regulation of Th2 inflammation emphasise the release of several epithelial derived cytokines: IL-25, IL-33, thymic stromal lymphopoietin (TSLP), as well as CC family chemokines as key proximal events. These mediators are discussed below with a particular emphasis on IL-33 – a cytokine of specific interest in this study.

1.23 Interleukin-33

IL-33 is a member of the IL-1 family of cytokines and signals through the ST2 receptor. It is expressed by several cell types most notably epithelial and endothelial cells and it can activate numerous other cells including Th2 cells, innate lymphoid cells, basophils, mast cells and eosinophils. The proposed role of IL-33 is as an alarmin with necrosis believed to be the principle way in which it is released from cells. The fact that full-length IL-33 is biologically active and does not require proteolytic cleavage for activation supports this role. However it seems increasingly likely that necrosis is not the only route to release. Indeed, Kouzaki et al recently demonstrated IL-33 release from epithelial cells exposed to the environmental allergen Alternaria alternata. This noncytolytic ‘stress’ did not lead to compromised cell membrane integrity even following high levels of IL-33 release.
Whilst the ST2 receptor is stably expressed on Th2 cells, it is not expressed on Th1 cells. This finding is supported by early experiments performed by Schmitz et al. demonstrating that Th2 but not Th1 cells from C57BL/6 mice respond to IL-33 with secretion of IL-5 and IL-13. This and other studies have led to IL-33 being described as a potent inducer of Th2 responses. It is important to note that Th2 cells are not the only source for the archetypal Th2 cytokines IL-4, IL-5 and IL-13. For example, basophils have been shown to release IL-4 and IL-13 following activation by IL-33, and the newly identified innate lymphoid cells also express ST2 and produce IL-5 and IL-13 in response to IL-33. In fact these lineage negative cells may be a principal cellular source of IL-5 and IL-13 and a previously unrecognised effector cell in asthma. Furthermore IL-33 activates dendritic cells (DCs) to produce the Th2 chemokine TARC/CCL17 as well as priming naïve T cells to produce IL-5 and IL-13. Taken together IL-33 is likely to be important for both innate and adaptive type 2 responses.

Yet IL-33 is a multi-faceted cytokine having far reaching effects beyond simply promotion of type 2 inflammation. A range of pro-inflammatory cytokines including TNF, IL-1β and IL-6 are induced following IL-33 activation of DCs, and recent studies have also shown that IL-33 appears to be the sole danger signal responsible for alarming mast cells of cell injury with subsequent release of similar pro-inflammatory mediators by mast cells as described above for DC’s. Furthermore, additional studies demonstrate both iNKT cells and NK cell production of IFN-γ in the presence of IL-33 illustrating that under certain conditions IL-33 can also promote Th1-type responses. IL-33 may therefore represent a general amplifier of inflammation with the outcome dependent on the local cellular and extracellular context.

1.24 Interleukin-33 and Asthma

In a genome-wide association study (GWAS) including over 10,000 asthmatics, a single-nucleotide polymorphism in the IL-33 gene, as well as IL18R1/ST2 (the IL-33 receptor) were two of the very few genes significantly associated with asthma. A second GWAS study published soon after delivered
similar findings. Elevated IL-33 mRNA and protein levels have been demonstrated in bronchial biopsies and bronchoalveolar lavage fluid respectively from severe asthmatics compared to healthy subjects. Moreover, IL-33 deficient mice sensitized and challenged with ovalbumin exhibit an attenuated metacholine response and reduced airway inflammation compared to wild type mice. Similar results were seen with an anti-ST2 blocking antibody or when ST2 deficient mice were studied.

1.25 Interleukin-33 and Virus Infections

Currently there is no human in vivo data suggesting a role for IL-33 in virus-induced asthma exacerbations. However, utilising a mouse model of RSV infection, Walzl demonstrated that monoclonal anti-ST2 treatment reduced lung inflammation and illness severity in mice with Th2 (but not Th1) immunopathology. A more recent French study using influenza A highlighted a significant induction of IL-33 in influenza-infected mouse lungs, both at the mRNA and protein level, whilst infection of a human lung epithelial cell line (A549) also led to increased expression of IL-33. A study published at the same time by Chang et al also using a mouse model of Influenza A infection demonstrated influenza-induced AHR independently of Th2 cells and adaptive immunity. Instead, influenza infection induced AHR through a previously unknown pathway that required the IL-13-IL-33 axis and innate lymphoid cells. Infection with influenza resulted in much more production of IL-33 by alveolar macrophages, which in turn activated natural helper cells producing substantial IL-13. Although IL-33 production following rhinovirus infection of BECs has been observed, this has not yet been shown in human in vivo studies.

1.26 Interleukin-25

IL-25 (also known as IL-17E) is a member of the IL-17 family and is produced by airway epithelial cells, alveolar macrophages, T cells, mast cells, basophils and eosinophils. Elevated expression of IL-25 and IL-25R transcripts are observed in asthmatic lung tissues. In mice, exogenous
administration of IL-25 or transgenic expression induces asthma-like features with mucus production and airway infiltration of macrophages and eosinophils.\textsuperscript{169,170} Blockade of IL-25 conversely reduces the airway inflammation and Th2 cytokine production.\textsuperscript{171} IL-25 induction by rhinovirus infection of BECs has been demonstrated,\textsuperscript{167} however, no in vivo human data is currently published.

\textbf{1.27 Thymic stromal lymphopoietin}

Thymic stromal lymphopoietin (TSLP) is an epithelial derived cytokine released in response to TLR-3 stimulation, inflammatory cytokines, IL-13 and bacterial peptidoglycan.\textsuperscript{172,173} Its overexpression in mice leads to asthma-type Th2-driven airway changes, whereas its inhibition attenuates the airway changes seen in mouse models of asthma.\textsuperscript{174} It is increased in bronchial biopsies as well as sputum from asthmatics and particularly subjects with severe disease.\textsuperscript{175–177} TSLP up-regulates OX40 ligand on DC’s, which then prime naïve CD4+ T cells for Th2 cell differentiation.\textsuperscript{178}

Interestingly, although viral nucleic acid analogs and inflammatory cytokines associated with viral infection are potent inducers of TSLP, whether or not TSLP plays a functional role during antiviral immune responses remains unclear. Yadava \textit{et al} has recently shown that influenza A induced TSLP five days after infection, suggesting TSLP may not regulate the early innate response to infection but rather adaptive immunity. In keeping with this, the early kinetic of viral propagation was unaffected in infected mice deficient in the TSLP receptor (TSLPR).\textsuperscript{179} TSLP was seen to play a role in viral clearance at later time-points, and the virus-specific CD8+ T cell response in the lung was reduced in the absence of TSLPR.

Using cultured human airway epithelial cells, Han showed increased production of TSLP by rhinovirus with a further increase seen following co-treatment with IL-4.\textsuperscript{167} Uller and colleagues also showed increased TSLP in asthmatic BECs this time in response to dsRNA (a surrogate of viral infection) whilst simultaneously observing lower expression of IFN-β.\textsuperscript{180} This dual abnormality of BECs from subjects with asthma was significantly different from non-asthmatic BECS and highlighted an
abnormal mediator profile that biases towards a Th2 immune response. There are currently no in vivo reports supporting these findings.

1.28 Th2 cell trafficking – the roles of CCL17, CCL22 and PGD₂.

Th2 cells are programmed during lineage differentiation to selectively express the chemokine receptors CCR4 and CCR8 as well as the prostaglandin D2 chemoattractant receptor DP2 or CRTH2. TARC/CCL17 and MDC/CCL22 are the two dominant chemokines involved in the recruitment of Th2 cells and are ligands for the CCR4 receptor. They are produced by epithelial cells, mast cells and dendritic cells and are increased following allergen challenge of mild asthmatics. It is not known whether these chemokines are induced during a virus-induced exacerbation in man however TARC/CCL17 has been shown to be increased in a mouse model of RSV infection with considerably more TARC/CCL17 induction observed in Th2-biased animals.

Another Th2 chemoattractant is PGD₂ – considered the major prostanoid released by mast cells and implicated in allergic diseases for the past 30 years. Airway mast cells are innate cells that play an essential role in the early phase recruitment of T cells and initiation of the asthmatic response. In humans, increases in nasal epithelial mast cells in allergic rhinitis and after an allergen challenge have been reported. They are a source of a range of cytokines and chemokines including I-309/CCL1, TARC/CCL17 and MDC/CCL22.

PGD₂ binds to the receptors GPCRs DP1 and DP2 - also known as CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) – which is expressed by human Th2 cells. PGD₂ is also produced in significant amounts by dendritic cells, eosinophils, macrophages, Th2 cells and endothelial cells and is the major mast cell product released during anaphylaxis. Increasing evidence is accumulating that it has a central role in the initiation and perpetuation of allergic inflammation. In a trial of a CRTH2 antagonist, Barnes et al recently provided the first clinical evidence that CRTH2 receptors contribute to airflow limitation, symptoms and eosinophilic
inflammation in asthma. In addition, in a large study of asthmatics in the Severe Asthma Research Program (SARP), increased BAL fluid PGD$_2$ was associated with a history of a recent asthma exacerbation and levels were found to be stronger predictors of severe asthma than FEV$_1$% predicted.

1.29 Biomarkers of type 2 inflammation

The measurement of biomarkers has been incorporated into studies of asthma to characterise the patient population and attempt to associate the disease process with therapeutic interventions. The emphasis on biomarkers has steadily increased in recent years following accumulating evidence of the existence of several distinct clinical and pathological asthma phenotypes marked by differing responses to therapy. A failure to recognise and discover the underlying mechanisms of these different asthma subtypes is considered by many to be a major limitation to progress. The need for personalised treatment programs is most clearly illustrated by the limited success of monoclonal antibodies targeting Th2 inflammation in an unselected population of asthmatics of which many have minimal evidence of active Th2 inflammation.

Some of the results presented in chapter 6 suggest that the novel sampling method we have utilised has the potential to identify asthmatics with significant type 2 inflammation by directly measuring the cytokine in question rather than relying on a biomarker of it. In view of this, the commonly used asthma biomarkers are briefly reviewed below.

1.30 IgE

The quantity of total serum IgE and presence of allergen-specific IgE are both important biomarkers for defining the atopic phenotype. However there is considerable overlap in serum IgE levels between atopic and nonatopic populations, which reduces its utility in identifying atopy. In vivo measurements of allergen-specific IgE in the skin (e.g. skin prick tests) are an alternative to serologic assays and appear to be the most commonly used tool for defining the presence or absence of atopy in clinical studies.
1.31 Eosinophils

Airway eosinophilia is an inflammatory characteristic of an asthma phenotype known to be responsive to corticosteroid treatment in many (but not all) asthmatics and has been used as a surrogate marker for the presence of Th2 inflammation due to the influence IL-5 has on eosinophil maturation, recruitment to the airways, and survival. Sputum cell counts are a well-validated method for assessing cellular inflammation of the airways and the concordance correlation coefficient for the 1-week repeatability of sputum eosinophils has been reported to be 0.82 (95%CI, 0.72-0.88). However a more recent study from the same group highlighted that almost a third of mild-to-moderate asthmatics had eosinophilia on only an intermittent basis following analysis of repeated sputum inductions.

Both sputum and peripheral blood eosinophils have been shown to inversely correlate with pulmonary function in asthma but neither accounts for all the variability in lung function measures. In fact it is notable that trials of Mepolizumab remove eosinophils without any effect on FEV1. It is also interesting to note that peripheral blood eosinophils cannot consistently be relied upon to identify eosinophilic asthma phenotypes, and no differences have been observed in the numbers of blood eosinophils between asthmatic patients with elevated IgE and those without elevated levels of IgE antibodies. Despite these inconsistencies, tailoring of asthma treatment based on sputum eosinophils is effective in decreasing asthma exacerbations. However the technical expertise required to perform it correctly makes it unsuitable for routine clinical practice.

1.32 Fractional Exhaled nitric oxide

Measuring fractional exhaled nitric oxide is simple to perform, offers an immediate result and has been regarded as an indirect marker for airway inflammation. However, values overlap greatly among healthy, atopic, and asthmatic persons (levels < 25ppb generally indicate a lower likelihood for eosinophilic inflammation and responsiveness to corticosteroids) and the sensitivity and specificity of FeNO for sputum eosinophilia is only approximately 70%. It has been proposed as a
possible tool to tailor asthma treatment however a recent systematic review and meta-analysis regarding its use in this context showed it not to be effective and concluded that there is insufficient justification to advocate its use in everyday clinical practice.\textsuperscript{197}

1.33 Periostin

Woodruff and colleagues have previously shown that 3 of the most differentially expressed bronchial epithelial genes between asthmatic and healthy subjects are periostin, chloride-channel accessory protein 1 (CLCA1), and SERPINB2. All 3 genes are induced in bronchial epithelial cells by recombinant IL-13 treatment\textsuperscript{199} and the presence or absence of coordinated high expression levels of these genes appear to act as a Th2 gene signature. This signature correlates with IL-5 and IL-13 expression in the bronchial mucosa as well as airway eosinophilia but not atopy. Subsequently an assay for periostin protein in peripheral blood has been developed and has recently been shown to be significantly increased in asthmatic patients with evidence of eosinophilic airway inflammation relative to those with minimal eosinophilia.\textsuperscript{200} In a separate trial periostin was used as a blood biomarker of Th2-high asthma and shown to be predictive of a positive effect of the anti-IL-13 mAb, Lebrikizumab.\textsuperscript{144}

1.34 Sampling airway mucosal lining fluid

One of the novel components of this study is the inclusion of a sampling technique to measure mediators in mucosal lining fluid (MLF). This method of sampling upper airway MLF uses a synthetic absorptive matrix (SAM) (Accuwick Ultra, SPR0730; Pall Life Sciences, USA) and was first described by Chawes.\textsuperscript{201} The SAM is a synthetic, fibrous, hydroxylated polyester medium designed for sample collection, storage and conjugate release. The material is hydrophilic with low biomolecular binding and protein-containing samples are stable during storage at -80\degree C. It is highly absorbent and the fibre surfaces have been modified to enhance water wettability.

The minimally invasive technique of sampling MLF using a SAM has been termed 'nasosorption' and
offers potential benefits over nasal lavage including improved sensitivity and sampling accuracy by overcoming the variable dilution of analytes associated with nasal lavage. This dilution often means many mediators of interest are diluted below the limits of detection of available assays.

Using nasosorption to sample nasal mucosal lining fluid of children with and without allergic rhinitis Chawes et al. highlighted elevated levels of IL-5 and IL-13 in the atopic children compared to healthy control subjects. In total 12 out of the 18 mediators measured were detectable in approximately two thirds of nasosorption samples analysed. Nicholson et al. used nasosorption following nasal allergen challenge with timothy grass pollen to assess the effect of anti-IL-13 therapy on nasal IL-13 levels and symptoms in hay fever patients out of season, whilst Folsgaard et al. more recently hypothesised that the atopic status of an infant’s parents had a differential effect on the nasal mucosal cytokine and chemokine profile at 1 month of age. In this large study of over 300 healthy infants, nasosorption permitted the measurement of 18 cytokines and chemokines and led the authors to define specific cytokine signatures in children with atopic parents. All three studies used Accuwick Ultra which unfortunately is no longer manufactured by Pall. The first section of the methods and results chapters discusses the experiments that I undertook to both firstly validate this sampling method and secondly find an alternative to Accuwick for sampling both upper and lower MLF in this study.

1.35 Sampling bronchial mucosal lining fluid

Bronchoalveolar lavage is an extremely useful sampling method in respiratory medicine and is used in a wide range of contexts most frequently as an aid to diagnosis. In respiratory research its use is also widespread both in human and animal models. However the same problems encountered with nasal lavage are often even more challenging with BAL and until now have been almost impossible to avoid. Most notably the variable dilution and recovery of BAL following instillation of large volumes of saline means that accurate comparisons of protein (or pathogen) levels taken at different time points or from different patient groups is difficult to achieve.
In an attempt to overcome this, Akiotishi Ishizaka (Keio University, Tokyo, Japan) in collaboration with Olympus developed a bronchial microsampling device (BC-401C, Olympus Co, Tokyo, Japan). Their probe consists of a 2.8mm outer-diameter polyethylene sheath and an inner 1.2mm cotton probe attached to a stainless steel guide wire (figure 1.1). This is inserted into the lungs through a 3.2mm diameter channel of a fibre optic bronchoscope and permits mucosal lining fluid to be sampled without the dilution and recovery issues inherent with BAL.

![Figure 1.1 Bronchial microsampling device manufactured by Olympus, Japan.](image)


In 2001 its successful use was first described in patients with acute respiratory distress syndrome (ARDS). Since then it has been utilised in a variety of settings including measurement of drug concentrations, detection of pathogenic bacteria, assessing the effects of smoking, and most recently as a tool in the early detection of lung cancer. However, unfortunately the Olympus bronchial microsampling device is only available to research groups located within Japan and has never been made available either for research purposes or commercially to clinicians in the UK. Despite our best efforts, we were unable to obtain any devices at Imperial College and so began the process of developing our own bronchoscopic sampling device. Dr Trevor Hansel has termed the
procedure ‘bronchosorption’ following his development of nasosorption. Publications using the Olympus device refer to their technique as bronchial microsampling.

1.36 The Nose and the United Airways Concept

The respiratory tract can be divided anatomically into the upper (nose, mouth and pharynx) and lower (trachea, bronchi and lungs) respiratory tract with the lymphoid tissue of Waldeyer’s ring representing the rather arbitrary line of demarcation. However it is also reasonable to consider the whole respiratory tract as a single entity, covered by ciliated epithelium and mucinous glands as far down as the smallest bronchi. As early as the 19th century a German scientist called Weber noticed a remarkable resemblance between nasal and bronchial mucosa. Since then many epidemiological studies have confirmed a link between asthma and allergic rhinitis (AR) with these conditions often coexisting in adults and children alike. Indeed rhinitis is present in >80% of patients with allergic asthma whilst patients with rhinitis are 3 times more likely to develop asthma than healthy control subjects. In a recent cross-sectional survey to determine the impact of AR on asthma control in children De Groot et al. found an ACQ ≥1.0 significantly more likely in children with AR than in those without (OR 2.74, 95% CI 1.28 to 5.91, p = 0.008). Both asthma and allergic rhinitis are characterised by chronic airway inflammation with eosinophils and T lymphocytes the predominant cells, and both are provoked by similar triggers including allergen exposure, viral infections, air pollution, and cold air. However, it is important to note that there are also clear differences between asthma and AR. In the first instance, bronchial obstruction is associated with smooth muscle contraction, whereas nasal obstruction is mostly secondary to capillary vessel dilatation. Secondly, collagen deposition is a feature of asthma but is not typically seen in AR.

Using nasal allergen challenge (NAC) as well as bronchial challenge Braunstahl et al. performed a series of experiments to address the question of whether the observed upper and lower airway similarities were due to generalised allergen exposure or due to nasobronchial interaction after local
exposure. In the first such study 9 subjects with AR sensitized to grass pollen and an equal number of healthy controls underwent NAC. This resulted in increased bronchial symptoms, lower PEF and increased eosinophils in subjects with AR. Using segmental bronchial challenge, the reversed experiment showed similar findings in the nose.

The precise mechanism(s) underlying the united airways concept are still debated however an increasing body of data suggests that the systemic circulation plays a role. In particular, IL-5 along with eosinophils are increased in peripheral blood following nasal and bronchial allergen provocation and may migrate not only to the challenged mucosa but further afield as well. To date, the relationship between cytokine production in the nose and lung during a rhinovirus infection in man has not been studied. However many studies have sampled the nose during experimental and naturally occurring infections, assuming in the process that this compartment provides an insight into pulmonary inflammatory and infectious mechanisms. The simultaneous sampling of the upper and lower respiratory tract performed in the current study has for the first time permitted an accurate assessment of this assumption.

1.37 Statement of Hypotheses

1. Moderately-severe and poorly-controlled asthmatics experience more severe lower respiratory symptoms and lung function impairments than mild and well-controlled asthmatics and during RV infection.

2. Sampling nasal and bronchial mucosal lining fluid with the techniques of nasosorption and bronchosorption will allow measurement of mediators that are below limits of detection in nasal and bronchoalveolar lavage.

3. The differences in outcome between asthma and healthy subjects relate to impaired type 1 and/or augmented type 2 inflammation.
1.38 Aims of Study

1. To develop a human RV challenge model of moderately-severe asthmatics with a range of baseline asthma control levels to provide insights into the roles that asthma severity and asthma control play in determining the outcome of a rhinovirus infection in asthma.

2. To develop a bronchial sampling device to permit measurement of mediators in bronchial mucosal lining fluid and to compare this technique with bronchoalveolar lavage sampling.

3. To sample nasal mucosal lining fluid for protein measurement over the course of infection

4. To determine whether there is evidence of deficient or dysregulated production of type 1 cytokines in asthma during a rhinovirus-induced exacerbation in vivo.

5. To determine whether there is evidence of augmented production of type 2 mediators in asthma during a rhinovirus-induced exacerbation in vivo.

6. To determine whether any of these deficiencies or augmented productions are related to symptom severity, virus load, lung function or airway inflammation in vivo.

7. To compare the immune response in the nose to the response in the lung during infection.
CHAPTER 2
MATERIALS AND METHODS

2.1 Validation of synthetic absorptive matrices for sampling airway mucosal lining fluid

Sampling airway mucosal lining fluid (MLF) using a synthetic absorptive matrix (SAM) aims to overcome the problems of mediator dilution and variable return of lavage fluid inherent to nasal and bronchoalveolar lavage sampling. Accuwick Ultra (catalog no. SPR0730; Pall Life Sciences, Ann Arbor, Mich), a synthetic, fibrous, hydroxylated polyester medium is one such SAM designed for sample collection, storage, and conjugate release.

At the start of this study in April 2009 Accuwick was commercially available in sheets 8 inches x 10 inches which was then cut into small strips by Parafix Tapes & Conversions Ltd (UK) for the purposes of this study. However, despite the apparent success of Accuwick in previously published studies it had not yet been properly validated. The experiments described below aimed to answer the following questions:

1. What were the cytokine recovery capabilities of Accuwick Ultra?
2. Did the recovery differ from one cytokine to another?
3. Was recovery reproducible?
4. Was there a more suitable SAM to Accuwick?

2.1.1 Assessment of Accuwick Ultra

Strips of Accuwick approximately 3cm x 0.5cm were spiked with 70µL of known concentrations of Meso Scale Device (MSD) protein standards (see section 2.18 for full details of the MSD platform). A pro-inflammatory 4-plex MSD plate was used measuring the following cytokines: IFNγ, IL-1β, IL-6, TNFα.
In total, 38 strips of Accuwick Ultra were spiked with the following concentrations: 2.4pg/mL (n=6), 9.8pg/mL (n=6), 39pg/mL (n=6), 156pg/mL (n=6), 625pg/mL (n=6), and 2500pg/mL (n=8).

These were then spun using a microcentrifuge filter system to isolate the spiked matrix. A volume of 70µL was chosen to ensure that a minimum of 50 µL (enough for 2 replicates on the MSD plate per strip of Accuwick) was recovered. The standard curve ran from 2500pg to 0.15pg using an eight point dilution series.

2.1.2 Comparison of absorptive matrices

A sputum supernatant from an individual with COPD (courtesy of Dr Joseph Footitt’s study, ethics approval 07/H0712/138) acted as the test sample for comparison of Accuwick Ultra with a second matrix: Whatman’s filter paper (GE Healthcare, UK). The Whatman’s paper was cut into similar sized strips to the Accuwick and spiked in an identical fashion to the Accuwick. In this instance a 7-plex pro-inflammatory plate was utilised allowing measurement of a wider range of cytokines namely: IFN-γ, IL-10, IL-12 p70, IL-6, IL-8 and TNFα.

To ensure that the cytokines were not being lost in transit through the spin filter itself, 40µl of the sputum supernatant was also spiked onto the spin filter directly without first being spiked onto the SAMs. The recoveries were then compared with the concentrations obtained following direct application of the sample to the well of the MSD plate (i.e. a neat sample).

Following discussion with Pall, a third SAM: ‘Leukosorb’ (Pall) was tested against Accuwick Ultra and Whatman’s filter paper. In addition, in order to assess the potential impact of differing biological sample matrices, both a sputum sample and a nasal sample were used to test the SAMs.

However, unfortunately there was insufficient sample volume to complete a full 3-way comparison and so the Leukosorb was tested against Whatman’s using the nasal sample and against Accuwick using the sputum sample.
2.1.3 Optimising protein recovery

I next investigated the potential for improving cytokine recovery by addition of a buffer to the SAM prior to spinning off the fluid. This was done in 2 parts, either by using a mix of Dulbecco’s PBS (H15-002, PAA) with 1% Bovine Serum Albumin (BSA) (A7906-500G, Sigma-Aldrich) or by using this mix with the addition of 1% Triton X-100 (9002-93-1, Sigma-Aldrich).

Finally, the possibility of improving recovery by allowing the sample to air dry on the SAM prior to eluting with a buffer was also a condition that was tested.

As in the previous experiment, a sputum supernatant from a subject with COPD was used in this series of experiments.

40µL of the sputum supernatant sample was spiked onto each of 24 strips of Leukosorb:

- 8 strips were placed in the spin filter whilst still wet and spun immediately with no addition of buffer
- 8 strips were eluted with buffer whilst still wet:
  Of these:
  - 4 had 60µL of PBS/ 1%BSA added prior to being placed in the spin filter and centrifuged
  - 4 had 60µL of PBS/ 1%BSA with 1% Triton X added prior to being placed in the spin filter and centrifuged
- 8 strips were left to air dry for 1 hour:
  - 4 then had 60µL of PBS/ 1%BSA added prior to being placed in the spin filter and centrifuged
  - 4 had 60µL of PBS/ 1%BSA with 1% Triton added prior to being placed in the spin filter and centrifuged.
Two MSD plates were used, each with a set of standards: Triton X was included in the standards of the plate in which Triton containing samples were analysed. The second plate did not include Triton in the standards or the samples.

25µL of sample was pipetted directly into 4 wells on each plate to act as a baseline level for calculation of % recoveries.

2.2 2

The bronchosorption device that was eventually used to sample bronchial MLF from the majority of subjects in this study was the end result of a series of stepwise revisions to earlier prototypes made by a medical device manufacturer called Hunt Development Ltd, (Midhurst, UK). Dr Onn Min Kon, (St Mary’s Hospital, London) and myself suggested a handle device similar to those found in other bronchoscopic sampling devices (such as bronchial biopsy forceps and bronchial brushes) to increase ease of use. The material of the catheter sheath had to be modified following initial testing to allow a more fluid advancement of the sampling probe, whilst the medical adhesive and site of attachment of the introducer to the handle had to be strengthened following a detachments in one of the first attempts. The design of the final device can be seen in figure 2.1.
2.3 Outline of clinical study and procedures

Fourteen healthy and 32 asthmatic volunteers meeting the inclusion criteria outlined in section 2.4 underwent baseline clinical sampling including lung function and bronchoscopy 2-4 weeks prior to inoculation on day 0 with RV16. Subjects were then seen on days 2,3,4,5,7,10 and 42 post-inoculation (p.i). Infection was induced with RV-16 via nasal spray into both nostrils. Nasal lavage
(NL) for virus load (VL) was performed at each visit along with sampling for nasal MLF ('nasosorption'). A second bronchoscopy was performed during the rhinovirus infection on day 4 p.i to permit measurement of a wide range of inflammatory mediators during the infection and allow comparison between healthy and asthmatic subjects.

The overall study design and timing of assessments is summarised below in figure 2.2

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<th>Day</th>
<th>-15</th>
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<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>42</th>
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<tbody>
<tr>
<td>Symptom diaries</td>
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<td>Virus inoculation</td>
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<td>Serum</td>
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<td>Nasal lavage</td>
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<td>Nasosorption</td>
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<td>Clinic Spirometry</td>
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<td>Histamine challenge</td>
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</table>

**Figure 2.2** Main study visits and assessments.

**2.4 Study subjects**

**2.4.1 Inclusion criteria for asthmatics**

Adults aged 18-55 years old, with no significant smoking or other respiratory history were eligible if they met diagnostic criteria for atopic asthma (physician diagnosis of asthma, bronchodilator reversibility >12%, bronchial hyperreactivity as measured by a provocative concentration of
histamine causing a 20% reduction in forced expiratory volume in 1 second (FEV₁) [PC₂₀] <8 mg/mL, and ≥1 positive skin prick test to a panel of aeroallergens) (listed below with additional criteria).

- Age 18-55 years
- Doctor diagnosis of asthma
- Histamine PC₂₀ < 8 mg/mL
- Mild-to-moderate disease based on 2004 GINA criteria
- Treatment with either short-acting β-agonists (SABA) alone or with maintenance inhaled corticosteroids (daily dose between 200 µg and 1000 µg BDP equivalent)
- Worsening asthma symptoms with infection since last change in asthma therapy
- Atopic on skin testing (≥ 1 positive skin prick test on a panel of 10 aeroallergens)

2.4.2 Inclusion criteria for healthy subjects

- Age 18-55 years
- No history or clinical diagnosis of asthma or any other significant respiratory disease
- No history of allergic rhinitis or eczema
- Negative responses on skin prick testing
- (PC)₂₀ > 8 µg/mL
- Absence of significant systemic disease

2.4.3 Exclusion criteria for asthmatics

- History of severe asthma defined by GINA
- Smoking history over past 6 months or > 5 pack year history
- Current symptoms of allergic rhinitis
- Current or previous history of significant respiratory disease (other than asthma)
- Any clinically relevant abnormality on screening or detected significant systemic disease
- Asthma exacerbation or viral illness within the previous 6 weeks
• Treatment with oral corticosteroids in the previous 3 months
• Current use of any nasal medication, anti-histamine, anti-leukotrienes, LABA, or anti-IgE therapy
• Presence of serum neutralising antibodies to rhinovirus-16 at screening
• Pregnant or breastfeeding women
• Contact with infants or elderly at home or at work

2.4.4 Exclusion criteria for healthy subjects

• History of atopy, asthma or any significant respiratory disease
• Smoking history over past 6 months or > 5 pack year history
• Current symptoms of rhinitis
• Any clinically relevant abnormality on screening or detected significant systemic disease
• Viral illness within the previous 6 weeks
• Current use of any nasal medication or anti-histamine
• Presence of serum neutralising antibodies to rhinovirus-16 at screening
• Pregnant or breastfeeding women
• Contact with infants or elderly at home or at work

2.4.5 Defining asthma severity status

Asthmatic volunteers were defined as mild or moderately-severe based on the GINA 2004 criteria (table 2.1).\(^22^1\) Despite the shift in asthma nomenclature in recent years away from a system based on disease severity toward one based on achievement of asthma control these definitions have been included to allow direct comparison with previous experimental inoculation studies as well as permitting a comparison between patients who would have traditionally been labelled as having mild and moderate asthma. However given shift in emphasis towards asthma control, subjects have also been defined according to their asthma control status (see section 2.4.6).
### Table 2.1 Classification system for asthma severity based on the GINA 2004 workshop report

<table>
<thead>
<tr>
<th>Patient symptoms and lung function</th>
<th>Current treatment step</th>
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<td>Step 1</td>
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<tr>
<td><strong>Step 1: Intermittent</strong></td>
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<tr>
<td>Symptoms ≤ 1 / week</td>
<td>Intermittent</td>
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<tr>
<td>Brief exacerbations</td>
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<tr>
<td>Nocturnal symptoms ≤ 2 / month</td>
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<tr>
<td>Normal lung function between episodes</td>
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<tr>
<td><strong>Step 2: Mild persistent</strong></td>
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<tr>
<td>Symptoms ≥ 1 / week but ≤ 1 / day</td>
<td>Mild persistent</td>
</tr>
<tr>
<td>Nocturnal symptoms ≥ 2 / per month but ≤ 1 / week</td>
<td></td>
</tr>
<tr>
<td>Normal lung function between episodes</td>
<td></td>
</tr>
<tr>
<td><strong>Step 3: Moderate persistent</strong></td>
<td></td>
</tr>
<tr>
<td>Symptoms daily</td>
<td>Moderate persistent</td>
</tr>
<tr>
<td>Exacerbations may affect activity and sleep</td>
<td></td>
</tr>
<tr>
<td>Nocturnal symptoms at least once per week</td>
<td></td>
</tr>
<tr>
<td>$\text{FEV}_1 &gt; 60$ and &lt;80% pred or PEF &gt; 60 and &lt;80% of personal best</td>
<td></td>
</tr>
<tr>
<td><strong>Step 4: Severe persistent</strong></td>
<td></td>
</tr>
<tr>
<td>Symptoms daily</td>
<td>Severe persistent</td>
</tr>
<tr>
<td>Frequent exacerbations</td>
<td></td>
</tr>
<tr>
<td>Frequent nocturnal asthma symptoms $\text{FEV}_1 ≤ 60%$ pred or PEF ≤ 60% of personal best</td>
<td></td>
</tr>
</tbody>
</table>

**2.4.6 Defining asthma control status**

In this study we chose to use the asthma control questionnaire (ACQ)\textsuperscript{16}, an instrument developed by Juniper and colleagues for assessing control in both clinical trials and clinical practise.

Questions rely on recall of the previous 7 days and comprise breathlessness, nocturnal waking, symptoms on waking, wheeze, activity limitation, frequency of SABA use, and pre-bronchodilator $\text{FEV}_1$% predicted. All seven items are scored on a 7-point scale without weighting (0= good control, 6 = poor control) and the overall score is the mean of the responses.
Asthma control groups are defined by ACQ cut-off points of ≤0.75 for well-controlled and ≥1.50 for poorly-controlled asthma respectively. Asthmatics that fell between these 2 categories have been termed ‘partially-controlled’.

2.5 Subject screening

The vast majority of subjects in this study were recruited through advertisements in the Metro and Evening Standard newspapers and from adverts placed on the Imperial College website. Additional paper adverts were placed throughout noticeboards at Imperial College and St Mary’s Hospital however the response to these was surprisingly poor.

Initial contact with volunteers was made by telephone and a pro forma screening questionnaire was completed (see appendix). Approximately 500 subjects were screened by telephone. Of these 183 attended Imperial College Respiratory Research Unit (ICRRU) at St Mary’s Hospital for the first of two potential screening visits. The remaining 317 subjects who initially replied to the advert either declined to enter the study (most commonly due to unwillingness to undergo two bronchoscopies and / or suffer a common cold) or were not suitable (most commonly due to smoking history, significant other chronic medical conditions or history of allergic rhinitis in non-asthmatic subjects).

The flow of participants through each stage of recruitment is illustrated in figure 2.3.

2.5.1 First screening visit

At the first screening visit a more in depth medical history was obtained followed by a full examination, spirometry, skin prick testing and lastly venepuncture for serum neutralising antibodies to RV16.

2.5.2 Second screening visit

Once the volunteers had been identified as seronegative and were otherwise suitable for study entry based on the first visit tests, they underwent a histamine challenge to assess bronchial
hyperresponsiveness. This was followed by a full set of screening bloods including full blood count, electrolytes, renal and liver function, C-reactive protein, clotting and blood group to ensure both general health and as a requirement prior to bronchoscopic sampling. Finally a chest X ray was carried out on all subjects prior to their first bronchoscopy.

**Figure 2.3** Study recruitment

### 2.6 Skin prick testing

All subjects attending screening were asked to withhold any use of antihistamine medication for the week prior to the screening visit. Atopy was determined by skin prick testing to common aeroallergens: six grass pollen mix; house dust mite; cat; dog; *Aspergillus fumigatus; Cladosporium*
herbarum; Alternaria alternata; birch, three tree and nettle pollen (ALK Abello). A drop of each aeroallergen was placed on the labelled inner forearm and lancets used to make the skin prick. Histamine (positive control) and 0.9% saline (negative control) were added to the above panel. At least one wheal ≥ 3 mm larger than the negative control was considered diagnostic of atopy.

2.7 Pulmonary function testing

Clinic spirometry was performed according to BTS/ARTP guidelines using a Micromedical MicroLab spirometer V002ML3500 (MicroMedical, Rochester, UK). Study participants were instructed to perform home spirometry on waking each morning, recording the best of three attempts of PEF and FEV₁ using a Piko-1 device (nSpire, UK).

Spirometry is one of the fundamental measures of asthma control and FEV₁ provides a highly reproducible and objective measure of airflow limitation. PEF is considered inferior to FEV₁ as it lacks accurate reference values for many populations, may underestimate airway obstruction in individuals with airway remodelling and confers no advantage in reproducibility. In this study both PEF and FEV₁ were recorded pre-bronchodilator on waking each morning.

The % change in morning FEV₁ and PEF from baseline during infection was calculated for each subject as the % fall from the mean of the 7 day period prior to inoculation. Maximal fall from baseline (%) for each subject represented the greatest fall from baseline over the 2 week period following inoculation.

2.8 RV-16 serology testing

Testing for the presence of serum RV16 neutralising antibodies was performed at the first screening visit and then repeated on the day of inoculation (day 0) and on day 42 for study participants to assess the degree of seroconversion. The identification of any degree of serum antibodies in the screening sample resulted in exclusion from study entry. RV16 serology was performed on heat
inactivated serum (samples placed for 30 minutes in a water bath at 56°C). Antibody levels were then assessed using a HeLa cell monolayer in a 96 well plate.

50μL of serum was serially diluted from 1:2 to 1:64 followed by addition of 50μL of RV16 stock virus containing 100 tissue infective dose 50% (TCID<sub>50</sub>) to each well. The TCID<sub>50</sub> quantifies the amount of virus required to produce a cytopathic effect (CPE) in 50% of inoculated tissue culture cells. The 96 well plate was shaken for 1 hour at room temperature followed by the addition of 100μL of freshly stripped HeLa cells at a concentration of 2 x 10<sup>5</sup> mL<sup>-1</sup>. Six wells each were reserved for positive controls (RV16 and cells in the absence of serum) and negative controls (media and cells in the absence of serum). The plate was then incubated for 72 hours before being read. The plate layout is shown in figure 2.3 accompanied by an example of a completed assay following the incubation period (figure 2.4). The antibody titre for each sample was defined by the greatest serum dilution where CPE was not identified. Seroconversion was defined as a titre of serum neutralizing antibodies to RV-16 of at least 1:4 at 6 weeks post-inoculation.
Figure 2.4 Serology plate layout. Each sample was tested in duplicate allowing 6 samples to be tested per plate (S1-S6). (S, neat serum; M, media alone; RV, virus alone; 1:2 to 1:64 serial serum dilution)

Figure 2.5 Example of a completed serology plate showing samples from day 0 and day 42 for subjects 81, 86 and 92. The cytopathic effect of virus can easily be identified by the pink colouration of the well however light microscopy was used in all cases to identify CPE. Subjects 86 and 92 demonstrate clear evidence of seroconversion, in contrast to subject 81 who failed to develop RV16 neutralising antibodies.
2.9 Histamine Challenge

AHR is an objective measure of variable airflow limitation and reflects the increased sensitivity of the airways to inhaled stimuli. Histamine diphosphate was used as the direct smooth muscle stimulus (maximum concentration 16 mg/mL) and performed according to guidelines by using the 2-min tidal breathing method. Subjects used a MicroLab Spirometer V002ML3500. The provocative concentration of histamine causing a 20% reduction in FEV$_1$ (PC$_{20}$) was calculated.

To be eligible for this study asthmatic subjects were required to have a (PC$_{20}$) < 8 mg/mL, and healthy subjects a (PC$_{20}$) >8 mg/mL. Asthmatic volunteers were asked to stop all bronchodilator medication at least 12 hours prior to the test. For safety reasons the test was not repeated on day 7 in subjects with an FEV$_1$ <70% predicted.

2.10 Virus inoculation.

Inoculation of volunteers was carried out in a specified room in ICCRU, St Mary’s Hospital and was performed in an identical fashion for the pilot and main studies (other than the dose of virus used: 10 TCID$_{50}$ in the pilot study and 100 TCID$_{50}$ in the main study).

RV16 was diluted in 250 µL of 0.9% saline and inoculated into both nostrils using an atomizer (no. 286; De Vilbiss Co., Heston, UK) (figure 2.5). Subjects were asked not to swallow during the procedure to ensure maximal pharyngeal contact. Subjects were also requested to sniff at each actuation to encourage delivery of particles to the lower airway.

Figure 2.6 Nasal atomiser used for inoculation of study volunteers
2.11 Symptom scores

Upper and lower respiratory symptoms were assessed by daily diary cards completed by study subjects at the end of each day. These were started 2 weeks before baseline sampling and continued until 4 weeks post-inoculation. Figure 2.6 shows the diary card used.

2.11.1 Upper respiratory tract symptoms

The daily ‘cold score’ was measured using the eight symptom Jackson scale and summated from individual scores (sneezing, headache, malaise, nasal discharge, nasal obstruction, sore throat, cough, fever) graded 0 (absent) to 3 (severe). This scoring system has been validated and used in many experimental RV infection studies. A clinical cold was considered to be present if 2 of the following 3 criteria were fulfilled:

1. A cumulative symptom score of at least 14 over a 6-day period.
2. The subjective impression of a cold.
3. Rhinorrhea present on at least 3 days

2.11.2 Lower respiratory tract symptoms

Lower respiratory tract symptoms were recorded in a similar fashion to cold symptoms and included cough on waking; wheeze on waking; daytime cough; daytime wheeze; daytime chest tightness; daytime shortness of breath; nocturnal cough, wheeze or shortness of breath. A grading of 0–3 also applied.

As previously reported, lower respiratory symptom scores were analysed in 2-week blocks as the baseline and acute infection stages both contained a bronchoscopy on the fourth day of each 2-week block (figure 2.7). Daily symptom scores during infection, corrected for baseline symptoms and the effects of bronchoscopy, were calculated by subtracting scores obtained during the baseline 2-week block from the corresponding days of the acute infection 2-week block. It is therefore possible
to have a negative score assigned to a study subject if baseline symptoms exceeded those during the acute infection block.

Total lower respiratory scores were calculated by summing the corrected daily scores for the 2-week infection period. Peak symptom score was the maximum corrected daily score in the 2-week block after infection.

**Figure 2.7** Example of symptom card

<table>
<thead>
<tr>
<th>0 = no symptoms</th>
<th>1 = mild</th>
<th>2 = moderate</th>
<th>3 = severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sneezing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Runny nose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blocked or stuffy nose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sore throat or hoarse voice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache or face pain</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Generally unwell</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Chill, fever or shivery</td>
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<td></td>
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<tr>
<td>Cough</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total cold score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cough on waking</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wheeze on waking</td>
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<td></td>
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<tr>
<td>Daytime Cough</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Daytime Wheeze</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Daytime chest tightness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daytime breathlessness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nocturnal cough, wheeze, breathlessness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Chest score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Medications (name and dose)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td></td>
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<tr>
<td>2.</td>
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<tr>
<td>3.</td>
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<tr>
<td><strong>Spirometry:</strong></td>
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<tr>
<td>Peak Flow am</td>
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<td></td>
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<tr>
<td>FEV₁ am</td>
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</table>
Figure 2.8 Baseline asthma symptom scores and any increase in symptoms following the first bronchoscopy were subtracted from the equivalent day symptom scores during the 2 week infection block.

2.12 Nasal Lavage

NL with sterile 0.9% sodium chloride was performed using a 10mL syringe attached to a hollow nasal adapter (‘olive’) used to obstruct the nostril and prevent leak of lavage fluid. A total of 5 mL of sterile normal saline was instilled into the left nostril and the fluid withdrawn into the 10mL syringe and flushed back into the nasal cavity 20 times over a 1 min period. Fluid was collected, aliquoted and stored at -80°C.

2.13 Sampling nasal mucosal lining fluid: ‘Nasosorption’

This minimally invasive technique samples upper airway mucosal lining fluid and offers potential benefits over NL including improved sensitivity through avoidance of significant analyte dilution (inherent with lavage). Nasosorption was performed prior to NL by placing strips of SAM (Leukosorb, Pall Life Sciences, Hampshire, UK) measuring 7mm x 35mm into each nostril for a duration of 2 minutes (figure 2.8). 100 μL of a PBS buffer containing 1% BSA and 1% Triton X (Sigma Aldrich, USA) was then applied to the SAM prior to elution by spin filter centrifugation (5 minutes at 16,000G at 4°C). The eluate was then immediately frozen at -80°C.
2.14 Bronchoscopy

All bronchoscopies were performed in the endoscopy unit at St Mary’s Hospital, UK using an Olympus PF260 bronchoscope. Subjects were administered nebulised salbutamol (2.5mg) prior to the procedure and sedation was achieved with intravenous midazolam. The bronchoscope was passed via the mouth and not the nose to avoid worsening nasal symptoms which may have affected the recording of upper respiratory symptom scores. In addition the potential for artificial transmission of virus and inflammatory mediators by the bronchoscope itself as it passed from the nose to the lung wished to be avoided.

2.14.1 Bronchoalveolar lavage

BAL was performed by instillation of 150mL sterile normal saline in 5 x 30 mL aliquots with a 10 second dwell time into the right upper lobe bronchus. An aliquot of unfiltered BAL was stored for qRT-PCR for virus load and the remaining BAL filtered, centrifuged and the supernatant immediately stored at -80°C.

2.14.2 Bronchial brushings

Six 10mm (BC-202D-5010 Olympus, Japan) disposable cytology brushes were used to collect epithelial cells from the right middle and lower lobe sub-segmental bronchi. All brushes were
immediately transferred into a tube of pre-warmed culture medium (BEGM) and their wires cut. Vigorous mixing of the brushes within the medium was performed to detach cells from the brushes, prior to pelleting of the cells with centrifugation.

2.14.3 Bronchial biopsies

Six bronchial biopsies were taken from the segmental and subsegmental bronchi of the right middle and lower lobe using 1.8mm radial jaw (1523, Boston Scientific, USA) serrated needle free biopsy forceps. Four biopsies were placed immediately in 4% paraformaldehyde and two placed in 500μL of the RNA stabilisation reagent RNAlater (Sigma-Aldrich) for future gene extraction.

2.14.4 Sampling bronchial mucosal lining fluid: ‘Bronchosorption’

Bronchosorption (Mucosal Diagnostics, Hunt Developments Ltd, Midhurst, UK) is a technique to sample bronchial mucosal lining fluid. The main benefit of this novel technique is the measurement of previously undetectable mediators through avoidance of the significant analyte dilution associated with bronchoalveolar lavage. The bronchosorption device is passed down the operating port of the bronchoscope. The distal end of the inner probe incorporates a folded strip of Leukosorb (Pall Life Sciences, Hampshire, UK) measuring 1.8mm x 30mm which is placed on the bronchial mucosa for a period of 30 seconds (figure 2.9). Following sampling, the bronchosorption device is withdrawn back into its sheath and then the complete device is removed from the bronchoscope. The sampling end of the probe is then cut off and treated in an identical way to the nasosorption strips of Leukosorb.
Figure 2.10 Sampling bronchial mucosal lining fluid using the technique of bronchosorption.
2.15 Virologic Confirmation of RV-16 Infection

Objective evidence of rhinovirus infection was established by either positive nasal lavage standard or quantitative PCR (qPCR) for rhinovirus or seroconversion defined as a titre of serum neutralizing antibodies to RV16 of at least 1:4 at 6 weeks post-inoculation (discussed in section 2.6).

RNA was extracted from nasal and bronchoalveolar lavage (QIAamp viral RNA mini kit; Qiagen Ltd, Crawley, UK) and reverse-transcribed (omniscript RT kit, Qiagen) with random hexamers. The Biosystems ABI Prism 7700 sequence detection system was used with AmplitaqGold DNA polymerase, a RV-16 specific primer pair (forward oligo 5’-GTG AAG AGC CSC RTG TGC T-3’, a reverse oligo 5’-GCT SCA GGG TTA AGG TTA GCC-3’) and a FAM/TAMRA labelled RV-16 probe (FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA). A master mix was made up consisting of Qiagen quantitect probe mix, forward primer (50nM), reverse primer (300nM), probe (100nM) and RNase inhibitor. 23μL of PCR mastermix was added to 2μL cDNA in each well of a 96 well Taqman plate.

The thermal cycle conditions used were: 50°C 2 mins, 95°C 10 mins then 45 cycles x 95°C 15s / 55°C 20s / 72°C 40s. Fluorescence data was collected for each cycle and the cycle number (Ct) at which fluorescence rose above threshold was calculated (threshold used for RV in data analysis was 0.11). Negative extraction (water), negative PCR (template only) and positive extraction (RV16 stock) was included. A standard curve was produced by using 2μL of RV plasmid serially diluted 10 fold from 10^7 to 10^0 copies. Results were expressed as copies/mL. The sensitivity was 10^3 copies/mL.

2.16 PCR for Additional Respiratory Viruses

Infection with viruses other than RV was excluded by testing nasal lavage by PCR on random hexamer primed cDNA for Mycoplasma and Chlamydia pneumoniae, adenoviruses, respiratory syncytial virus, influenza AH1/AH3/B, parainfluenza 1–3, human metapnuemoviruses (HMPV), and
coronaviruses 229E and OC43 as described. This PCR work was performed with the assistance of several technicians in our laboratory, notably Julia Aniscenko, Leila Gogsadze and Eteri Bakhsoliani.

2.17 Differential Cell Counting

Bronchial brushing cells and the BAL cell pellet were resuspended in PBS and cytospin slides were prepared (Shandon cytospin; 400 rpm, 5 min) and stained using Shandon Diffquick kit (Thermo Shandon Ltd, Cheshire, UK). Differential cell counts were counted blind to study status and expressed as a percentage of at least 400 inflammatory cells.

2.18 Cytokine and chemokine measurement

Mediators were measured in extracted nasal and bronchial mucosal lining fluid as well as nasal lavage and BAL supernatant samples. They included IFN-γ, IL-4, IL-5, IL-12p40, IL-13, IL-15, IL-25, IL-33, TSLP, CCL17 (TARC), CCL22 (MDC), CXCL10 (IP-10) and CXCL11 (I-TAC).

The MSD platform (Meso Scale Discovery, Gaithersburg, MD, USA) along with their Ultrasensitive Multi-spot Human cytokine assays and Sector Imager 6000 plate reader was used for these measurements. Measurement of mediators during the preliminary SAM validation and optimisation experiments (section 2.1) was also carried out on this platform.

The technique allows quantitative detection of up to 10 mediators per well in a 96 well plate format and utilises a capture antibody attached to each spot within the well which enables measurement of multiple mediators simultaneously. Electrochemiluminescence of detection antibody is then recorded with an internal high sensitivity camera – the Sector Imager.

In addition to the advantage of measuring multiple mediators simultaneously in a single well, the MSD platform only requires 25μL of sample volume per well. In view of the small volumes generated by naso- and broncosorption this makes MSD particularly attractive in this study.
Following an initial step in which 25μL of blocking solution is added and incubated for 30 minutes, the samples are added to the wells for an incubation period of 2 hours. Repeat washing is followed by addition of 25μL of detection antibody and a further incubation for 1 hour. Finally 150μL of read buffer is added before the plate is placed in the Sector imager for reading.

The lower limit of detection was 0.6 pg/mL for all mediators except for IL-33 (3pg/mL), IP-10/CXCL10 (10pg/mL), TARC/CCL17 (2.7 pg/mL), MDC/CCL22 (2.7pg/mL), IL-12p40 (2.4pg/mL), IL-25 (10pg/mL), and TSLP (10pg/mL). All samples with values under the detection limits were given a value of 0pg/mL for the subsequent statistical analysis.

Whilst I carried out all the MSD work in the preliminary experiments, the mediator analysis generated from the main study was carried out and funded by Novartis Institute for Biomedical Research, UK. The data generated by them was done blinded to subject group.

2.19 Statistical analysis and software

It was not possible to perform a power calculation for this study as no data exists on the outcomes of interest using the population studied. However important related observations have been made on smaller numbers than enrolled here.68

Data are presented as mean (±SEM) values for normally distributed data or as median (interquartile range) values for nonparametric data. Differences between groups were analysed by unpaired t tests or Mann-Whitney tests. Within-group comparisons were analysed with Wilcoxon’s signed rank test. Correlations between data sets were examined using Pearson’s correlation for normally distributed data and Spearman's rank correlation coefficient for nonparametric data. Data was log-transformed for presentation in many of the scatter plots, however, where the original dataset was non-parametric in distribution, the Spearman's rank correlation is shown. The Bland-Altman test was used to determine the repeatability of nasosorption sampling. Differences were considered significant for all statistical tests at P values less than 0.05. All reported P values are two-sided.
The strategy for analysis of clinical, virological, and cytokine measures for which data was collected across multiple time points post-inoculation included the identification of both peak (maximal) and total (area under the curve) levels for these variables. Peak symptom score, maximal fall in peak expiratory flow, peak virus load and the peak cytokine level has been used previously by both the Johnston group and others. All data analyses were conducted using SPSS version 20.0 (IBM Corp, NY, USA). Microsoft Office Excel was used to record all study results. Zotero was used for bibliographic database management.
Chapter 3
Results Chapter 1
Development and Validation of Nasosorption and Bronchosorption

3.1 Introduction

A new method for sampling upper airway mucosal lining fluid (MLF) was first described by Chawes et al. in 2010. This minimally invasive technique known as 'nasosorption' offers potential benefits over nasal lavage including improved sensitivity and sampling accuracy, by overcoming the variable dilution of analytes associated with nasal lavage. Using this technique Chawes highlighted elevated levels of IL-5 and IL-13 in children with allergic rhinitis, Nicholson et al. used nasosorption after nasal allergen challenge, whilst Folsgaard et al. more recently reported its successful use in neonates to define cytokine signatures in children with atopic parents. In all 3 studies a synthetic absorptive matrix (SAM) (Accuwick Ultra, Pall Life Sciences, Hampshire, UK) was used. However, none of the published studies directly assessed cytokine recovery from Accuwick or directly compared concentrations measured by nasosorption with those obtained by nasal lavage.

At the start of this study in 2009 Accuwick was commercially available in sheets 8 inches x 10 inches which was then cut into small strips by Parafix Tapes & Conversions Ltd (UK) for the purposes of this study. During the course of the initial experiments to validate Accuwick, Pall halted its manufacture requiring the identification and validation of an alternative matrix for nasosorption. Following several further experiments it became clear that an alternative matrix for nasosorption was Leukosorb (also made by Pall Life Sciences). Like Accuwick, this synthetic, fibrous, hydroxylated polyester medium was designed for sample collection, storage, and conjugate release. The material is hydrophilic with low biomolecular binding, and protein-containing samples are stable during storage at -80°C. It is highly absorbent, and the fibre surfaces have been modified to enhance water wettability.
The first set of results presented in this chapter report on the steps taken to validate Accuwick and subsequently Leukosorb. Results from a direct comparison between nasosorption using Leukosorb and nasal lavage to formally assess the theoretical advantages of this new technique are then described.

As with nasal lavage, bronchoalveolar lavage can be an extremely useful sampling method and is used in a wide range of contexts most frequently as an aid to diagnosis. However the same problems encountered with nasal lavage are often even more challenging with BAL. Most notably the variable dilution and recovery of BAL following instillation of large volumes of saline means that accurate comparisons of protein levels taken at different time points or from different patient groups is extremely difficult to achieve. In an attempt to overcome this, Ishizaka in collaboration with Olympus developed a bronchial microsampling device consisting of a 2.8mm outer-diameter polyethylene sheath and an inner 1.2mm cotton probe attached to a stainless steel guide wire (see chapter 1, figure 1.2). However, despite its first use in 2001, it has never been made available either for research purposes or commercially to clinicians in the UK. Therefore, the second main aim of this study was to adapt the nasosorption matrix in size and shape and devise a probe that would allow the safe sampling of bronchial mucosal lining fluid using Leukosorb. The actual manufacturing of the probe that followed the initial design phase was undertaken by Hunt Developments Ltd, a company that specialises in the design and manufacture of medical devices using precision equipment under fully sterile conditions.

3.2 Assessment of Accuwick

Thirty-eight strips of Accuwick were spiked with 70µl of known concentrations of Meso Scale Device (MSD) protein standards for IFNy, IL-1β, IL-6, and TNFα. The concentrations used were: 2.4pg/mL (n=6), 9.8pg/mL (n=6), 39pg/mL (n=6), 156pg/mL (n=6), 625pg/mL (n=6), and 2500pg/mL (n=8). Of the four cytokines tested, a good recovery of protein was only observed for IL-1β (% recovery ~100% at each of the concentration). In contrast, the recovery of IFN-γ at concentrations above 10 pg/mL
ranged from 12% at [2500 pg/mL] to 35% at [39 pg/mL]. The % recovery of IL-6 was also inversely related to the concentration ranging from 43% at [625 pg/mL], to 71% at [39 pg/mL], and (94%) at [9.8 pg/mL]. A similar pattern was observed for TNF-α (Table 3.1a and b). However, it should be noted that the coefficients of variation (CV’s) achieved indicate that these results were reproducible, with the majority of the CVs being below 15%. (CV is the standard deviation represented as a percentage of the calculated concentration for the sample).

Table 3.1  Calculated concentrations (a) and % recovery (b) of four cytokines achieved following spiked samples on Accuwick

<table>
<thead>
<tr>
<th></th>
<th>Calc Conc. Mean pg/mL</th>
<th>Calc. Conc. CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calc Conc. Mean pg/mL</td>
<td>IFNγ, IL-1β, IL-6, TNFα</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFNγ, IL-1β, IL-6, TNFα</td>
</tr>
<tr>
<td>2500 pg/mL</td>
<td>309.66</td>
<td>261.17</td>
</tr>
<tr>
<td>625 pg/mL</td>
<td>89.76</td>
<td>666.91</td>
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<tr>
<td>156 pg/mL</td>
<td>28.87</td>
<td>163.89</td>
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<tr>
<td>39 pg/mL</td>
<td>13.54</td>
<td>43.08</td>
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<tr>
<td>9.8 pg/mL</td>
<td>11.29</td>
<td>12.07</td>
</tr>
<tr>
<td>2.4 pg/mL</td>
<td>9.90</td>
<td>3.33</td>
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<table>
<thead>
<tr>
<th></th>
<th>% Recoveries achieved</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IFNγ, IL-1β, IL-6, TNFα</td>
</tr>
<tr>
<td>2500 pg/mL</td>
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</table>
3.3 Comparison of absorptive matrices and optimisation of protein recovery

In order to exclude the possibility that the MSD standards used to spike the SAM in the first experiment may behave differently from an actual human sample in a matrix such as sputum or nasal lavage, a sputum supernatant from an individual with COPD (courtesy of Dr Joseph Footitt’s study, ethics approval 07/H0712/138) acted as the test sample. In this experiment measurement of a wider range of cytokines namely: IFN-γ, IL-10, IL-12 p70, IL-6, IL-8, and TNF-α was performed.

A direct comparison between Accuwick and Whatman’s filter paper (GE Healthcare, Buckinghamshire, UK) was conducted (n = 8 for each matrix). Whatman cellulose filters have been used for many years in a large number of settings and benefit from high wet strength enabling efficient sampling of mucosal surfaces.

In addition, to ensure that the cytokines were not being lost in transit through the spin filter itself, 40µl of the sputum supernatant was also spiked onto the spin filter directly without first being spiked onto the SAMs. The recoveries were then compared with the concentrations obtained following direct application of the sample to the well of the MSD plate (i.e. a neat sample) (table 3.2).

Table 3.2 Comparison of Accuwick with Whatman’s filter paper. Percentage recoveries are shown for 7 cytokines measured from a sputum supernatant sample applied directly on to Accuwick, Whatman’s or the filter of the polypropylene tube itself prior to elution.

<table>
<thead>
<tr>
<th>% Recoveries achieved</th>
<th>IFNγ</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>IL-10</th>
<th>IL-12 p70</th>
<th>TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample + spin filter</td>
<td>104</td>
<td>86</td>
<td>74</td>
<td>100</td>
<td>108</td>
<td>123</td>
<td>116</td>
</tr>
<tr>
<td>Sample + Accuwick</td>
<td>6</td>
<td>53</td>
<td>10</td>
<td>49</td>
<td>6</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Sample + Whatman</td>
<td>31</td>
<td>96</td>
<td>22</td>
<td>91</td>
<td>35</td>
<td>59</td>
<td>48</td>
</tr>
</tbody>
</table>
Concentrations of cytokines in the sputum sample ranged from 18 pg/mL of IL-10 to over 3ng/mL in the case of IL-8. This experiment confirmed the findings of the first experiment highlighting the poor recovery of cytokines by Accuwick: Only 2/7 cytokines (IL-1β and IL-8) demonstrated recoveries greater than 15% with a mean recovery of 21% across the 7 proteins. Whatman’s filter paper significantly outperformed Accuwick with an average recovery of 55% however the volume of sample returned following centrifuging was lower by an average of 25%. In addition Whatman’s was considerably more friable when wet than Accuwick and would therefore be unsafe for bronchosorption given the potential for paper breakage during sampling. In general, very little protein was lost in the actual spin filter itself excluding this as the main source of lost recovery.

The next series of experiments firstly compared Accuwick with Leukosorb and secondly examined the potential for improving cytokine recovery. This first involved the addition of a buffer to Leukosorb prior to elution, and was performed in 2 parts, either by using a mix of Dulbecco’s PBS (H15-002, PAA) with 1% Bovine Serum Albumin (BSA) (A7906-500G, Sigma-Aldrich) or by using this mix with the addition of 1% Triton X-100 (9002-93-1, Sigma-Aldrich).

Comparison of Accuwick with Leukosorb in the absence of a buffer revealed a similarly poor recovery of cytokines (with the exception of IL-1β) which appeared to differ from cytokine to cytokine in a consistent manner to the previous experiment. Overall Leukosorb did not lead to statistically significant improvements over Accuwick ($P = 0.510$). However addition of the buffer containing protein and detergent resulted in a substantial improvement in recovery which was observed across the range of cytokines (mean recovery with buffer: 62%, range 28-100%; $P = 0.003$) (Figure 1). Overall a 14 fold improvement in recovery was observed compared to sampling without buffer. Importantly this improved recovery was not observed when a PBS buffer containing 1% BSA was used in the absence of Triton ($P = 0.17$).

A further experiment was carried out to assess the impact of allowing Leukosorb to air dry first prior to addition of a buffer versus addition when still moist from recent sampling. This showed that
drying led to no statistically significant difference in recovery across the range of cytokines tested when compared to addition of buffer whilst the Leukosorb was still moist. This was true for both the buffer without Triton (dry versus wet, \( P = 0.812 \)) and for the buffer with Triton (dry versus wet, \( P = 0.639 \)).

**Figure 3.1** Comparison of cytokine recovery using Accuwick and Leukosorb synthetic absorptive matrices (SAMs) with recovery from Leukosorb following addition of a protein buffer containing 1% Triton X.

3.4 Reproducibility of nasosorption

Subjects recruited to this study underwent nasosorption sampling at eight time-points including two either side of the infection: the first on day 0 prior to inoculation, and the second 6 weeks post-inoculation at the time of serum antibody testing. It has therefore been possible to assess the reproducibility of this technique by comparing measurements on these two days. IL-33 has been
chosen as the cytokine for comparison as this was one of the only mediators analysed in this study that had measurable levels above the lower limit of detection for all 39 study subjects at both time-points. It should however be noted that the presence of a new infection or the onset of clinically significant allergic rhinitis on day 42 was not an exclusion criteria for this study and was not recorded in the study record of each subject. It is therefore possible that differing measurements for an individual could be explained by either of these two scenarios.

The degree of agreement between IL-33 levels is illustrated in the Bland Altman plot (figure 3.2). The mean difference and 95% limits of agreement between the two sets of measurements is 2.8 ± 85.6 pg/mL. The fact that the mean difference is close to zero suggests there is a lack of any significant bias between the two measurements (i.e. day 42 levels are not consistently higher than day 0 levels). However there is no single statistical test to accurately judge if the 95% limits of agreement are acceptable in this instance. Cohen’s kappa coefficient is a measure of agreement for categorical and not continuous variables and is therefore unsuitable in this context. The wide range of baseline IL-33 levels across the study subjects (ranging from < 10 pg/mL to > 100 pg/mL) adds further complexity to this assessment. Additional experiments are required to shed further light on the reproducibility of nasosorption. This could most easily be accomplished by measuring cytokine levels in an individual on several consecutive days or possibly even several times within a single day. This and other experiments to further validate naso- and bronchosorption are planned in the future.

3.5 Comparison of nasosorption with nasal lavage

Measurement of the pro-inflammatory cytokines IL-1β and IL-6 in nasal lavage and nasosorption samples of 10 healthy subjects was performed and illustrates the benefits of this novel technique. Significantly greater levels of both IL-1β and IL-6 were present in the nasosorption samples compared with the nasal lavage samples. The median level of IL-1β in nasosorption was 165.7 pg/mL (range 83.8 - 337.7 pg/mL) compared to 4.9 pg/mL (range 0.7 - 8.0 pg/mL) in nasal lavage (P =0.005). The median level of IL-6 in nasosorption was 43.0 pg/mL (20.3 – 94.5 pg/mL) compared to 0.8 pg/mL.
(0.4-7.1 pg/mL) in nasal lavage. Indeed only 7/10 subjects had levels of IL-6 greater than the lower limit of detection in NL (0.6 pg/mL). In contrast, all subjects had levels within the limits of detection when measured by nasosorption (figure 3.3 a and b). Reassuringly, protein levels measured by nasosorption correlated strongly with levels in nasal lavage (IL-1β, r=0.88, P=0.001; IL-6, r=0.84, P=0.002) (figure 3.3 c and d).

Figure 3.2 Bland Altman plot to assess reproducibility of Nasosorption. IL-33 measured by nasosorption on day 0 pre-inoculation was compared with levels on day 42 post inoculation.

Figure 3.3 Comparison of upper airway sampling techniques. Levels of IL-1β (a) and IL-6 (b) in nasal mucosal lining fluid measured by nasosorption are significantly greater than in nasal lavage.
3.6 Development of bronchosorption

Once the experiments to find a suitable alternative to Accuwick outlined above were complete, the process of developing a device with a sampling tip composed of Leukosorb could begin. The bronchosorption device currently in use is the end result of a series of stepwise revisions to earlier prototypes that had failed for one reason or another. These failures unfortunately meant that a few of the first subjects to enter the study did not have bronchosorption performed at the time of their bronchoscopy.

We based the device handle on other tried and tested bronchoscopic sampling devices (such as bronchial biopsy forceps and bronchial brushes) to increase ease of use and reliability. Following initial failings a number of improvements to the design had to be introduced. The material of the catheter sheath had to be modified to allow a more fluid advancement of the sampling probe, whilst the medical adhesive and site of attachment of the introducer to the handle had to be strengthened following a detachment in one of the first attempts. The sampling tip itself was a piece of Leukosorb folded and compressed several times to enable it to maintain a degree of rigidity when wet following bronchial sampling. The dimensions were limited by the operating channel of the bronchoscope but were made as large as possible to maximise the surface area for sampling.

Hunt developments Ltd are deserving of a great deal of gratitude for not only manufacturing the device to the highest possible level but also for making the adjustments I proposed in a timely manner to avoid critical delays. The final design of the device can be seen in figure 2.9.

3.7 Comparison of bronchosorption with bronchoalveolar lavage

As with nasal sampling, IL-6 serves as a good cytokine to compare the two bronchial techniques as it was measurable in all study subjects at baseline. The Th1 chemokine CXCL10/IP-10 was also measured at levels above the lower limit of detection in all subjects using both sampling techniques. Correlations between BAL levels and bronchosorption levels for these cytokines revealed close correlations (IL-6, r=0.65, P< 0.001; CXCL10/IP-10, r=0.47, P=0.004). The median level of IL-6 in BAL
was 5.4 pg/mL compared to 38.5 pg/mL in bronchosorption, whilst the median level of CXCL10/IP-10 in BAL was 144 pg/mL compared to 1014 pg/mL in bronchosorption. In both cases this is equivalent to a 7-8 fold increase in concentration over what can be measured in BAL. When dealing with cytokines near the lower limits of detection with BAL, the benefits of bronchosorption can be readily appreciated.

**Figure 3.4** Comparison of lower airway sampling techniques. Levels of IL-6 and CXCL10/IP-10 in bronchial mucosal lining fluid measured by bronchosorption are significantly greater than bronchoalveolar lavage.
3.8 Discussion

One of the main novel aspects of this study is the inclusion of a new sampling technique to measure secreted mediators in airway mucosal lining fluid, and in so doing provide an opportunity to measure previously undetectable inflammatory mediators and advance our understanding of disease mechanisms.

However, despite the successes of nasosorption in the three previously published studies\textsuperscript{201–203}, it appears the use of Accuwick for sampling nasal mucosal lining fluid has never been formally assessed or properly validated. At the time of writing, the results presented in this chapter represent the first such assessment of using these matrices for this purpose.

In view of the fact that Accuwick is no longer being manufactured, it has also been possible to identify an alternative sampling matrix in Leukosorb that shows equivalence in protein binding and release characteristics to Accuwick and is of an appropriate strength for safe sampling of the bronchial mucosa. Furthermore it has been possible to demonstrate that protein recovery can be greatly improved by the addition of a buffer containing both protein (1% BSA) and detergent (1% Triton) prior to elution, a finding that will greatly aid future projects using these sampling techniques. The additional finding that protein recovery is not significantly affected by the matrix drying out prior to application of the buffer is also a useful finding as there can frequently be a delay between taking a human sample and it arriving in the lab for initial processing.

The finding that some cytokines are more easily recovered than others was unexpected and cannot easily be explained. In view of the near complete recovery of IL-1β and the demonstration of good reproducibility (irrespective of the percentage recovery) it does not appear to be secondary to a sampling error. It also appears not to be related to the molecular weights of the cytokines as both IL-1β and IFN-α are 17KDa yet their initial recoveries on Accuwick varied greatly from one another. Even TNF-α at 57KDa is still small enough to have easily passed through the spin filter with ease, and
the subsequent experiments performed to assess retention by the cellulose acetate filter present in the polypropylene tubes excluded this as a possible cause definitively. However, it is possible that the differences relate to the charge of the proteins as some of the cytokines are known to be more charged than others and therefore may bond to the matrix more robustly. Further work and additional experiments are clearly needed to better understand the reasons for this and assess how other cytokines differ in their protein binding / release properties.

Nasosorption has been shown for the first time to be a reproducible technique that correlates well with nasal lavage but is able to consistently and reliably measure greatly increased concentrations of mediators. This technique can therefore offer insights into pathways that have not been amenable to investigation previously. Moreover, it is well-tolerated and easy to perform which makes it suitable for sampling both children and adults. Similarly, the development of the bronchosorption sampling device is a significant advance for respiratory medicine. It correlates well with BAL and should permit previously undetectable proteins to be accurately measured across a wide range of airway diseases providing valuable insights into mechanisms of disease.

Over the course of this study approximately 100 bronchoscopies and 200 bronchosorptions have been performed. The technique is well-tolerated, quick to complete, does not appear to have the potential to cause bronchospasm in the same way as bronchoalveolar lavage is recognised to, and does not appear to be associated with the fever that sometimes follows lavage. The theoretical danger of SAM detachment whilst sampling never materialised despite often vigorous coughing on the part of exacerbating asthmatics. It seems unlikely that this technique will not become in widespread use in both the research and healthcare setting.
Chapter 4
Results chapter 2

Clinical and Virological Outcomes of Experimental Rhinovirus Infection in Asthma

4.1 Introduction

The human model of experimental rhinovirus infection permits the study of a rhinovirus infection under controlled conditions, allows comparison of symptoms, airway physiology, lung function, airway inflammation, and use of asthma medication to name but a few. Its evolution to include the study of asthma has led to a significant increase in our understanding of the processes that underlie virus-induced exacerbations and offers a model with which new medications can be tested. To date, this model has demonstrated that mild asthmatics experience increased rhinovirus-associated morbidity compared to healthy subjects, with increased lower respiratory symptoms of a mild exacerbation, falls in lung function, and airway hyperresponsiveness. However the model has been limited by the inclusion of only mild asthmatics, adequately-controlled at study entry. This has meant that the findings are not directly relevant to the group of asthmatics that new asthma therapies are most often targeted to. Another consequence has been that the influence of baseline asthma severity and asthma control on the outcome of rhinovirus infections in asthma has remained unknown. This is in the context of an increased appreciation in recent years of the link between 'current control' and 'future risk' in asthma - the idea that good current asthma control can reduce the future risk of adverse outcomes. Reference to respiratory virus infections with regard to this literature has been completely absent, despite their role as the dominant trigger for exacerbations.

Secondly, to date the current model of experimental infection has used a high-dose virus inoculum (1000 to 30,000 TCID$_{50}$) to inoculate study subjects with. However this may not truly reflect the onset of a naturally-occurring infection, which would start with a low dose natural inoculation,
followed (if immune responses do not succeed in preventing) by replication of virus to high virus loads, leading to consequent expression of symptoms and signs.

The aim of the work presented in this chapter has therefore been to firstly test the hypothesis that rhinovirus infection leads to greater morbidity in moderately-severe and inadequately-controlled asthma compared to subjects with milder and well-controlled disease; and secondly to test the hypothesis that a low-dose virus-inoculum (100 TCID₅₀) is as effective in inducing clinical infection in asthmatic and healthy subjects as the higher doses used in previous studies.

4.2 Subject demographics

Thirty two patients with asthma and 14 healthy volunteers were recruited and inoculated with RV-16. 7/46 (15%) subjects (3/14 mild asthmatics, 1/18 moderate asthmatic and 3/14 healthy subjects, \( P=\text{NS} \)) failed to develop an infection as evidenced by absent serum neutralising antibody to RV-16 at 6 weeks post inoculation and no detection of RV-16 in nasal lavage or BAL at any point after inoculation. These subjects appeared similar in baseline characteristics to those subjects successfully infected and the reason they failed to develop evidence of infection remains unclear. These subjects were therefore excluded from further analysis. The 39 successfully infected subjects included 28 asthmatics (11 mild, 17 moderate) and 11 healthy volunteers. Defining these asthmatics by baseline asthma control gave 12 well-controlled, 8 partially-controlled, and 8 poorly-controlled asthmatics. Baseline characteristics of the successfully infected subjects with the asthmatics subjects classified according to asthma severity and asthma control are shown in Table 4.1a & b.

There were no statistically significant differences in age and gender between healthy and asthmatic groups, and no significant difference between the asthma groups (whether defined by severity or baseline control) in baseline airway hyperresponsiveness, total IgE, or baseline eosinophil count in bronchoalveolar lavage. Baseline FEV₁ (% predicted) differed significantly between healthy and asthma groups and also according to asthma category (mild 93 ± 11, moderate 82 ± 10, \( P = 0.008 \);
well-controlled 95 ± 10, partially-controlled 78 ± 7, poorly-controlled 82 ± 10, \( P = 0.001 \) (Table 4.1 and figure 4.1).

All the mild asthmatics enrolled in this study were steroid naive and managed on intermittent short-acting bronchodilators alone. 15/17 (88%) moderate asthmatics were taking regular maintenance inhaled corticosteroids at an average dose of 427±71 mcg BDP. The remaining two asthmatics not on regular inhaled corticosteroids met criteria for inclusion in to the moderate group due to an FEV\(_1\) < 80% predicted at study entry. The proportion of asthmatics using inhaled steroids was greatest in the poorly-controlled group (7/8) compared to either the well-controlled (4/12) or partially-controlled group (5/8), however this difference was not statistically significantly despite a strong trend towards significance (\( P = 0.057 \)).

**4.3 Virologic Confirmation of RV-16 Infection.** All subjects were seronegative (absent neutralizing antibodies) for RV-16 at screening and on repeat serology performed on day 0 prior to inoculation. Rhinovirus infection was confirmed by positive nasal lavage qPCR for rhinovirus or seroconversion defined as a titre of serum neutralizing antibodies to RV16 of at least 1:4 at 6 weeks post-inoculation. Serology performed at 6 weeks showed a rise in RV16 neutralising antibody titre to \( \geq \) 1:4 for all but 3 subjects (2 healthy, 1 mild asthmatic). A more robust antibody response of \( \geq \)1:16 was seen in 31/39 subjects (8/11 healthy, 8/11 mild, 15/17 moderate asthmatics). See Appendix Table 1 for full results.

**4.4 Adverse Responses to Rhinovirus Inoculation**

After inoculation there were no adverse events, subject withdrawals nor any requirement for oral or systemic corticosteroids, antibiotics or hospital admission.
### Table 4.1a Baseline Demographic and Clinical Characteristics of Study Volunteers According to Asthma Severity

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy (N =11)</th>
<th>Mild Asthma (N = 11)</th>
<th>Moderate Asthma (N = 17)</th>
<th>P value Across all groups</th>
<th>Between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>31±12</td>
<td>33±11</td>
<td>37±10</td>
<td>0.317</td>
<td>-</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>36</td>
<td>64</td>
<td>47</td>
<td>0.434</td>
<td>-</td>
</tr>
<tr>
<td>Male</td>
<td>74</td>
<td>36</td>
<td>53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline FEV(_1) Percent of predicted value</td>
<td>104±18</td>
<td>93±11</td>
<td>82±10</td>
<td>&lt;0.001</td>
<td>H v’s mild: 0.04 H v’s mod: &lt;0.001 mild v’s mod: 0.008</td>
</tr>
<tr>
<td>Baseline histamine PC(_{20}) (mg/mL)</td>
<td>-</td>
<td>1.24±1.98</td>
<td>1.26±2.10</td>
<td>0.966</td>
<td></td>
</tr>
<tr>
<td>Baseline asthma control (ACQ)</td>
<td>-</td>
<td>0.69±0.44</td>
<td>1.38±0.54</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Use of inhaled corticosteroids (% of subjects)</td>
<td>-</td>
<td>0</td>
<td>15 (88)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Daily dose of ICS BDP or equivalent (mcg)</td>
<td>-</td>
<td>-</td>
<td>427±71</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IgE IU/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median Interquartile range</td>
<td>16</td>
<td>207</td>
<td>132</td>
<td>&lt;0.001</td>
<td>H v’s mild: &lt;0.001 H v’s mod, &lt;0.001 mild v’s mod: 0.46</td>
</tr>
<tr>
<td>BAL Eosinophilia (%)</td>
<td>0</td>
<td>0.7</td>
<td>0.3</td>
<td>0.006</td>
<td>H v’s mild: 0.005 H v’s mod: 0.02 mild v’s mod: 0.71</td>
</tr>
<tr>
<td>Skin prick test responses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Number of positive responses ≥3mm (% subjects)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grass</td>
<td>-</td>
<td>6 (55)</td>
<td>10 (59)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDM</td>
<td>-</td>
<td>10 (91)</td>
<td>11 (65)</td>
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<tr>
<td>Mugwort</td>
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<td>1 (9)</td>
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<td>Cladosporium</td>
<td>-</td>
<td>0 (0)</td>
<td>1 (6)</td>
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<td>Alternaria</td>
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<td>1 (9)</td>
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<td>Birch</td>
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<td>3 (27)</td>
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<td>Three tree</td>
<td>-</td>
<td>1 (9)</td>
<td>3 (18)</td>
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<tr>
<td>Cat</td>
<td>-</td>
<td>4 (36)</td>
<td>11 (65)</td>
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<tr>
<td>Dog</td>
<td>-</td>
<td>1 (9)</td>
<td>4 (24)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
# Table 4.1b Baseline Demographic and Clinical Characteristics of Study Volunteers According to Asthma Control

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Well controlled ACQ: ≤0.75 (N = 12)</th>
<th>Partially Controlled ACQ: 0.76-1.49 (N = 8)</th>
<th>Poorly controlled ACQ ≥1.50 (N = 8)</th>
<th>P value Across all groups</th>
<th>Between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>33±12</td>
<td>38±10</td>
<td>36±10</td>
<td>0.542</td>
<td>-</td>
</tr>
<tr>
<td>Sex (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>75</td>
<td>50</td>
<td>25</td>
<td>0.087</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline FEV₁ Percent of predicted value</td>
<td>95±10</td>
<td>78±7</td>
<td>82±10</td>
<td>0.001</td>
<td>well v’s part: 0.001 well v’s poor: 0.02 part v’s poor: 1.0</td>
</tr>
<tr>
<td>Baseline histamine PC₂₀ (mg/mL)</td>
<td>1.55±1.95</td>
<td>0.29±0.61</td>
<td>1.78±2.78</td>
<td>0.277</td>
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<tr>
<td>Baseline asthma control (ACQ)</td>
<td>0.56±0.27</td>
<td>1.18±0.15</td>
<td>1.86±0.32</td>
<td>&lt;0.001</td>
<td>Well v’s part: &lt;0.001 Well v’s poor:&lt;0.001 Part v’s poor: &lt;0.001</td>
</tr>
<tr>
<td>Use of inhaled corticosteroids (% of subjects)</td>
<td>4(33)</td>
<td>5 (63)</td>
<td>7 (88)</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>IgE IU/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>95</td>
<td>356</td>
<td>152</td>
<td>0.183</td>
<td></td>
</tr>
<tr>
<td>Interquartile range</td>
<td>65-212</td>
<td>122-1105</td>
<td>52-710</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL Eosinophilia (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>0.3</td>
<td>1.3</td>
<td>0.3</td>
<td>0.225</td>
<td></td>
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<tr>
<td>Interquartile range</td>
<td>0-1.0</td>
<td>0.3-3.0</td>
<td>0-1.8</td>
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<td></td>
</tr>
<tr>
<td>Skin prick test responses Number of positive responses ≥3mm (% subjects)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grass</td>
<td>8 (67)</td>
<td>4 (50)</td>
<td>4 (50)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>HDM</td>
<td>8 (67)</td>
<td>6 (75)</td>
<td>7 (88)</td>
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<td>2 (17)</td>
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Figure 4.1 Baseline FEV1 according to asthma severity (a) and asthma control category (b). +P<0.05; ++P<0.01; +++P<0.001.
4.5 Upper Respiratory Symptom Scores

The classic symptoms of a common cold became evident in the majority of healthy and asthmatic subjects within 48 hours of inoculation and most commonly began with a sore throat. Symptoms were significantly elevated above baseline on days 3-10 in the healthy group (all \( P < 0.05 \)) and on days 2-12 in the asthma group (all \( P < 0.05 \)). The peak intensity of symptoms in both groups occurred on day 4 post-inoculation, however, mean symptom scores on days 4-6 as well as day 8, and days 11-14 were significantly higher in the asthma group than in healthy subjects (figure 4.2) suggesting both a more intense and also more prolonged cold for many asthmatics.

Figure 4.2 Daily upper respiratory symptom score following rhinovirus inoculation. Asthma v\'s Healthy, +\( P<0.05 \); ++\( P<0.01 \); +++\( P<0.001 \).

Analysis of peak upper respiratory symptom scores along with total 14 day scores (equivalent to area under the curve) for each subject revealed similar peak levels for mild and moderate asthma (figure 4.3a), and for each of the asthma control groups (figure 4.4a). However a clear trend for
greater total scores for moderately severe and poorly-controlled asthmatics can be seen suggesting an overall greater burden of symptoms over the course of infection (figures 4.3b and 4.4b).

**Figure 4.3** Peak and total upper respiratory symptom scores according to asthma severity. 
$+P < 0.05; ++P < 0.01$. 

![Graph showing peak and total upper respiratory symptom scores](image-url)
Figure 4.4 Peak and total upper respiratory symptom scores according to baseline asthma control. $+P<0.05$; $++P<0.01$. 
4.6 Lower Respiratory Symptom Scores

Asthmatics experienced substantially greater lower respiratory symptom scores compared to healthy subjects following inoculation with RV-16. Compared to healthy subjects, daily lower respiratory symptom scores were significantly greater on days 3-6 for the mild asthmatics and on days 3-13 for the moderate asthmatics (all $P<0.05$) (figure 4.5a). In addition, symptom scores were significantly greater for the moderate asthmatics compared to the mild asthmatics on days 8, 10, 11 and 13 highlighting a more prolonged, severe exacerbation in these subjects (all $P<0.05$).

Defined instead by asthma control category, the poorly-controlled sub-group experienced increased daily lower respiratory symptoms following inoculation compared to the well- and partially-controlled asthmatics, with an earlier symptom peak (day 4) and more sustained duration of symptoms (days 7-13) (figure 4.5b). Indeed the peak symptom score for poorly-controlled subjects was significantly greater than observed in asthmatics with better baseline control (all $P<0.05$). The difference in peak lower respiratory symptom score was not statistically significant between moderate and mild asthmatics (figure 4.6).

In contrast, total lower respiratory symptoms scores (summation of daily scores over the 14 day period post inoculation) were significantly greater in moderate asthmatics than in both healthy subjects (45.2±6.2 vs 1.45±3.5, $P<0.001$) and mild asthmatic subjects (45.2±6.2 vs 20.3±4.9, $P=0.004$) and in mild asthma vs healthy subjects (20.3±4.9 vs 1.45±3.5, $P=0.005$) (figure 4.7a).

Total lower respiratory scores in the poorly-controlled sub-group (59.8±8.6) were almost three times the scores of the well-controlled (21.3±5.5, $P=0.001$), and almost twice those of the partially-controlled (32.5±6.3, $P=0.02$) groups (figure 4.7b). It is also noteworthy that the mean total lower respiratory score for poorly-controlled asthmatics was substantially higher than for the larger moderate asthmatic group (from which they were also part) suggesting that these subjects were at the most severe end of the moderate group.
Figure 4.5  Daily lower respiratory symptom scores in healthy and asthmatic subjects defined by baseline asthma severity (a) and baseline asthma control category (b).

Panel a: *P<0.05; **P<0.01; ***P<0.001 asthma v’s healthy subjects; †P<0.05 moderate v’s mild asthma. Panel b: ††P<0.05, †††P<0.01 poorly-controlled v’s well-controlled asthma; #P<0.05; ##P<0.01 poorly-controlled v’s partially-controlled asthma.
Figure 4.6  Peak lower respiratory symptom scores in healthy and asthmatic subjects defined by baseline asthma severity (a) and baseline asthma control category (b).

+\(P<0.05\); ++\(P<0.01\); +++\(P<0.001\).
Figure 4.7 Total 14 day lower respiratory symptom scores in healthy and asthmatic subjects defined by baseline asthma severity (a) and baseline asthma control category (b). 

$+P<0.05; ++P<0.01; +++P<0.001$. 

![Diagram showing total 14-day lower respiratory symptom scores in healthy and asthmatic subjects defined by baseline asthma severity and control category.](image-url)
4.7 Changes in lung function with infection

A similar relationship between asthma severity, control and exacerbation severity was seen for changes in lung function during rhinovirus infection. Asthmatics experienced greater virus-induced falls in morning PEF and FEV\(_1\) than healthy subjects which were significantly greater for moderate-severe asthmatics than healthy subjects on days 3-9, 11 and 13 (all \(P<0.05\)). The greatest fall in PEF in the moderate asthmatic group occurred on day 5 and this was significantly greater than the fall in mild asthmatics on that day (17.4 ± 3.1% v 7.2 ± 3.5%, \(P = 0.045\)) (figure 4.8). The maximal fall in FEV\(_1\) during the infection period was also greatest for moderately-severe asthmatics compared to mild asthmatics (20.8 ± 2.04% v's 14.3 ± 1.3%, \(P = 0.032\)) and healthy subjects; (6.2 ± 1.2%, \(P<0.001\)) figure 4.9a).

The poorly-controlled asthmatics also had the greatest maximal fall in FEV\(_1\) (24.6 +/- 3.1% compared to 16.3 ± 1.7%, \(P = 0.025\) for well-controlled and 14.9 +/- 2.0%, \(P = 0.021\) for partially-controlled asthmatics) (figure 4.9b). This is despite the fact the partially-controlled and poorly-controlled asthmatics had similar baseline FEV\(_1\) at study entry (figure 4.1 and Table 4.1).

Unsurprisingly, a strong correlation was observed between maximal falls in PEF and FEV\(_1\) in asthma (\(r = 0.614, P = 0.001\)).
**Figure 4.8** Change in morning peak expiratory flow from baseline during the 14 day period following rhinovirus inoculation. Asthmatic subjects defined by baseline asthma severity (a) and baseline asthma control category (b).

Panel a: *P<0.05; **P<0.01; ***P<0.001 asthma v’s healthy subjects; †P<0.05 moderate v’s mild asthma. Panel b: †P<0.05 poorly-controlled v’s well-controlled asthma; ‡P<0.05 poorly-controlled v’s partially-controlled asthma.
Figure 4.9 Maximal fall in morning FEV₁ from baseline during the 14 day period following rhinovirus inoculation. Asthmatic subjects defined by baseline asthma severity (a) and baseline asthma control category (b). ++P<0.05; +++P<0.01; ++++P<0.001.
4.8 Effects of Virus Infection on Airway Hyperresponsiveness in Asthma

38/39 subjects performed a baseline histamine challenge however due to low FEV₁ levels (<70% predicted) on day 7 post-inoculation the test was not attempted in 6 asthmatics (1 mild, 5 moderate). None of the 11 healthy subjects achieved a 20% drop in FEV₁ following the top histamine dose of 16mg/mL at either time-point. Of the 22 asthmatics that completed baseline and day 7 tests, 16 (73%) had evidence of increased BHR on day 7 compared to baseline ($P = 0.003$). This increase was statistically significant in both mild ($P = 0.045$) and moderate ($P = 0.022$) asthma groups. However no significant differences between mild and moderate asthmatics (or between asthma control groups) were seen despite 88% of the moderate asthmatics using regular inhaled corticosteroid therapy throughout the infection. In view of the range of baseline asthma control of these subjects and the fact that it was not possible to perform a day 7 test in several of the most severely affected asthmatics, it is difficult to draw any firm conclusions from these results other than the general finding of virus-induced AHR in asthma.

**Figure 4.10** Bronchial hyperresponsiveness at baseline and during RV infection. +,$P<0.05$. 

![Histamine PC20 chart](image)
4.9 Assessment of Virus load

Virus load was measured in both bronchoalveolar lavage (BAL) on day 4 and in nasal lavage on days 2, 3, 4, 5, 7, and 10 post-inoculation. Unfortunately, levels in BAL were in general very low with only 3/11 healthy and 11/28 asthmatics measuring over 10,000 copies / mL. Consequently identifying any differences between asthma and healthy subjects and between the individual asthma sub-groups has not been possible.

Virus load in nasal lavage was seen to peak on day 3 in asthmatic subjects but not until day 4 in healthy subjects. On day 3, levels in asthma were significantly greater (nearly 250-fold) compared to healthy subjects (median day 3 copies/mL: $1.68 \times 10^6$ [1.60 x $10^4$ - 1.28 x $10^7$] v's 6.92 x $10^3$ [1.50 x $10^3$ -3.21x$10^6$], $P=0.042$). A trend for greater (~10-fold) day 2 levels was also seen in asthma ($P=0.058$) (figure 4.11a). The peak level of virus load was greater in asthma but again this was not statistically significant (figure 4.11b).

No statistically significant differences in virus load were observed when comparing asthma severity or control categories however greater median levels were seen in the moderately severe and poorly-controlled groups compared to milder and well-controlled subjects (figure 4.12).
Figure 4.11  Virus Load in nasal lavage. +, P<0.05 compared to healthy subjects; NS = non-significant.
Figure 4.12 Peak virus Load according to asthma sub-group. NS = non-significant
### 4.10 Relationships between virus load and clinical outcome

Relationships were observed between day 2 virus load and both upper and lower respiratory symptoms between days 2 - 4. These included relationships with upper respiratory symptoms on day 2 (all subjects, \( r=0.550, P<0.001 \); asthma only, \( r=0.623, P=0.001 \)), and day 3 (all subjects, \( r=0.485, P=0.002 \); asthma only, \( r=0.548, P=0.003 \)), whilst relationships with lower respiratory symptom scores in asthma were most significant on day 3 \( (r=0.640, P<0.001) \) and day 4 \( (r=0.547, P=0.003) \). No relationships between day 4 BAL virus load and symptom scores were observed.

In asthma, significant relationships were also observed between virus load and falls in lung function. Specifically, day 3 virus load correlated with maximal fall in PEF \( (r=-0.586, P=0.001) \), and day 7 PEF \( (r=-0.405, P=0.036) \), whilst peak virus load correlated with maximal fall in PEF \( (r=-0.501, P=0.008) \), as well as PEF on days 4 \( (r=-0.461, P=0.016) \), and 7 \( (r=-0.397, P=0.040) \). Trends towards significance were seen at several other time-points.

There were no relationships between virus load and other baseline clinical features including baseline FEV\(_1\) (% predicted), eosinophil count, total IgE or SPT results, and no correlation with virus load and the degree of AHR in asthma was observed.

Relationships between virus load and inflammatory mediators are discussed in chapter 5 (type 1 immune mediators) and chapter 6 (type 2 immune mediators).

### 4.11 Inflammatory cell measurements

Lower airway inflammation (measured in BAL) was increased during infection compared to baseline in asthma, and in asthma relative to healthy subjects, however, this was only statistically significant for eosinophils (figure 4.13). Median BAL eosinophil percentages: baseline asthma \( (0.5 [0.0-1.7]) \) v's day 4 \( (1.2 [0.0-3.8]), P=0.025 \); day 4 asthma v's day 4 healthy subjects \( (0.0 [0.0-0.7]), P=0.046 \). There were no significant differences in inflammatory cell number according to baseline severity, asthma control or treatment. Inflammatory cell proportions can be seen in full in the appendix.
The rhinovirus-induced eosinophilia is in keeping with significant inductions observed in both nasal and bronchial IL-5 in the asthmatic subjects in this study (chapter 6) and highlights the potential benefits of anti-IL-5 therapies such as Mepolizumab in reducing this element of virus-induced inflammation.

It is noteworthy that nine asthmatics (35%) had no eosinophils present at either time point (which included six subjects on maintenance inhaled corticosteroid therapy) stressing the need to target Mepolizumab at selected asthmatics only.

**Figure 4.13** Virus-induced bronchoalveolar lavage eosinophilia in asthma.

![Graph showing eosinophil levels in asthma and healthy subjects](image)

4.12 Discussion

Up until the time of this study, experience with the human model of experimental rhinovirus-induced asthma exacerbations has been limited to mild asthmatics adequately managed on intermittent short-acting bronchodilator therapy. One of the main aims of this study was to advance this model by challenging more severe and less well-controlled asthmatics. This therefore is the first time moderately-severe and poorly-controlled asthmatics have been challenged with rhinovirus in a
controlled setting permitting novel insights into the roles of severity and asthma control in the pathogenesis of virus-induced asthma exacerbations. The inoculation of 28 asthmatics also makes this the largest study of its kind to date, whilst the use of a low dose inoculum offers the advantage of mimicking a naturally-occurring infection more closely and therefore potentially making the findings more meaningful.

A significant degree of variability in the clinical, virological, and inflammatory response to rhinovirus was evident in this study. This is in keeping with both published prospective longitudinal cohort studies as well as controlled experimental infection studies. The observation that asthmatics developed more severe upper respiratory symptoms is however in contrast to the majority of studies of this kind which have on the whole reported similar increases in upper respiratory tract symptoms in both healthy subjects and asthmatics. Zambrano also reported significantly higher cold scores in asthmatics following experimental infection with rhinovirus, however Corne observed similar frequency, severity and duration of cold symptoms in asthmatic and normal subjects in a prospective study of naturally acquired rhinovirus colds, while Message and deMore both reported no significant increases in cold severity in asthmatic compared to normal subjects during experimental rhinovirus infections. The observations of more severe and prolonged colds in the asthmatic group reported in this study are therefore consistent with the Zambrano study and suggest that many asthmatics do indeed experience more severe colds than healthy subjects. The underlying mechanisms responsible for this are unclear but an increasing body of evidence points to a deficiency within the asthmatic airway of anti-viral type 1 (IFN-α, and -β) and type 3 (IFN-λ) interferons (discussed in chapter 1). Such a deficiency would be in keeping with the greater virus loads in asthma during the early stages of infection demonstrated here for the first time. Increased rhinovirus replication in asthma has been previously observed ex vivo and related to impaired production of anti-viral interferons, however this is the first experimental or natural infection study to observe statistically significantly increased virus loads in asthma during virus induced
exacerbations \textit{in vivo}. These data, along with relationships of multiple outcomes with virus load in this and other studies,\textsuperscript{68} highlight anti-viral approaches as alternative promising approaches for novel therapies for asthma exacerbations.

In previously published work (using virus inocula of between 50-300 x the dose used in this study) Grunberg\textsuperscript{80}, Cheung\textsuperscript{60} and Message\textsuperscript{68} all observed a peak in cold symptoms on day 2-4 post-inoculation with symptoms returning to baseline levels within one week in most subjects. In the present study, a day 4 peak suggests that the significantly smaller inoculum used has not greatly altered the kinetics of infection with at most a 24 hr delay to peak upper respiratory symptoms.

Interestingly, the peak of lower respiratory symptoms over days 4-7 in this study does not appear substantially different to published work either: Cheung recorded an increase in asthma symptoms on day 4 with a peak on days 6-11, whilst Message observed a peak between days 4-6. Grunberg did report an earlier peak between days 2-4 serving to highlight the significant variability in clinical response to rhinovirus that exists.

A return to baseline levels by day 13 was observed in all previous studies, with many subjects returning to normal levels within a week of inoculation. This is consistent with the results presented here from the healthy subjects and the milder, well-controlled asthmatics but is in contrast to the results for moderately-severe and poorly-controlled asthmatics who remained significantly more symptomatic than healthy subjects 2 weeks post-inoculation.

The variability that accompanies symptom scores is also evident with virus-induced changes in lung function. The asthmatics in this study experienced the greatest falls in lung function between days 5-7. This is in keeping with data from Bardin who observed a significant fall in PEF from baseline on days 4-9,\textsuperscript{65} from Message who reported a maximal fall in PEF on day 5 post-inoculation,\textsuperscript{68} and from Grunberg who also observed a significant decrease in FEV\textsubscript{1} after RV16 infection.\textsuperscript{80} However others have not shown any significant impact of rhinovirus infection on lung function: following the
inoculation of 7 mild asthmatics with RV16, Cheung did not observe any significant change in FEV₁ although a strong trend was observed ($P=0.06$); Fleming inoculated 11 mild asthmatics who similarly did not experience significant falls in FEV₁ or PEF, although did record a significant increase in asthma symptoms. It is interesting to note that the mean baseline FEV₁ (% pred) for the asthmatic subjects in the Cheung and Fleming studies were greater (95% and 92% predicted respectively) than in the Grunberg study (86% predicted) indicating that perhaps the baseline severity of the asthmatic subjects recruited to these studies influenced the clinical outcome in a similar fashion to the impact baseline severity has played in the current study. Indeed the 7 asthmatics recruited by Cheung were so well-controlled that only 4 of them required sporadic SABA for relief of symptoms – the remaining subjects were so mild that even occasional SABA was unnecessary.

A large number of relationships were observed between virus load and both symptom severity and falls in lung function suggesting virus load is driving exacerbation severity and highlighting the need for development of effective antiviral approaches.

Airway hyperresponsiveness is one of the cardinal features of asthma. Virus-induced AHR has been previously shown following both naturally occurring URTIs (of which 82% were RV), as well as in the context of experimental rhinovirus infection. Cheung observed increased responses to methacholine on days 2, 7 and 15 in atopic asthmatics compared to non-infected asthmatics; Grunberg observed increased responses to histamine on day 4 which was most pronounced in those with a severe cold; whilst Message noted similar findings when a histamine challenge was repeated on day 6 post-inoculation. Interestingly, in a study of atopic non-asthmatics with active allergic rhinitis, Gern observed similar results to those seen in asthma indicating that allergy in the absence of asthma was also sufficient to influence lower airway responses to rhinovirus.

The current study is the first to have measured virus-induced AHR in moderately-severe and poorly-controlled asthmatics, and the first to attempt an analysis of how severity and control affect this.
Unfortunately, due to safety concerns it was not possible to perform the histamine challenge on several of the most-severely affected asthmatics making this analysis incomplete. It has been possible to confirm the previously published reports of increased AHR during rhinovirus infection and at the same time to show that in many cases treatment with inhaled corticosteroids failed to prevent this increase. However, as many of the asthmatics were on inadequate doses of inhaled corticosteroids by the very nature of them being inadequately controlled at baseline one cannot infer the complete ineffectiveness of inhaled corticosteroids in this regard. In addition whilst adherence to the inhaled corticosteroids was stressed at every possible opportunity, there is no way to be 100% certain that all the asthmatics were fully compliant with their medication.

It has also been possible to demonstrate a significant increase in BAL eosinophilia in asthmatic but not healthy subjects on day 4 post-inoculation. This is consistent with the IL-5 induction by rhinovirus shown in chapter 6 and supports the use of anti-IL-5 therapies such as Mepolizumab in selected asthmatics. As there was only a single infection time-point for bronchial sampling in this study it is likely that a significant increase in other inflammatory cells may have been shown had sampling been a day or two later. At the same time the extent of the eosinophilia may have been considerably greater later in the infection than was seen on day 4.

The observations presented here of more severe and more prolonged exacerbations in moderately-severe and poorly-controlled asthmatics compared to those with mild and well-controlled disease are completely novel yet are consistent with the results of a multitude of unrelated studies including various trials of asthma medications. Indeed if one accepts that the majority of exacerbations are secondary to virus infections, how then can the ability of asthma medications (including inhaled steroids, long-acting β-agonists and others with no significant anti-viral properties) to reduce the frequency of severe exacerbations be explained? In all cases where exacerbation frequency is reduced, asthma control is also improved over the course of the study - either because of the drug or simply by better adherence to their standard medication (hence placebo effect). Extrapolating
these findings to the current study, it is reasonable to suggest that the same asthmatics recruited here would experience less severe exacerbations if their asthma was more optimally controlled first. The results presented here are also supported by a recent retrospective pooled analysis of 5 studies by Bateman et al. demonstrating the influence of current asthma control on the risk of future exacerbations.\textsuperscript{227} The patients in those studies represent the more severe end of the asthma spectrum and the trigger of the exacerbations in those studies was unknown. Our results also support findings of the GOAL study in which unscheduled healthcare resource utilisation for exacerbations related to the level of control achieved rather than the treatment received.\textsuperscript{228} Taken together, the data presented here demonstrates that whilst all asthmatics (including well-controlled) have increased virus-induced lower respiratory morbidity compared to healthy subjects, poor control and increased asthma severity are each associated with even greater virus-induced respiratory morbidity. This highlights the importance of maintaining good asthma control in reducing future risk of severe asthma exacerbations.
5.1 Introduction

Viruses stimulate the differentiation of antigen-activated CD4+ T cells into Th1 effectors. The innate immune reactions that they elicit are associated with the production of a number of cytokines including (and perhaps most importantly) IL-12 which promote Th1 development. The principal sources of IL-12 are activated dendritic cells and macrophages. In addition to promoting differentiation of Th1 cells, it stimulates IFN-γ production by T cells and NK cells, and enhances NK cell and CTL-mediated cytotoxicity.

Th1 cells secrete IFN-γ which promotes further Th1 differentiation amplifying the reaction whilst at the same time acting on dendritic cells and macrophages to induce more IL-12 secretion. Both IFN-γ and IL-12 stimulate Th1 differentiation by activating the transcription factors T-bet, STAT1 and STAT4. Of these T-bet is considered the master regulator of Th1 differentiation. T-bet is induced in naive CD4+ cells in response to IFN-γ. IFN-γ also activates STAT1 which in turn stimulates further expression of T-bet setting up a positive amplification loop. In addition IFN-γ inhibits the differentiation of naive CD4+ T cells to the Th2 (and Th17) subset promoting polarisation of the immune response.

Such polarisation of the Th1 subset leads to up-regulation of the chemokine receptor CXCR3 on the surface of the T cells which serve to bind the chemokines MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11. All three chemokines are induced by IFN-γ and are therefore involved in the promotion of the Th1 response.

IL-15 is an additional cytokine that promotes Th1 polarisation. It serves important growth-stimulating and survival functions for both T cells and NK cells, and not only is it induced by IFN-γ but
when presented by dendritic cells to NK cells activates signalling pathways that promote NK cell IFN-γ production.

Therefore, the cytokines IL-12, IL-15, and IFN-γ as well as the chemokines IP-10/CXCL10 and I-TAC/CXCL11 together make up a major part of the mediator network involved in virus-induced type 1 inflammation. However, our knowledge of these mediators in relation to rhinovirus infection is asthma is extremely limited with almost no published human in-vivo data in this area with the exception of IP-10/CXCL10.

Interestingly there is some data to suggest a deficient type 1 immune response in response to respiratory virus infection in asthma. Specifically, deficient production of IFN-γ and IL-12 in peripheral blood and/or nasal lavage has been hypothesised to be relevant to the pathogenesis of virus induced wheezing illness in infants. Whilst studies in adults with asthma or allergic rhinitis have also implicated relative deficiencies in type 1 immune responses in both sputum and blood in increasing severity of rhinovirus induced illness or in impaired RV clearance. However these studies failed to identify deficient type 1 immune responses in asthmatic relative to healthy subjects.

More recently, Message identified deficient IFN-γ and IL-12 production from bronchoalveolar lavage cells from asthmatic subjects, following in-vitro stimulation with virus. Thus, measurement of the type 1 immune response to rhinovirus in-vivo was a major aim of this study.

Using the techniques of nasosorption and bronchosorption to measure IL-12, IL-15, IFN-γ, IP-10/CXCL10, and I-TAC/CXCL11, the hypotheses that rhinovirus induces these mediators in-vivo and that their levels relate to cold and exacerbation severity in asthma were assessed. The additional hypothesis that there is evidence of a dysregulated / deficient type 1 immune response in asthma was also tested.
5.2 Levels of IL-12

In the lung, IL-12 levels were similar in asthma and healthy subjects both at baseline (healthy baseline bronchial 32.2 pg/mL [17.4 - 39.8] v's asthma 25.8 pg/mL [22.8 - 44.36], \( P = 0.97 \)) and on day 4 (healthy day 4 bronchial 24.1 pg/mL [18.3 - 31.5] v's asthma 35.9 pg/mL [18.8 - 47.2], \( P = 1.00 \)) with no significant induction on day 4 in either the healthy (\( P = 0.33 \)) or asthma (\( P = 0.14 \)) group. In contrast, analysis of nasal IL-12 during infection revealed a significant induction in both groups with similar peak levels (healthy baseline nasal 10.8 pg/mL [7.3 - 20.4] v's infection peak 24.9 pg/mL [14.9 - 35.7], \( P = 0.005 \); asthma baseline nasal 11.7 pg/mL [8.0 - 16.1] v's infection peak 20.3 pg/mL [14.3 - 28.2], \( P < 0.001 \); healthy peak v's asthma peak, \( P = 0.39 \)) (figure 5.1). In both groups a significant induction above baseline was seen on day's 2 and 3 only (kinetics discussed in section 5.18). Although median levels were greater on these (and all other) days during infection in healthy subjects compared to asthma, these differences were not statistically significant.

**Figure 5.1** Levels of IL-12 in nasal and bronchial mucosal lining fluid. +++\( P < 0.01 \); +++\( P < 0.001 \).
5.3 Relationships between IL-12 and clinical outcome in asthma

In the nose, IL-12 on day 4 correlated significantly with both peak upper \( r = 0.500, P = 0.008 \), total upper \( r = 0.411, P = 0.033 \), and peak lower respiratory score \( r = 0.472, P = 0.013 \) (figure 5.2). Significant relationships with symptom scores were also observed for IL-12 on day 3 and 7 (all \( P < 0.05 \)). In the lung, day 4 bronchial IL-12 failed to show any significant associations with clinical outcome measures.

Figure 5.2 Relationships between IL-12 and respiratory symptom scores during rhinovirus infection in asthma.

5.4 IFN-γ levels

Baseline nasal and bronchial levels of IFN-γ were similar between asthmatic and healthy subjects, however a trend towards greater baseline bronchial levels was seen in healthy subjects (healthy, 1.0 pg/mL [0.7 - 1.5] v’s asthma 0.7 pg/mL [0.6 - 1.2], \( P = 0.08 \)). In both healthy and asthmatic subjects peak infection levels in the nose were significantly increased above baseline (healthy day 0, 0 pg/mL [0 - 1.8], peak nasal 22.8 pg/mL [1.0 - 72.4], \( P = 0.16 \); asthma day 0, 0 pg/mL [0 - 0.9], \( P < 0.001 \)), however there were no significant differences in the daily or peak median nasal levels between the
two groups. In asthma, the increase with infection observed in the nose was already significant on
day 2, and remained significantly elevated until day 10 post-inoculation (all $P < 0.01$). In the lung, a
significant increase with infection above baseline was only observed in asthma (healthy, $P = 0.8$;
asthma $P = 0.014$) (figure 5.3).

Figure 5.3 Levels of IFN-γ in nasal and bronchial mucosal lining fluid. +$P<0.05$; +++$P<0.001$.

5.5 Relationships between IFN-γ and clinical outcome in asthma

In asthma, relationships between IFN-γ and clinical outcome were observed for both nasal and
bronchial levels. In the nose, peak nasal IFN-γ correlated significantly with total upper ($r = 0.406$, $p =
0.036$), total lower ($r = 0.502$, $p = 0.008$) and peak lower respiratory symptom scores ($r = 0.454$, $p =
0.018$), with levels on each of days 2 to 4 also significantly correlating with lower respiratory
symptom scores (all $P < 0.05$).

In the asthmatic lung, day 4 bronchial IFN-γ was again seen to correlate with both upper and lower
respiratory symptom scores including total upper ($r = 0.463$, $p = 0.02$), total lower ($r = 0.368$, $p =
0.07$), and peak lower ($r = 0.463$, $p 0.02$) (figure 5.4).
5.6 Levels of IL-15

Analysis of IL-15 revealed similar baseline nasal and bronchial level in asthma and healthy groups. In asthma, a trend towards a significant induction in the lung was observed (asthma baseline bronchial 0.8 pg/mL [0.6 - 1.3], day 4, 1.2 pg/mL [0.8 - 1.7], $P = 0.058$), which was not seen in healthy subjects (healthy baseline bronchial 0.9 pg/mL [0 - 1.2], day 4 1.1 pg/mL [0.6 - 1.2], $P = 0.96$). However, as can be seen, levels were extremely low in both groups and at both time-points.

In the nose, a significant induction was seen in both groups (healthy baseline nasal 0.9 pg/mL [0.7 - 2.1] v’s peak infection 4.0 pg/mL [1.9 - 5.7], $P = 0.004$; asthma baseline nasal 1.7 pg/mL [1.0 - 2.1], $P = 0.005$).
peak infection 5.0 pg/mL [3.1 - 7.4], \( P < 0.001 \). In asthma, this increase was significant from days 3 - 10 (all \( P < 0.05 \)) with day 4 levels being significantly higher than in the healthy group (day 4 nasal, healthy 1.2 pg/mL [0.8 - 4.8 pg/mL]; asthma 2.7 pg/mL [1.5 - 4.9 pg/mL], \( P = 0.046 \)) (figure 5.5).

**Figure 5.5** Levels of IL-15 in nasal and bronchial mucosal lining fluid. +++\( P < 0.01 \); +++\( P < 0.001 \).

### 5.7 Relationships between IL-15 and clinical outcome in asthma

In asthma, day 4 nasal IL-15 correlated significantly with total upper respiratory symptom score \( (r = 0.485, P = 0.010) \), whilst a strong trend towards significance was observed for total lower respiratory symptom score \( (r = 0.378, P = 0.052) \) (figure 5.6). However a significant relationship with lower respiratory symptoms was reached with peak nasal IL-15 levels in asthma \( (r = 0.420, P = 0.029) \).
**Figure 5.6** Relationships between day 4 nasal IL-15 and respiratory symptom scores during rhinovirus-induced asthma exacerbations.

5.8 Levels of IP-10/CXCL10

Baseline levels of IP-10/CXCL10 in nasal and bronchial mucosal lining fluid levels were similar in asthma and healthy groups. In the lung, a significant increase in IP-10/CXCL10 levels were observed during infection in asthma but not healthy subjects (healthy baseline 1016.6 pg/mL [665.9 - 3413.5], day 4 2277.7 pg/mL [1650.9 - 3770.3], \( P = 0.33 \); asthma baseline 1011.7 pg/mL [471.2 - 1458.2], day 4 1998.8 pg/mL [1103.9 - 4832.1], \( P < 0.001 \)), however despite this the day 4 bronchial level were not significantly different between the two groups, with median levels actually being numerically greater in healthy subjects compared to asthma (healthy day 4 bronchial, 2277.7 pg/mL v’s asthma 1998.8 pg/mL, \( P = 0.77 \)). In contrast to the lung, a significant induction of nasal IP-10/CXCL10 was seen in both healthy and asthmatic subjects with a trend towards greater peak levels in asthma reflecting more severe infections (healthy baseline nasal 945.7 pg/mL [381.9 - 2083.0] v’s infection peak 4058 pg/mL [3017.0 - 4182.0], \( P = 0.004 \); asthma baseline nasal 1966.1 pg/mL [1230.3 - 3483.2] v’s infection peak 4859 pg/mL [3985.8 - 7287.5], \( P < 0.001 \); healthy peak to asthma peak, \( P = 0.07 \)) (figure 5.7). Comparison of daily nasal levels saw significantly greater levels in asthma on days 2-4 (all \( P < 0.05 \)) with the greatest levels seen on day 3 (healthy 2256.6 pg/mL [598.7 – 4096.9 pg/mL] v’s asthma 3660.6 pg/mL [2616.7 – 6473.7 pg/mL], \( P = 0.046 \).
**Figure 5.7** Levels of IP-10/CXCL10 in nasal and bronchial mucosal lining fluid. ++P<0.01; +++P<0.001.

5.9 Relationships between IP-10/CXCL10 and clinical outcome in asthma

In contrast to the mediators already discussed for which relationships with cold and exacerbation severity were most frequently evident for cytokine levels early in the course of infection (between days 2-4 post-inoculation), the most significant relationships between IP-10/CXCL10 and clinical outcome measures were observed on days 5 - 10 suggesting that persistently elevated levels (rather than an early peak) were more relevant to infection severity. For example, nasal levels of IP-10/CXCL10 on days 2 - 4 each failed to correlate with either peak or total upper respiratory symptom score in asthma, whereas significant relationships with peak scores were seen on day 5 (r = 0.434, P = 0.024), day 7 (r = 0.471, P = 0.015), and day 10 (r = 0.392, P = 0.043). Similarly, significant relationships with total upper respiratory symptom scores were also observed on day 5 (r = 0.407, P = 0.035), day 7 (r = 0.527, P = 0.006), and day 10 (r = 0.546, P = 0.003). Furthermore levels on these days also correlated with exacerbation severity in asthmatics: Relationships with total lower respiratory symptom scores were observed on day 5 (r = 0.423, P = 0.028), day 7 (r = 0.485, P = 0.012), and day 10 (r = 0.418, P = 0.030) (figure 5.8).
Figure 5.8  Relationships between IP-10/CXCL10 and respiratory symptom scores during rhinovirus infection in asthma
5.10 Levels of I-TAC/CXCL11

Both baseline nasal and bronchial levels of I-TAC/CXCL11 as well as day 4 bronchial levels were similar between asthmatic and healthy groups. However the day 4 bronchial level in asthma did represent a significant increase over baseline levels (healthy baseline 24.5 pg/mL [16.5 - 163.6] v’s day 4 77.8 pg/mL [42.5 - 203.9], P = 0.29; asthma baseline 19.4 pg/mL [11.9 - 33.4] v’s day 4 119.8 pg/mL [24.8 - 561.3], P < 0.001). Peak nasal levels showed a similar induction over baseline in both groups (healthy baseline nasal, 14.5 pg/mL [3.5 - 36.7] v’s infection peak, 1243 pg/mL [294.0 - 2768.0], P = 0.008; asthma baseline nasal, 39.3 pg/mL [14.3 - 97.4] v’s infection peak, 2070.5 pg/mL [1165.5 - 2830.3], P < 0.001; healthy peak to asthma peak, P = 0.32). However, individual daily levels were greater in asthma than healthy subjects on 2 (healthy 28.8 pg/mL [1.8 - 58.4 pg/mL]; asthma 113.9 pg/mL [29.4 - 492.5 pg/mL], P = 0.032), day 4 (healthy, 87.8 pg/mL [8.5 - 771.1 pg/mL]; asthma 681.2 pg/mL [85.5 - 2492.8 pg/mL], P = 0.027), and day 5 (healthy 285.1 pg/mL [25.4 - 436.4]; asthma 627.0 pg/mL [186.4 - 2103.6], P = 0.031).

Figure 5.9 Levels of I-TAC/CXCL11 in nasal and bronchial mucosal lining fluid. ++P<0.01; +++P<0.001.
5.11 Relationships between I-TAC/CXCL11 and clinical outcome in asthma

In asthma, levels of I-TAC/CXCL11 related significantly to the severity of the cold and asthma exacerbation: On day 4 post-inoculation, significant relationships were observed between total upper respiratory symptom score and both nasal ($r = 0.438, P = 0.022$) and bronchial I-TAC/CXCL11 ($r = 0.438, P = 0.22$) levels. Nasal levels on days 2 and 3 also related significantly to total lower respiratory symptoms in asthma (both $P < 0.05$) (figure 5.10).

**Figure 5.10** Relationships between I-TAC and respiratory symptom scores during rhinovirus-induced asthma exacerbations.
5.12 Relationships between type 1 mediators and respiratory symptoms in healthy subjects

Of the Th1 mediators discussed so far, only IL-12 was found to correlate significantly with upper respiratory symptoms: Peak upper respiratory symptom score correlated with nasal IL-12 on day 2 ($r = 0.694$, $P = 0.018$), day 3 ($r = 0.745$, $P = 0.009$), and day 5 ($r = 0.779$, $P = 0.005$), whilst peak nasal IL-12 level correlated with both peak upper respiratory symptoms ($r = 0.782$, $P = 0.004$) as well as total upper respiratory symptoms ($r = 0.609$, $P = 0.047$) (figure 5.11).

Additional trends towards significance were noted between peak upper respiratory score and nasal I-TAC/CXC11 on day 3 and 4 (both $P = 0.054$) as well as for day 3 IFN-$\gamma$ ($P = 0.09$).

Although multiple relationships were evident between nasal IL-12 and cold severity, none were observed for bronchial IL-12 levels during infection (despite measurable levels in all healthy subjects). This may simply reflect limited induction in the lung of healthy subjects associated with the minimal lower respiratory symptomatology that they experience, however significant relationships were observed between peak lower respiratory symptom score and both day 4 bronchial IP-10/CXCL10 and I-TAC/CXCL11 in healthy subjects (both $r = 0.798$, $P = 0.006$). These relationships were driven by 3/11 healthy subjects that had recorded lower respiratory symptoms during infection.

**Figure 5.11** Relationships between peak IL-12 levels and cold severity in healthy subjects.
5.13 Relationships between type 1 mediators and virus load

In the healthy lung, trends towards significance were observed between BAL virus load and both day 4 bronchial IFN-γ (r = 0.626, P = 0.053) and day 4 bronchial IP-10/CXCL10 (r = 0.571, P = 0.084).

In the asthmatic lung, these relationships reached significance (BAL VL and day 4 bronchial IFN-γ, r = 0.443, P = 0.027; BAL VL and day 4 IP-10/CXCL10, r = 0.515, P = 0.008). Additionally BAL VL correlated with day 4 bronchial I-TAC/CXCL11 (r = 0.525, P = 0.007).

In addition, peak nasal virus load levels in asthma correlated significantly with several Th1 mediators in the lung on day 4 including IFN-γ (r = 0.547, P = 0.005), IL-15 (r = 0.425, P = 0.034), and IL-12 (r = 0.439, P = 0.028) strengthening the association further between virus replication and induction of type 1 immunity.

Figure 5.12 Relationships between type 1 mediators and virus load.

5.14 Relationships between type 1 mediators and atopy

There were no significant relationships between the degree of atopy at study entry (as measured by serum total IgE and skin prick test responses) and baseline levels of type 1 mediators in the airway. However, in asthma, the degree of atopy correlated inversely with the extent of type 1 mediator
induction by rhinovirus. Statistically significant relationships were observed between serum total IgE and day 2 nasal IFN-γ (r = -0.460, P = 0.016), day 3 nasal IFN-γ (r = -0.386, P = 0.042), day 3 nasal IL-12 (r = -0.470, P = 0.012), day 4 nasal IL-12 (r = -0.424, P = 0.025), day 5 nasal IL-15 (r = -0.386, P = 0.043), and peak nasal IL-15 levels (r = -0.423, P = 0.025) (figure 5.13). There were no relationships between IgE levels in healthy subjects.

Similar inverse relationships were also observed between total SPT response (mm) and various measurements of type 1 mediators during infection including day 5 IL-15 (r = -0.395, P = 0.037), peak nasal IL-15 (r = -0.392, P = 0.039), and day 7 I-TAC/CXCL11 (r = -0.437, P = 0.023).

Figure 5.13  Relationships between baseline IgE and Th1 mediator levels during infection.

5.15 Relationships between baseline asthma severity and control and type 1 mediator levels

No significant differences in median levels were observed between mild and moderate asthmatics or between the asthma control groups for any of the type 1 mediators measured. The ACQ score also failed to correlate with mediator levels either at baseline or during infection.
5.16 Relationship between nasal and bronchial levels of type 1 mediators

Basal levels of type 1 mediators in both the nose and lung were in many case below limits of detection and so relationships for some cytokines were impossible to assess. However levels of the Th1 chemokines IP-10/CXCL10 and I-TAC/CXCL11 were detectable in all study subjects at baseline. Analysis of all baseline bronchial measurements with baseline nasal levels revealed a significant relationship (all subjects, IP-10/CXCL10 \( r = 0.407, P = 0.014 \); I-TAC/CXCL11, \( r = 0.356, P = 0.033 \)) (figure 5.14). These relationships remained significant when asthmatics were analysed in isolation (IP-10/CXCL10 \( r = 0.402, P = 0.046 \); I-TAC/CXCL11, \( r = 0.445, P = 0.026 \)).

During infection, a strong relationship was also apparent between inductions of type 1 mediators in the nose with that in the lung. Specifically, in asthma, a significant relationship was observed between day 4 bronchial and peak nasal levels for IFN-\( \gamma \) (\( r = 0.695, P < 0.001 \)), IL-12 (\( r = 0.452, P = 0.023 \)), IL-15 (\( r = 0.639, P = 0.001 \)), IP-10/CXCL10 (\( r = 0.489, P = 0.013 \)) and I-TAC/CXCL11 (\( r = 0.452, P = 0.023 \)) (figure 5.14). Such relationships were absent during infection in healthy subjects reflecting the limited lower respiratory involvement in non-asthmatics.

**Figure 5.14** Relationships between bronchial and nasal IP-10/CXCL10 levels at baseline and during infection.
5.17 Relationship between baseline type 1 inflammation and virus-induced type 1 inflammation

In the lung, there were no significant relationships between the basal level of type 1 mediators and the degree of their induction on day 4 post-inoculation. This was the case both for healthy and asthmatic groups. Interestingly however, there was a relationship between baseline nasal levels of IP-10/CXCL10 and their peak induction levels. This was seen in both healthy \( (r = 0.793, P = 0.004) \) and asthmatic \( (r = 0.423, P = 0.025) \) groups (figure 5.x). In asthma, a similar relationship was seen for IL-12 \( (r = 0.431, P = 0.022) \). There were no such relationships for any of the other type 1 mediators.

**Figure 5.15** The relationship between baseline and peak infection levels of IP-10/CXCL10.

5.18 Kinetics of type 1 mediator induction with rhinovirus in asthma

In asthma, nasal levels of IL-12 and IFN-γ both reach a peak on day 3 post-inoculation with a significant rise above baseline already observed by day 2 for both cytokines. However whereas IL-12 returned to near baseline levels on day 4, IFN-γ remained significantly elevated above baseline for the duration of nasal sampling (until at least day 10). IL-15 appeared delayed by approximately 24 hours in comparison to IFN-γ and IL-12 with a significant increase above baseline not seen until day 3. As with IFN-γ, levels remained significantly elevated above baseline until at least day 10.
Levels of both chemokines were significantly elevated above baseline by day 2, peaking on day 3 and remaining significantly elevated above baseline for the duration of infection. In contrast to the cytokines, near peak levels were maintained for approximately 3 days.

It should be noted that a degree of variation in the kinetic profile of these mediators exists between study subjects. This is illustrated in figure 5.x and discussed further in chapter 6.

5.19 Relationships between the type 1 mediators

In support of the close functional relationship between the different type 1 mediators, extremely strong relationships were observed in both the lung (in asthma) and in the nose (both healthy and asthma). It is noteworthy that the strongest relationship in this entire study was between the two Th1 chemokines ($r = 0.967, P < 0.001$) followed closely by relationships between the Th2 chemokines MDC and TARC, and the relationship between IL-5 and IL-13 (discussed in chapter 6).

Very strong relationships were also seen between the other type 1 cytokines including day 4 bronchial IL-12 and IFN-γ ($r = 0.856, P < 0.001$), IFN-γ and IL-15 ($r = 0.856, P < 0.001$), as well as between IFN-γ and the chemokines (IP-10/CXCL10, $r = 0.690, P < 0.001$; I-TAC/CXCL11, $r = 0.779, P < 0.001$) (figure 5.x).
Figure 5.16  Kinetics of type 1 mediator induction following rhinovirus infection in asthma. All asthmatics (A); examples of individual asthmatics (B,C).
Figure 5.17 Relationships between different mediators of type 1 inflammation during RV infection in the asthmatic lung.

5.20 Discussion

The results reported in this chapter represent the most extensive assessment to date of how mediators of type 1 inflammation in the asthmatic airway respond to rhinovirus infection in-vivo. The findings presented here include the observations that:

1. There is a significant induction by rhinovirus of IL-12, IL-15, IFN-γ, IP-10/CXCL10, and I-TAC/CXCL11 in the upper airway of both healthy and asthmatic individuals.
2. There is a significant induction of IFN-γ, IP-10/CXCL10, and I-TAC/CXCL11 (with additional non-significant increases in IL-12 and IL-15 in the asthmatic but not healthy lung during rhinovirus infection.

3. Increased levels of IL-12, IL-15, IFN-γ, IP-10/CXCL10, and I-TAC/CXCL11 during infection with rhinovirus are associated with increased virus load.

4. Increased IL-12, IL-15, IFN-γ, IP-10/CXCL10, and I-TAC/CXCL11 in the asthmatic airway during infection with rhinovirus are associated with more severe colds and asthma exacerbations.

5. Increased IL-12 is also associated with more severe colds in healthy subjects whilst greater bronchial IP-10/CXCL10 levels are observed in the minority of healthy subjects who also experience additional lower respiratory symptoms.

6. In asthma and healthy subjects at baseline, the extent of type 1 inflammation in the lung relates to the inflammation seen in the nose. During infection this relationship continues to be found in asthmatic but not healthy subjects.

7. Levels of each of the type 1 mediators are very closely associated with each other during infection in support of their close functional roles.

Taken together, the findings of type 1 mediator induction with levels relating to virus load and symptom severity suggest these mediators are intimately involved in the pathogenesis of virus-induced exacerbations in asthma. The absence of a significant induction in the healthy lung in all but the few subjects who were experiencing additional lower as well as upper respiratory symptoms is further support of this.

Induction of IP-10/CXCL10 both in-vitro and in-vivo has previously been reported following human experimental infection with RV-16, along with relationships to symptom severity and viral titre.233 This controlled human data is also supported by studies of naturally-occurring infections in which IP-10 has also shown to be increased in nasal lavage from asthmatic children during a viral infection234,
whilst serum IP-10 levels were increased to a greater extent in those with acute virus-induced asthma than in those with non-virus-induced acute asthma.235

The induction and relationships of this key Th1 chemokine demonstrated in the present study confirm published findings and add to the body of data indicating a role for IP-10 in the pathogenesis of RV-induced colds and exacerbations of asthma. Indeed IP-10/CXCL10 has been suggested as a marker of respiratory virus infection by some authors.236,237

The demonstration of I-TAC/CXCL11 induction in-vivo, along with the associations of greater cold and exacerbation severity, and its known role as a ligand for the CXCR3 receptor found on activated Th1 lymphocytes and natural killer cells also positions this chemokine as potentially central to cold / exacerbation pathogenesis. Yet there is currently no published human data reporting the induction of this chemokine in the exacerbating asthmatic airway.

The recent study by Lewis of naturally-occurring viral infections in asthmatic children also reported greater levels of IFN-γ protein in nasal aspirates during the periods of infection, with levels correlating with symptoms.234 Whilst other studies have attempted to measure IFN-γ RNA in sputum samples during infection, the current study is the first to demonstrate bronchial IFN-γ protein induction by rhinovirus in asthma, the absence of this induction in the healthy lung (but presence in the nose), and the relationships of bronchial IFN-γ levels with asthma exacerbation severity. These results do not confirm the findings by Message who reported the absence of a significant IFN-γ induction following virus stimulation of cells from asthmatic subjects whilst showing inverse relationships between CD4+ IFN-γ production, virus load and clinical outcome. However in contrast to the in-vivo data reported in the current study, the peripheral blood and BAL CD4+ IFN-γ levels in the Message study were from a small number of subjects and were assessed at baseline before experimental RV infection and related to subsequent clinical and virological outcome.238
Message also reported IL-12 induction by virus in healthy but not asthmatic cells, which again is in contrast to the *in-vivo* findings reported in this chapter of significant induction in both healthy and asthmatic subjects in the nose with a non-significant increase in median levels in the asthmatic lung during infection. It is however noteworthy that despite greater upper respiratory symptom scores and virus loads in asthma compared to healthy subjects, the median upper airway IL-12 levels on each day post-inoculation were less than observed in the healthy group. This would in some respects support the idea of a somewhat deficient Th1 response in the asthmatic airway.

The literature surrounding IL-15 in virus-induced asthma exacerbations is even more sparse than for the other type 1 mediators and is limited to only a small handful of *in-vitro* studies. As such the results reported here of IL-15 protein induction and relationships to cold and exacerbation severity are the first of its kind. These findings are supported by work by Zdrenghea and colleagues who reported that rhinovirus leads to up-regulation of IL-15 in macrophages and cultured epithelial cells \(^{238,239}\) and that higher levels of IL-15 was released when RV-16 infection of macrophages was carried out in the presence of IFN-γ.\(^{240}\) The very close correlation between IL-15 and IFN-γ levels in the current study is also supportive of this finding.

In summary, the results presented in this chapter appear to demonstrate a robust type 1 immune response to rhinovirus in asthma with the possible exception of IL-12. Induction of this pathway correlates with virus load as well as symptom severity. The absence of significant type 1 pathway induction in the healthy lung raises the possibility that its activation in asthma may be in part responsible for the differing clinical responses to rhinovirus in asthma. However the relative balance with type 2 inflammation (the focus of the next chapter) may be more critical.
Chapter 6

Results Chapter 4

Type 2 Inflammation During Rhinovirus-Induced Exacerbations of Asthma

6.1 Introduction

Until now measurement of the Th2 cytokines (IL-4, -5, and -13) in airway samples has been extremely difficult with the conventional techniques of nasal and bronchoalveolar lavage due to dilution of these mediators below detection limits of assays. This has led to a reliance upon RNA levels\textsuperscript{63} or indirect measures such as eosinophils (for IL-5)\textsuperscript{141,142} or periostin (for IL-13)\textsuperscript{145} but not upon direct measurement of protein. It is therefore not known whether respiratory virus infection in asthma leads to amplification of Th2 inflammation \textit{in-vivo}, but studies reporting powerful synergistic interactions between allergen exposure and virus infection in increasing risk of asthma exacerbations\textsuperscript{51,52}, as well as studies reporting reductions in exacerbations using monoclonal antibodies targeting Th2 cytokines\textsuperscript{141–144,241} both suggest that Th2 cytokines may be important in the pathogenesis of asthma exacerbations.

In addition, \textit{ex-vivo} human data from Message that baseline IL-4, IL-5, and IL-13 production by BAL T cells were all associated with more severe virus-induced asthma symptoms following infection,\textsuperscript{68} and mouse data from Bartlett & Walton showing a significant induction of IL-4 and IL-13 in rhinovirus infected, ova-sensitised mice compared with ova-sensitised controls together lend further support for a central role of augmented type 2 immunity in rhinovirus-induced asthma exacerbation pathogenesis.

Therefore the \textit{in-vivo} analysis of type 2 inflammation during rhinovirus-induced exacerbations of asthma was one of the main focuses of this study. Sampling nasal and bronchial mucosal lining fluid has permitted the first successful protein analysis of type 2 inflammatory mediators during a virus-induced exacerbation of asthma \textit{in-vivo}. Measurement of a large number of mediators known to be important to this pathway has been made both at baseline and during infection including the
epithelial-derived Th2-inducing cytokine IL-33, the Th2 chemokines TARC/CCL17 and MDC/CCL22, and the classic Th2 cytokines IL-4, IL-5, and IL-13. It had been hoped that measurement of the other epithelial-derived cytokines IL-25 and TSLP would also be possible, however problems were encountered with both the TSLP and IL-25 assays raising serious doubts about the accuracy of the results. Therefore these cytokines are not presented here.

6.2 Levels of Th2 cytokines in airway mucosal lining fluid

IL-4: IL-4 levels in both the upper and lower airway were measured at very low levels in this study and were close to, or below the lower limit of detection (0.6 pg/mL) for many subjects. Indeed, at baseline, bronchial IL-4 was measurable in only 9/28 asthmatics and 5/11 healthy subjects (only 2 healthy subjects with levels > 1pg/mL). On day 4, bronchial IL-4 was detectable in 11/28 asthmatics and only 1/11 healthy subject. In the nose, baseline IL-4 was measurable in 19/28 asthmatics and 2/11 healthy subjects with levels increasing above the LLD during infection in 26/28 asthmatics and in 4/11 healthy subjects. As such any correlation involving IL-4 (excluding those using nasal levels during infection in asthma) should be interpreted with this in mind.

In the nose, median levels of IL-4 were significantly higher both at baseline and during infection in asthma (baseline healthy v's asthma, 0 pg/ml [0 - 0] v's 0.9 pg/mL [0 - 1.7], $P = 0.04$; infection peak healthy v's asthma, 0 pg/mL [0 - 1.5] v's 1.8 pg/mL [0.9 - 3.3], $P = 0.004$). In asthma, this increase with infection was to a significant degree ($P < 0.001$).

In the lung, day 4 bronchial levels were statistically higher in asthma than healthy subjects despite both groups having median levels of 0 pg/mL (bronchial day 4 healthy v's asthma, 0 pg/ml [0 - 0] v's 0 pg/mL [0 - 1.0], $P = 0.05$). No significant induction in bronchial IL-4 was observed in either group (figure 6.1 a&b).
There was no significant difference in either baseline or infection levels of IL-4 according to asthma severity, asthma control, or treatment group. However, median levels were higher in moderate and poorly-controlled subjects (median peak infection IL-4: mild asthma, 1.1 pg/mL; moderate asthma, 2.0 pg/mL; well-controlled asthma, 1.7 pg/mL; poorly-controlled asthma, 2.2 pg/mL).

**IL-5:** In the nose, median levels of IL-5 were significantly higher both at baseline and during infection in asthma (baseline healthy v's asthma, 0 pg/ml [0 - 0.8] v's 1.8 pg/mL [0.6 - 7.0], \(P = 0.008\); infection peak healthy v's asthma, 0.8 pg/mL [0 - 5.0] v's 13.0 pg/mL [4.5 - 33.9], \(P = 0.002\)). In asthma, this increase with infection was of a significant amount (\(P < 0.001\)).

In the lung, there were trends for greater baseline and day 4 bronchial IL-5 levels in asthma, (baseline bronchial healthy v's asthma, 0 pg/ml [0 - 0.6] v's 0.7 pg/mL [0 - 1.3], \(P = 0.084\); day 4 bronchial healthy v's asthma, 0 pg/ml [0 - 0.8] v's 1.1 pg/mL [0 - 1.9], \(P = 0.052\)). In asthma, this increase with infection was significant (\(P = 0.044\)) (figure 6.1 c&d).

There was no significant difference in either baseline or infection levels of IL-5 according to asthma severity, asthma control, or treatment group. However, median levels were again higher in the nasal samples from moderate and poorly-controlled subjects (median nasal peak infection IL-5: mild asthma, 10.1 pg/mL; moderate asthma, 13.1 pg/mL; well-controlled asthma, 7.3 pg/mL; poorly-controlled asthma, 18.4 pg/mL; day 4 bronchial IL-5: mild asthma, 1.1 pg/mL; moderate asthma, 0.8 pg/mL; well-controlled asthma, 0.7 pg/mL; poorly-controlled asthma, 1.6 pg/mL).

**IL-13:** As with IL-4 and IL-5, median nasal levels of IL-13 were significantly higher both at baseline and during infection in asthma (baseline healthy v's asthma, 0 pg/ml [0 - 0.8] v's 2.0 pg/mL [0.8 - 3.8], \(P = 0.001\); infection peak healthy v's asthma, 0.8 pg/mL [0 - 2.2] v's 4.9 pg/mL [2.6 - 16.0], \(P < 0.001\)). In asthma, this increase with infection was significant (\(P < 0.001\)).

In the lung, there were greater baseline and day 4 bronchial IL-13 levels in asthma however this was significant only at day 4 (baseline bronchial healthy v's asthma, 0 pg/ml [0 - 0] v's 0.6 pg/mL [0 - 1.2], \(P = 0.018\); day 4 bronchial healthy v's asthma, 0 pg/ml [0 - 0.8] v's 2.6 pg/mL [1.2 - 4.8], \(P = 0.008\)).
$P = 0.068$; day 4 bronchial healthy v's asthma, 0 pg/ml [0 - 1.5] v's 0.7 pg/mL [0 - 1.2], $P = 0.009$). In asthma, the increase with infection did not reach significance ($P = 0.156$) (figure 6.1 e&f).

There was no significant difference in either baseline or infection levels of IL-13 according to asthma severity, asthma control, or treatment group. However, median levels were generally higher in moderate and poorly-controlled subjects (median nasal peak infection IL-13: mild asthma, 10.1 pg/mL; moderate asthma, 13.1 pg/mL; well-controlled asthma, 7.3 pg/mL; poorly-controlled asthma, 18.4 pg/mL; day 4 bronchial IL-13: mild asthma, 1.1 pg/mL; moderate asthma, 0.8 pg/mL; well-controlled asthma, 0.7 pg/mL; poorly-controlled asthma, 1.6 pg/mL).

### 6.3 Relationships between Th2 cytokines and virus-induced respiratory symptoms in asthma

**IL-4:** No significant correlations were observed between IL-4 and either upper or lower respiratory symptoms scores. However, because of low IL-4 levels across the infection in all but a few asthmatics these results should be viewed with a degree of caution. A trend towards significance was seen for day 3 nasal IL-4 and peak lower respiratory symptom score ($r = 0.378$, $P = 0.052$).

**IL-5:** In the nose, significant correlations between IL-5 and symptoms were observed on each of days 2, 3, 4, and peak infection level: Day 2 IL-5 correlated with total upper respiratory symptoms ($r = 0.415$, $P = 0.035$) with trends for relationships with both peak lower ($r = 0.364$, $P = 0.067$) and total lower respiratory symptom scores ($r = 0.351$, $P = 0.079$). Nasal day 3 IL-5 tended to correlate with total upper ($r = 0.362$, $P = 0.064$), peak upper ($r = 0.366$, $P = 0.060$), and correlated significantly with peak lower respiratory symptoms ($r = 0.410$, $P = 0.034$); whilst both day 4 and peak IL-5 correlated with peak upper and peak lower respiratory score respectively (day 4, $r = 0.417$, $P = 0.030$; peak IL-5, $r = 0.420$, $P = 0.029$).

In the lung, a trend towards significance was seen for between day 4 bronchial IL-5 and total lower respiratory symptom score ($r = 0.383$, $P = 0.059$) (figure 6.2 a&b).
**IL-13:** In the nose, day 2, -3, -4, and peak infection IL-13 all correlated significantly with peak upper respiratory symptom scores (day 2, $r = 0.391$, $P = 0.048$; day 3, $r = 0.484$, $P = 0.010$; day 4, $r = 0.516$, $P = 0.006$; peak infection, $r = 0.475$, $P = 0.012$), whilst a relationship between day 3 nasal IL-13 and peak lower respiratory symptom score was also observed ($r = 0.387$, $P = 0.046$).

In the lung, day 4 bronchial IL-13 correlated significantly with total lower respiratory symptoms ($r = 0.420$, $P = 0.036$) (figure 6.2 c&d).
Figure 6.1 Rhinovirus infection leads to induction of the Th2 cytokines IL-4, -5, and -13 in the nose in asthma but not healthy subjects. Bronchial levels of IL-4 and IL-13 were significantly greater on day 4 in asthma compared to healthy subjects. Bronchial IL-5 was significantly induced by rhinovirus in asthma. (+P<0.05; ++P<0.01; +++P<0.001)
Figure 6.2 Relationships between Th2 cytokines and respiratory symptom scores during rhinovirus infection in asthma.

6.4 Relationships between Th2 cytokines and virus-induced respiratory symptoms in healthy subjects

No correlations with day 4 bronchial Th2 cytokine levels were performed in healthy subjects given the minority of subjects with measurable bronchial levels (1/11 subject had measurable IL-4 levels, 3/11 had measurable IL-5, and 2/11 had measurable IL-13). It is however noteworthy that those few subjects did in fact have the greatest lower respiratory symptom scores.

In the nose, 4/11 had measurable IL-4 at some point during the infection, 6/11 had measurable IL-5, whilst 8/11 had measurable IL-13 over the course of infection. Correlations between peak IL-5 and
clinical outcomes in healthy subjects revealed significant relationships with both total upper respiratory symptom score \( (r = 0.791, P = 0.004) \) (figure 6.3a) as well as with peak upper respiratory scores \( (r = 0.791, P = 0.004) \). Peak nasal IL-13 levels also correlated significantly with total upper respiratory symptom score \( (r = 0.606, P = 0.048) \) (figure 6.3b).

**Figure 6.3** Relationships between Th2 cytokines and upper respiratory symptom scores during rhinovirus infection in healthy subjects.

![Diagram](image)

### 6.5 Relationships between Th2 cytokines and airway hyperresponsiveness

Airway hyperresponsiveness (AHR) in asthma was assessed using the histamine \( PC_{20} \) test performed at baseline prior to the first bronchoscopy and on day 7 post-inoculation to assess for any virus-induced change. In asthma, the baseline level of AHR correlated significantly with baseline nasal IL-5 \( (r = -0.407, P = 0.031) \) with increased IL-5 associated with lower concentrations of histamine required to cause a 20% fall in FEV\(_1\). A trend towards significance was also seen between baseline nasal IL-13 and baseline \( PC_{20} \) \( (r = -0.358, P = 0.061) \) (figure 6.4a).
Significant relationships with day 7 $PC_{20}$ in asthma were also observed for baseline nasal IL-13 ($r = -0.475$, $P = 0.026$) (figure 6.4b), baseline nasal IL-5 ($r = -0.438$, $P = 0.042$), and day 2 nasal IL-5 ($r = -0.460$, $P = 0.036$).

**Figure 6.4** Relationship between IL-13 and airway hyper-responsiveness

6.6 Relationships between Th2 cytokine levels in asthma

Although in many ways expected, the relationships between the different Th2 cytokines in both the upper and lower airway were amongst the strongest in the whole study. In particular, the induction of IL-5 almost exactly mirrored that of IL-13 with correlation coefficients approaching 1.0 (peak nasal IL-5 and IL-13, $r = 0.918$, $P < 0.001$; day 4 bronchial levels, $r = 0.871$, $P < 0.001$) (figure 6.5a&b). Relationships were less impressive but still highly significant for IL-4 and both IL-5 (peak nasal, $r = 0.551$, $P = 0.002$) and IL-13 (peak nasal, $r = 0.487$, $P = 0.014$) (figure 6.5c-f).
Figure 6.5 Relationships between the different Th2 cytokines in nasal and bronchial mucosal lining fluid during rhinovirus infection in asthma.
6.7 Levels of IL-33 in airway mucosal lining fluid

At baseline, median levels of IL-33 in both nasal and bronchial samples were similar in healthy and asthmatic groups (nasal IL-33, healthy: 45.5 pg/mL [29.0 - 190.4] v's asthma 26.5 pg/mL [20.4 - 56.8]; bronchial IL-33, healthy 1.50 ng/mL [1.13 - 3.02] v's asthma 1.83 ng/mL [1.16-4.62] and no statistically significant differences were seen with regard to asthma severity, treatment or asthma control status.

During rhinovirus infection, nasal IL-33 increased from baseline in both healthy and asthmatic groups, but this only reached a significant level in asthma (healthy, median peak nasal IL-33: 129.3 pg/mL [90.0 - 451.0], \( P = 0.08 \); asthma, median peak nasal IL-33: 139.0 pg/mL [47.3 - 319.3], \( P <0.001 \)) (figure 6.6a).

Peak nasal levels were generally higher in moderately severe and poorly-controlled asthmatics compared to mild and well-controlled subjects but these differences were not statistically significant (mild asthma, 100.0 pg/mL [47.0 - 272.0]; moderate asthma, 145.0 pg/mL [41.5 - 337.0]; well-controlled asthma, 116.0 pg / mL [38.0 - 242.5]; poorly-controlled asthma, 252.0 pg/mL [ 75.3 - 368.3]).

In the lung, no significant inductions of IL-33 were observed in either the asthma or healthy group when comparing day 4 with the baseline bronchoscopy sample (figure 6.6b) and no significant differences were seen between asthma severity and control categories. However, a trend towards a relationship between day 4 bronchial IL-33 and baseline ACQ was observed (\( r = 0.384, P = 0.058 \)) (figure 6.7).

6.8 Relationship between IL-33 and respiratory symptom scores

In asthma, day 4 bronchial IL-33 correlated with peak and total upper respiratory symptom scores (peak upper, \( r = 0.490, P = 0.013 \); total upper, \( r = 0.417, P = 0.038 \)) as well as with peak and total lower respiratory symptoms (peak lower, \( r = 0.575, P = 0.003 \); total lower, \( r = 0.419, P = 0.037 \))
(figure 6.8). Significant relationships between nasal day 3, day 4 and peak nasal IL-33 with both peak and total lower respiratory symptom scores were also observed (all $P < 0.05$) strengthening the association between increased airway IL-33 and infection / exacerbation severity.

In healthy subjects (and despite measurable levels during infection), day 4 bronchial IL-33 did not correlate with any of the clinical outcome measures. In contrast day 2 nasal IL-33 did correlate with both total upper respiratory symptom score ($r = 0.618$, $P = 0.043$) and peak upper respiratory symptom score ($r = 0.662$, $P = 0.026$). Peak nasal IL-33 was also seen to correlate with peak upper respiratory symptoms ($r = 0.658$, $P = 0.028$) (figure 6.9).

**Figure 6.6** IL-33 in nasal (a) and bronchial (b) mucosal lining fluid. (+++P<0.001)
**Figure 6.7** The relationship between baseline asthma control level and rhinovirus-induced bronchial IL-33 levels. An individual with an ACQ of ≤0.75 is considered to be well-controlled; an ACQ ≥ 1.5 represents poor control.

**Figure 6.8** Relationships between day 4 bronchial IL-33 levels and upper and lower respiratory symptoms during rhinovirus infection in asthma.
6.9 Relationship between IL-33 and other mediators of type 2 inflammation

IL-33 correlated with many of the mediators of Th2 inflammation both at baseline and during infection in asthma. In the lung, baseline IL-33 correlated significantly with bronchial IL-5 ($r = 0.617$, $P = 0.001$), IL-13 ($r = 0.612$, $P = 0.001$), TARC/CCL17 ($r = 0.558$, $P = 0.004$), and MDC/CCL22 ($r = 0.656$, $P <0.001$). The absence of a relationship with IL-4 both at baseline ($r = 0.335$, $P = 0.102$) and on day 4 ($r = 0.032$, $P = 0.881$) may simply be a function of low / undetectable levels of this cytokine in the bronchial samples. However, no significant relationships with IL-33 were seen for any of the nasal IL-4 post-inoculation time-points either (including peak level) despite all but 2 asthmatics having detectable IL-4 at some point during their infection.

On day 4, similar relationships between bronchial IL-33 and IL-5 ($r = 0.587$, $P = 0.002$), IL-13 ($0.522$, $0.007$), (figure 6.10) and MDC/CCL22 ($r = 0.598$, $P = 0.002$) were observed. In contrast to pre-infection bronchial samples, no relationship appeared to exist between day 4 IL-33 and TARC/CCL17 levels ($r = 0.216$, $P = 0.299$). However, significant relationships were observed between day 2 nasal
TARC/CCL17 and day 4 bronchial IL-33 ($r = 0.569$, $P = 0.004$), as well as with both day 2 nasal IL-33 ($r = 0.457$, $P = 0.016$), and day 3 nasal IL-33 ($r = 0.445$, $P = 0.02$). In the nose, the relationships between IL-33 and the other Th2 mediators matched those seen in the lung: Day 2 nasal IL-33 correlated with day 2 IL-5 ($r = 0.544$, $P = 0.003$), IL-13 ($r = 0.388$, $P = 0.046$), and MDC/CCL22 ($r = 0.458$, $P = 0.016$).

**Figure 6.10** Relationships between bronchial IL-33 and Th2 cytokine levels during rhinovirus infection in asthma.

6.10 Levels of TARC/CCL17 in airway mucosal lining fluid

In the nose, median levels of TARC/CCL17 were significantly higher both at baseline and during infection in asthma (baseline healthy v’s asthma, 8.2 pg/ml [4.5 - 16.1] v’s 17.1 pg/mL [11.3 - 25.4], $P = 0.009$; infection peak healthy v’s asthma, 17.0 pg/mL [10.2 - 26.5] v’s 29.4 pg/mL [17.0 - 51.3], $P = 0.01$). In asthma, this increase with infection was significant ($P < 0.001$), but in contrast to to the Th2 cytokine results, a significant induction was also seen in the healthy nose ($P < 0.05$) (figure 6.11a).
In the lung, both baseline and day 4 bronchial TARC/CCL17 levels were similar in asthma and in healthy subjects (baseline, 26.6 pg/ml [20.6 - 30.9] v's 24.0 pg/mL [21.3 - 40.5], \( P = 0.38 \); day 4, 24.5 pg/mL [20.4 - 29.2] v's 28.0 pg/mL [21.0 - 39.5], \( P = 0.26 \)). No significant induction of TARC/CCL17 was observed on day 4 in either group (figure 6.11b).

**6.11 Levels of MDC/CCL22 in airway mucosal lining fluid**

In the nose, median levels of MDC/CCL22 were higher (but not significantly) at baseline and significantly higher during infection in asthma (baseline healthy v's asthma, 15.7 pg/ml [9.6 - 31.4] v's 25.2 pg/mL [16.1 - 43.2], \( P = 0.070 \); infection peak healthy v's asthma, 27.0 pg/mL [16.0 - 59.0] v's 58.0 pg/mL [33.3 - 89.8], \( P = 0.041 \)). In asthma, this increase with infection was significant (\( P < 0.001 \)) (figure 6.11c). In a similar fashion to what was observed with the other Th2 chemokine TARC/CCL17, a significant induction of of MDC/CCL22 was also seen in the healthy nose during infection (\( P < 0.01 \)).

In the lung, both baseline and day 4 bronchial MDC/CCL22 levels were greater in asthma than in healthy subjects, however these differences were not statistically significant (baseline, 18.2 pg/ml [13.8 - 47.9] v's 22.6 pg/mL [15.8 - 36.8], \( P = 0.93 \); day 4, 20.3 pg/mL [16.3 - 46.0] v's 24.7 pg/mL [13.5 - 51.4], \( P = 0.74 \)). In the lung, no significant induction of MDC/CCL22 was observed on day 4 in either group (figure 6.11d). There were no significant differences in Th2 chemokine levels according to asthma severity, treatment group, or asthma control category, however, when asthma control was analysed as a continuous rather than categorical variable, day 2 nasal MDC/CCL22 did significantly correlate with baseline ACQ (\( r = 0.384, P = 0.048 \)).

**6.12 Relationships between Th2 chemokine levels in asthma**

Strong relationships were observed between levels of the two Th2 chemokines in both the nose and in the lung, and both at baseline and during infection (peak nasal, \( r = 0.761, P < 0.001 \); baseline bronchial, \( r = 0.626, P = 0.001 \); day 4 bronchial, \( r = 0.570, P = 0.003 \)) (figure 6.12).
Figure 6.11 Rhinovirus infection leads to induction of the Th2 chemokines MDC/CCL22 and TARC/CCL17 in the nose in both asthma and healthy subjects. However nasal levels during infection are significantly higher in asthma. No significant induction in bronchial levels was observed on day 4. (+P<0.05; ++P<0.01; +++P<0.001).
6.13 Relationships between Th2 chemokines and respiratory symptoms in asthma

In contrast to the cytokines already discussed, significant relationships were also observed in asthma between pre-infection levels of both TARC/CCL17 and MDC/CCL22 and severity of the infection: Baseline (day 0) nasal TARC/CCL17 levels correlated significantly with total upper respiratory symptom score \( (r = 0.403, P = 0.037) \), whilst baseline bronchial MDC/CCL22 correlated with total lower respiratory symptom scores \( (r = 0.418, P = 0.042) \) (figure 6.13). In other words those asthmatics with the greatest levels of the Th2 chemokines prior to infection had the most severe
asthma exacerbations and colds following inoculation with rhinovirus. A trend towards significance for peak lower respiratory symptom score was also seen for baseline bronchial MDC/CCL17 ($r = 0.400, P = 0.053$).

Increased day 4 bronchial MDC/CCL22 in asthma was similarly associated with a more severe clinical picture, correlating with both total upper ($r = 0.407, P = 0.043$), total lower ($r = 0.441, P = 0.028$) and peak lower respiratory symptom scores ($r = 0.526, P = 0.007$) (figure 6.14). In the nose, day 2 nasal MDC / CCL22 also correlated with the same clinical outcome measures (total upper, $r = 0.467, P = 0.016$; total lower, $r = 0.445, P = 0.023$; and peak lower respiratory symptoms, $r = 0.405, P = 0.04$).

Interestingly associations with TARC/CCL17 were limited to the upper airway only with day 2 and day 3 nasal TARC/CCL17 correlating significantly with total upper respiratory score in asthma (day 2, $r = 0.531, P = 0.005$; day 3, $r = 0.446, P = 0.020$).

**Figure 6.13** The relationship between baseline bronchial MDC / CCL22 and lower respiratory symptoms in asthma.

![Graph showing the relationship between baseline bronchial MDC and total lower respiratory symptom score.](image)
6.14 Relationships between Th2 chemokines and respiratory symptoms in healthy subjects

Levels of both TARC/CCL17 and MDC/CCL22 were measurable in bronchial samples from healthy subjects, however, levels did not relate to any of the clinical outcome measures. In contrast, levels of both of these chemokines in the nose on day 2 did correlate with peak upper respiratory symptom score (TARC/CCL17, $r = 0.612, P = 0.046$; MDC/CCL22, $r = 0.621, P = 0.042$) (figure 6.15).
6.15 Relationships between Th2 chemokines and Th2 cytokines in asthma

In the lung, strong relationships between the Th2 chemokines and cytokines were observed both at baseline and during infection: TARC/CCL17 and IL-5 (baseline, \( r = 0.548, P = 0.005 \); day 4, \( r = 0.661, P < 0.001 \)); TARC/CCL17 and IL-13 (baseline, \( r = 0.596, P = 0.002 \); day 4, \( r = 0.671, P < 0.001 \)); MDC/CCL22 and IL-5 (baseline, \( r = 0.535, P = 0.006 \); day 4, \( r = 0.726, P < 0.001 \)); and MDC/CCL22 and IL-13 (baseline, \( r = 0.546, P = 0.005 \); day 4, \( r = 0.620, P = 0.001 \)). This is very much in keeping with the functional role these chemokines have in attracting IL-5 and IL-13 producing Th2 cells (figure 6.16).

In the nose, where IL-4 was more readily measurable, significant relationships were also seen with IL-4: Day 2 IL-4 (TARC/CCL17, \( r = 0.597, P = 0.001 \); MDC/CCL22, \( r = 0.538, P = 0.004 \)) and day 3 IL-4 (TARC/CCL17, \( r = 0.623, P < 0.001 \); MDC/CCL22, \( r = 0.436, P = 0.020 \)) (figure 6.16).

As described earlier (6.9), highly significant relationships were also seen between MDC/CCL22 and IL-33 (figure 6.17).
Figure 6.16 Relationships between bronchial Th2 chemokine and cytokine levels during rhinovirus infection in asthma.
6.16 Relationships between type 2 mediators and virus load

Of the type 2 mediators discussed in this chapter, a relationship with virus load was seen only for IL-33. In asthma, peak nasal IL-33 levels correlated significantly with nasal virus load on day 2 ($r = 0.499, P = 0.007$), day 3 ($r = 0.554, P = 0.002$), and peak infection level ($r = 0.441, P = 0.019$) (figure 6.18). Day 3 nasal IL-33 in particular correlated significantly with both day 2 ($r = 0.429, P = 0.023$) and day 3 virus load ($r = 0.495, P = 0.007$). There were no significant relationships between virus load and these mediators in healthy subjects.

Figure 6.18 The relationship between IL-33 levels and virus load during rhinovirus infection in asthma.
6.17 Relationship between type 2 mediators and markers of atopy

There is no single variable that can accurately quantify the degree of atopy. In this study, three variables were recorded as markers of atopy including serum total IgE, number of positive skin prick test responses to a panel of common aeroallergens, and the combined size (in millimetres) of all positive responses. Analysis of any relationships between these variables and levels of baseline or virus-induced type 2 mediators was performed.

In asthma, nasal IL-4 measured on days 0, 2, 3, and infection peak all correlated significantly with both number of positive SPTs and combined size of the responses (Peak IL-4 and number of positive SPTs, $r = 0.530, P = 0.004$; total size of SPTs, $r = 0.560, P = 0.002$). Significant relationships were also seen for IL-13 on day 2 (IL-13 and number of positive SPTs, $r = 0.401, P = 0.038$; total size of SPTs, $r = 0.423, P = 0.028$), and day 3 (IL-13 and number of positive SPTs, $r = 0.373, P = 0.050$; total size of SPTs, $r = 0.457, P = 0.014$).

Interestingly, despite the very similar inductions of IL-5 and IL-13 seen in this study and the exceptionally strong correlations between these two cytokines, there were no significant relationships between the markers of atopy described above and levels of IL-5. In addition, no significant relationships were observed between baseline serum total IgE and levels of IL-4 or other type 2 mediators either at baseline or during infection. However, this could be explained by the very low levels of IL-4, especially pre-inoculation.

6.18 Relationships between nasal and bronchial type 2 mediator levels in asthma

Whether or not the patterns of inflammation in the nose in asthma reflect the inflammatory pathways in the lung has been debated for many years. Table 6.1 shows the correlations between the upper and lower airway both at baseline and during infection for the mediators discussed in this chapter. As IL-4 levels were below limits of detection in the majority of subjects, correlations of IL-4 are absent. Several of the correlations are also shown in figure 6.19.
At baseline, nasal and bronchial IL-5 ($r = 0.454$, $P = 0.022$) and IL-33 ($r = 0.517$, $P = 0.008$) correlated significantly. Whilst the absence of a relationship in the case of IL-13 may be explained by low baseline IL-13 levels (frequently below the LLD), baseline levels of both TARC/CCL17 and MDC/CCL22 were measurable in the majority of cases yet evidence of a relationship between levels of these chemokines in the nose and lung at baseline was also absent.

Analysis of the relationships during infection demonstrated extremely strong relationships for all mediators with the exception of TARC/CCL17, which again stood apart from the other cytokines. It is interesting to note that where relationships were observed between upper and lower airway mediators during infection, the strongest associations were consistently between day 2 nasal levels and day 4 bronchial levels suggesting a 2 day lag between infection and cytokine induction in the upper and lower airway (day 2 nasal v’s day 4 bronchial: IL-5, $r = 0.655$, $P = 0.001$; IL-13, $r = 0.567$, $P = 0.004$; IL-33, $r = 0.490$, $P = 0.015$; MDC/CCL22, $r = 0.561$, $P = 0.004$) (figure 6.19).

**Figure 6.19** Relationships between bronchial and nasal Th2 cytokine levels in asthma.
**Table 6.1** Correlations between nasal and bronchial cytokine levels in asthma. Underlined correlations represent the day of nasal sampling most closely related to day 4 bronchial levels. Bold type represents statistical significance.

<table>
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<th>Correlation Coefficient</th>
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<tr>
<td>IL-5</td>
<td><em>r = 0.454, P = 0.022</em></td>
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<tr>
<td>IL-13</td>
<td><em>r = 0.319, P = 0.120</em></td>
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<tr>
<td>IL-33</td>
<td><em>r = 0.517, P = 0.008</em></td>
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<tr>
<td>TARC/CCL17</td>
<td><em>r = -0.318, P = 0.121</em></td>
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<tr>
<td>MDC/CCL22</td>
<td><em>r = -0.038, P = 0.858</em></td>
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<tr>
<td>IL-5</td>
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<td>Bronchial day 4 v’s nasal day 2</td>
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<td>IL-13</td>
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<td>Bronchial day 4 v’s nasal day 2</td>
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<td>Bronchial day 4 v’s nasal day 2</td>
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<td><em>r = 0.388, P = 0.056</em></td>
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6.19 Relationship between baseline type 2 inflammation and virus-induced type 2 inflammation

The question of whether or not the basal level of type 2 mediators in the airway prior to the infection relates to the degree of their induction during a virus-induced exacerbation is unknown and was evaluated.

In the nose, strong relationships were observed between baseline (day 0) mediator level and the peak infection level: IL-4 \( (r = 0.652, P < 0.001) \), IL-5 \( (r = 0.680, P < 0.001) \), IL-13 \( (r = 0.807, P < 0.001) \), IL-33 \( (r = 0.478, P = 0.010) \), TARC/CCL17 \( (r = 0.731, P < 0.001) \), and MDC/CCL22 \( (r = 0.703, P < 0.001) \). The correlations for IL-5 and IL-13 are shown in figure 6.20.

In the lung, baseline levels of these mediators also correlated strongly with levels on day 4 post-inoculation: IL-5 \( (r = 0.661, P = 0.006) \), IL-13 \( (r = 0.529, P = 0.009) \), IL-33 \( (r = 0.678, P < 0.001) \), TARC/CCL17 \( (r = 0.584, P = 0.003) \), and MDC/CCL22 \( (r = 0.619, P = 0.002) \). However, as bronchial day 4 samples frequently failed to show a significant change from baseline, these latter relationships may simply reflect the reproducibility of the technique rather than any predictability of mediator induction by virus according to basal levels.

Figure 6.20 Relationships between baseline and peak infection levels of Th2 cytokines in asthma.
6.20 Kinetics of nasal type 2 mediator induction in asthma

Finally, in addressing the kinetics of nasal type 2 mediator induction by rhinovirus in asthma, it is apparent that a significant amount of variation exists from subject to subject. In the first instance, analysis of the group averages reveals levels of IL-33 doubling from baseline by day 2 post-inoculation, reaching a peak on day 3 before returning to near basal levels on day 7. A similar kinetic profile is seen for MDC/CCL22. TARC/CCL17 on the other hand appears to peak 24 hours later on day 4, at the same time as IL-13. IL-5 is seen to peak between days 3 - 4 (figure 6.21a).

However, analysis of the data on a subject by subject basis highlights markedly different kinetics. Examples of this are seen in figure 6.21 b-c. In subject DJ148, a very clear induction of IL-33 occurs with levels reaching a peak on day 3 before returning to baseline on day 5. This induction is then followed 24 hours later by induction of TARC/CCL17, MDC/CCL22, IL-4, and IL-13. IL-5 reaches a peak between days 3 - 4. In contrast, subject DJ104 shows a very early induction on day 2 of TARC/CCL17, MDC/CCL22, IL-5, and IL-13 with induction of IL-33 occurring on day 3 with levels peaking on day 4. This disparity could be explained by several factors (see discussion 6.21) however it does serve to highlight the differences that can be seen between interpreting group statistics and examining individual data.
Figure 6.21  Kinetics of nasal type 2 mediator induction following rhinovirus infection in asthma. All asthmatics (A); examples of individual asthmatics (B,C).
6.21 Chapter Discussion

At the time of writing, this study is the first to have succeeded in measuring type 2 mediators during a virus-induced asthma exacerbation in-vivo, and in so doing has identified an entire inflammatory pathway that until now has not been considered a significant contributor to the pathophysiology of virus-induced asthma exacerbations. It has been possible to demonstrate that rhinovirus infection leads to the induction of the key Th2 inducing cytokine IL-33 along with both the Th2 chemokines TARC/CCL17 and MDC/CCL22 and Th2 cytokines IL-4, IL-5, and IL-13. Increased levels of these Th2 mediators during infection correlate with asthma exacerbation severity and the degree of airway hyperresponsiveness. These results offer a clear rationale for the success of recent drug trials targeting Th2 cytokines in asthma. Indeed the reduction in exacerbation frequency during these trials suggests that the associations observed in the current study are functionally relevant. Moreover the prospect of being able to inhibit the induction of all three Th2 cytokines through the upstream targeting of IL-33 is tantalising.

It has also been possible to demonstrate that virus-induced Th2 inflammation in the nose reflects levels in the asthmatic lung for all mediators with the exception of TARC/CCL17. Following inoculation with rhinovirus, an approximate 2 day lag appears to exist between induction of mediators in the nose and induction in the lung with day 4 bronchial samples correlating most closely with day 2 nasal samples in all cases (with the exception of TARC/CCL17). Additionally basal levels of all 5 mediators are strongly related to the degree of their induction during rhinovirus infection offering a potential opportunity to identify suitable asthmatics for Th2 targeted therapies through the use of nasosorption in the clinic setting.

The demonstration of Th2 cytokine induction in-vivo by rhinovirus and the associations with exacerbation severity supports the observations by Message et al. who found that baseline IL-4, IL-5, and IL-13 production by BAL T cells were all associated with more severe virus-induced asthma symptoms following infection in-vivo. In addition, Bartlett & Walton observed significant induction
of IL-4 (day 1) and IL-13 (days 1 and 2) expression in rhinovirus infected, ova-sensitised mice compared with ova-sensitised controls using the mouse model of RV-induced exacerbations.\textsuperscript{57}

In this study the significant induction of IL-33, along with the Th2 chemokines and Th2 cytokines in the nose during infection was in most cases not replicated to a significant degree in the lung (although median levels in the asthmatic lung on day 4 always exceeded those at baseline). This most likely relates to the timing of the bronchial sample, with day 4 one or two days too premature to assess the magnitude of cytokine induction fully. In support of this are the findings that in the nose, Th2 mediators reached their peak on day 3 or 4, and that a 2 day lag appeared to exist between induction in the nose and the lung. It would therefore seem likely that the optimal day for sampling the lung would be either day 5 or 6 depending on the mediator(s) of interest. Unfortunately, due to the invasive nature of bronchoscopy for lower airway sampling and the difficulty with which this can therefore be performed more than once during the acute infection period, a single 'snap-shot' of bronchial cytokine levels was all that was feasible in this study.

It was somewhat surprising to find that a degree of type 2 mediator induction was also evident in those non-asthmatic subjects with the most severe colds. This suggests that activation of this pathway is associated with an exaggerated response to rhinovirus whether asthmatic or not. The absence of measurable Th2 cytokines in the lung of healthy subjects is consistent with their lack of lower respiratory symptoms. It is unclear whether other viruses are also capable of inducing Th2 inflammation, however some data from mouse models suggest RSV may also be capable of doing so\textsuperscript{182}. Indeed to date, much of the published work relating respiratory viral infections to the induction of type 2 immunity has focused on the relationship between RSV infection in early life and development of persistent wheezing and asthma.\textsuperscript{243–246}

The observation that exacerbation severity was significantly associated with increased levels of IL-33, as well as the downstream Th2 chemokines and cytokines rather than any single one of these mediators alone adds further weight to the case that this pathway is functionally related to
exacerbation pathogenesis. The relationship between virus load and IL-33 lends further support to this and is in keeping with the respiratory epithelium being both the site of virus infection and the main source of IL-33. But perhaps some of the most compelling findings to support the observations described in this chapter are the results of the recent drug trials targeting IL-5 and IL-13 with monoclonal antibodies. The DREAM study was a large multicentre, double-blind placebo-controlled trial of the anti-IL-5 monoclonal antibody, mepolizumab. Over 600 asthmatics with a history of severe exacerbations and signs of eosinophilic inflammation were recruited, with the rate of clinically significant exacerbations as the primary outcome measure. Whilst the specific trigger for these exacerbations was not recorded, it is highly probable that the majority of cases were triggered by viruses with rhinoviruses being the dominant cause (in keeping with nearly all epidemiological studies of asthma exacerbations). By blocking IL-5, mepolizumab reduced the rate of exacerbations by approximately 50%. Lebrikizumab, a monoclonal antibody directed against IL-13 was assessed in patients with uncontrolled asthma over a period of 24 weeks. In subjects with 'Th2 high' disease at baseline (defined as a total IgE > 100 IU/mL and an eosinophil count > 0.14 x 10^9 cells/L) a 60 % reduction in exacerbations compared to placebo was demonstrated. When these studies are considered together with the data presented in this chapter and the huge body of published data highlighting viruses (and in particular rhinoviruses) as the dominant trigger for exacerbations, it is reasonable to suggest that the success of these therapies relate to their ability to prevent virus-induced Th2 cytokine production and the downstream effects that result from this.

Phenotyping asthma has shown that approximately half of asthmatics have a Th2-driven phenotype. Therefore these therapies are unlikely to be successful in all asthmatics. Given the significant costs associated with these treatments the ability to accurately identify patients most likely to benefit from these drugs is highly desirable. Until now, the difficulty in directly measuring IL-5 and IL-13 protein in the airway has been too great and there has therefore been a reliance on markers of these cytokines. Traditionally, the use of eosinophil counts and total IgE have been used for this task. More recently periostin, an extracellular matrix protein induced by IL-4 and IL-13 in
airway epithelial cells$^{247}$ and lung fibroblasts$^{248}$ has been measured. However, all of these tests have limitations and further validation of periostin is required before it can become in widespread use.

The results presented in this chapter do however offer a potential alternative. They show that baseline levels of both IL-5 and IL-13 correlate significantly with the degree of induction by rhinovirus, and that virus-induced Th2 inflammation in the nose reflects levels in the asthmatic lung. It therefore seems possible to identify at baseline those asthmatics who will go on to have the most marked Th2 response to virus using nasosorption. Not only is this sampling method quick, cheap, and minimally invasive, but it directly measures the cytokine of interest rather than biomarkers of that cytokine. These findings need to be confirmed in further larger studies potentially involving naturally-occurring exacerbations but offer great promise if reproduced. Further work must also be undertaken to establish the stability of the Th2 phenotype in asthma, i.e. does the degree of asthma control or other factor significantly affect it over time?

The demonstration of IL-33 induction by rhinovirus during an asthma exacerbation is a novel finding. Despite the fact that we utilised techniques to directly sample the mucosa we were surprised at the high levels of IL-33 observed in the lungs. However, since IL-33 correlated with virus load, asthma symptom scores, and levels of mediators reported to be induced by it (IL-5 and IL-13), and these relationships were exclusive to asthma, it is reasonable to suggest that increased levels during infection are likely related to asthma exacerbation pathogenesis.

It is interesting to note the absence of a relationship between IL-33 and IL-4 both at baseline and on day 4, and both in the nose and the lung. This in contrast to the strong relationships observed between IL-33 and the other Th2 cytokines IL-5 and IL-13 as well as with TARC/CCL17 and MDC/CCL22 but is in keeping with several published studies in mouse models showing production of IL-5 and IL-13 but not IL-4 by innate lymphoid cells / nuocytes in response to IL-33.$^{152}$ In fact these lineage negative cells are considered by many to be the principal cellular source of IL-5 and IL-13 and
a previously unrecognised effector cell in asthma. In addition, IL-33 has been shown to activate
dendritic cells to produce TARC/CCL17 as well as priming naïve T cells to produce IL-5 and IL-13.
The IL-33 observations presented in this chapter highlight this cytokine as a potential novel target for
the prevention and/or treatment of asthma exacerbations. Indeed, this approach may be more
effective than blocking individual Th2 cytokines given that IL-33 is reported to induce multiple Th2
cytokines.
Chapter 7

Discussion

This study set out to advance our understanding of the pathophysiology of virus-induced exacerbations of asthma. The appreciation that rhinovirus is the single most common trigger for asthma exacerbations and the subsequent development several years later of a human model of experimental rhinovirus-induced asthma exacerbations has allowed us to examine these acute events in a controlled setting. To date, published studies using this model have demonstrated that compared to healthy volunteers, rhinovirus leads to increased lower respiratory symptoms, falls in lung function, and increased airway hyperresponsiveness in mild, well-controlled asthmatics in keeping with a mild exacerbation. The exclusion in previous studies of moderately-severe asthmatics and those not well-controlled at the time of infection has meant that the influence of asthma severity and control on the outcome of a rhinovirus infection in asthma has remained unknown.

Additional published in-vitro and ex-vivo data have suggested that although the Th1 response is considered the classical immune response to virus infection, the exaggerated clinical response to rhinovirus seen in asthma might be related to an augmented Th2 and/or a deficient Th1 immune response to this virus. The finding in recent drug trials using monoclonal antibodies directed against the Th2 cytokines IL-4, -5, and -13 of a reduction in the frequency of exacerbations in selected asthmatics has provided a clear demonstration of the relevance of this pathway to exacerbation pathogenesis. Yet until the current study there has been no actual demonstration of Th2 cytokine induction during a virus-induced asthma exacerbation in-vivo. This has been in part due to the difficulty in measuring these and many other inflammatory mediators in the airway due to their dilution to undetectable levels following the conventional sampling techniques of bronchoalveolar and nasal lavage.
Therefore the findings presented in this thesis both provide novel insights into clinical factors that influence the outcome of a rhinovirus infection as well as furthering our understanding of the underlying immunological mechanisms involved in exacerbation pathogenesis. In addition the development of a novel bronchial sampling device has the potential to greatly increase our understanding of a wide range of respiratory diseases by permitting the analysis of previously undetectable mediators.

Specifically, the major findings of this study include the first demonstration that:

1. Asthmatics with moderately-severe disease experience more severe rhinovirus-induced exacerbations than those with milder disease.

2. Asthmatics with poorly-controlled disease at the time of rhinovirus infection experience more severe exacerbations than those with well-controlled disease.

3. Asthmatics have significantly greater virus load than healthy subjects following rhinovirus infection supporting a possible deficiency in their anti-viral immune response.

4. Nasosorption and bronchosorption are well-tolerated airway sampling techniques that have significant advantages over conventional techniques.

5. Rhinovirus induces multiple members of the type 2 immune pathway in the asthmatic airway in-vivo and that these inductions relate to virus load, exacerbation severity, and bronchial hyperresponsiveness.

6. Rhinovirus induces comparable type 1 immune responses in the upper airway of both healthy and asthmatic subject’s in-vivo, but induction of type 1 mediators in the lower airway of asthmatics only.

7. The inflammatory response to rhinovirus in the upper airway is mirrored in the lower airway in asthma.
8. Basal levels of many cytokines including IL-5 and IL-13 relate to the degree of their induction during the exacerbation, highlighting nasosorption as a potential tool for identifying asthmatics best suited to targeted therapies.

The relationship between baseline asthma severity and clinical outcome following rhinovirus infection observed in this study raises several interesting questions worthy of future study. Firstly, do asthmatics with poor baseline lung function have a history of more frequent and / or severe virus-induced exacerbations which are at the heart of their progressive loss of lung function over time? If so, this may be secondary to a genetic predisposition in a similar fashion to that reported for the anti-viral protein IFITM3 in the context of influenza\textsuperscript{250}, or due to other co-factors that may be relevant such as a significant degree of underlying allergic inflammation (e.g. due to house-dust mite and / or other sensitisations).\textsuperscript{251} If this were the case, in recruiting a cohort of asthmatics with more severe airflow obstruction, one may be inadvertently selecting asthmatics with a propensity for more frequent and / or severe virus-induced infections. This could explain the findings of increased virus-induced morbidity in the moderately-severe asthmatics presented in this study. Indeed it is possible that there are virus-induced remodelling factors that are up-regulated upon infection in certain asthmatics that could explain the significant decline in lung function observed over time with exacerbations.\textsuperscript{252} In a mouse model of RSV infection, Tourdot and Lloyd have highlighted fibroblast growth factor (FGF)-2 as one possible protein.\textsuperscript{253} In addition a role for IL-25 in orchestrating airway remodelling has recently been shown by highlighting a link between Th2 inflammation (including IL-33), and airway remodelling.\textsuperscript{254}

In addition to the novel finding of a relationship between baseline asthma severity and clinical outcome, a further novel finding of this study has been the demonstration that the level of asthma control at the time of infection relates to the severity of the virus-induced exacerbation. The idea that a link between current control and future exacerbation risk exists has been increasingly appreciated in the last few years and reported in the context of naturally occurring exacerbations in
longitudinal studies. To date, no author has suggested that this may be related to the influence of improved control on reducing the severity of disease following virus infection (despite most exacerbations being secondary to respiratory virus infections). Taken together, the relationships between asthma control and exacerbation risk in both the longitudinal study and this controlled study highlight the vital need for maintaining good asthma control in reducing the risk of severe exacerbations.

This study did not set out to assess the effect of asthma medication on clinical, virological, or inflammatory outcomes, and no accurate conclusions can be safely drawn about the efficacy of inhaled corticosteroids in reducing exacerbation severity. This is primarily because all of the inadequately controlled asthmatics were on suboptimal doses of their inhaled corticosteroid, and full adherence to their treatment could not be guaranteed (despite regular encouragement to do so). In addition the effect of treatment on virus-induced airway hyperresponsiveness was heavily biased by the inability to perform the day 7 PC20 on several of the most severely affected asthmatics.

A potential confounding factor in this and previous studies that is regularly commented upon is the extent to which atopy in general, rather than asthma, is responsible for the differential response to rhinovirus demonstrated here. On the one hand, the appreciation that non-asthmatic individuals with evidence of sensitisation to one or more aeroallergens do not experience an asthma exacerbation when they fall ill with a common cold suggests that the presence of asthma and not atopy is the key differentiating factor. Furthermore anecdotal clinical experience of severe non-atopic asthmatics under the care of the Royal Brompton Hospital suggests that these asthmatics can have life-threatening exacerbations when they develop a respiratory viral infection despite the absence of demonstrable atopy. A recent study by Baraldo and colleagues attempted to address this question by recruiting children with asthma, atopy, neither, or both and infecting cultured epithelial cells obtained at bronchoscopy with rhinovirus. The authors found that deficient type 1 (β) and type 3 (λ) interferon responses to rhinovirus were present in asthma whether atopic or not but in
addition were found in atopic patients without asthma. It is interesting to note however, that those subjects with atopy but not asthma had as much IL-4 positivity and epithelial damage in their bronchial biopsies, as did those children with asthma, suggesting the common factor is the degree of underlying airway inflammation. This is supported by the finding of interferon deficiency in cystic fibrosis and COPD. The precise make-up of this increased inflammation is unclear and is likely to differ from subject to subject from a significant eosinophilia in some asthmatics to a marked neutrophilia in others to simply an increase in levels of pro-inflammatory (or other) cytokines.

Whilst we did not assess type 1 and type 3 interferon innate immune responses in this study, the finding of greater virus loads, and greater levels of virus-induced morbidity particularly in those with poorly-controlled asthma supports this hypothesis. Possibilities for future work to address the role of atopy in the asthmatic response to virus are discussed at the end of this chapter.

The development of bronchosorption has been one of the overriding successes of this study. It has permitted the measurement of the key mediators of type 1 and 2 inflammation including several that have never been measured in the asthmatic lung during an exacerbation before. The combination of this sampling technique with the human model of experimental rhinovirus infection in asthma has allowed numerous relationships between the degree of induction of these mediators and cold / asthma exacerbation severity to be revealed. The findings themselves support the enhanced accuracy of these novel techniques as not only are there differences where one would naturally expect them (as with Th2 cytokine levels in asthma compared to healthy subjects), but also extremely strong relationships between mediators known to be closely related functionally (as with IL-5 and IL-13, TARC/CCL17 and MDC/CCL22, as well as IP-10/CXCL10 and I-TAC/CXCL11.

However, many unknowns remain regarding this sampling technique and only limited validation experiments have been possible in the time-frame of this study. In addition at a time when the current study was drawing to a close, Scadding and colleagues performed a three way comparison of Accuwick with a cellulose matrix known as 111 (Pall), and a synthetic polyurethane sponge. The
results suggested that the sponge was superior to the other two matrices. Unfortunately no comparison with Leukosorb was performed and no assessment following application of buffer was carried out to assess protein recovery. Future experiments need to address this and other questions concerning these sampling techniques (this is discussed in full at the end of this chapter).

The protein analysis of the upper and lower airway performed in this study has provided the first demonstration of type 2 cytokine and chemokine induction by rhinovirus in asthma in-vivo. Moreover, levels of these mediators relate to virus load, exacerbation severity, and airway hyperresponsiveness and in so doing support the previous observations that baseline IL-4, IL-5, and IL-13 production by BAL T cells were all associated with more severe virus-induced asthma symptoms following subsequent infection in-vivo. The recent findings of a reduction in exacerbations with the use of monoclonal antibodies directed against the Th2 cytokines confirms the functional relevance of the current observations.

The demonstration of IL-33 induction by rhinovirus in the asthmatic airway in-vivo and the relationships to virus load, exacerbation severity, as well as levels of the Th2 mediators (IL-5 and IL-13) reported to be induced by it are all novel findings in man. The observations are consistent with results from mouse models of RSV infection in which monoclonal anti-ST2 (IL-33 receptor) treatment reduced lung inflammation and illness severity in mice with Th2 but not Th1 immunopathology, as well as the results of a mouse model of Influenza A infection which demonstrated production of IL-33 which in turn activated innate lymphoid cells producing substantial amounts of IL-13 and leading to airway hyperresponsiveness. Taken together, the results highlight IL-33 as a potential novel target for the prevention and/or treatment of asthma exacerbations.

This is also the first study to demonstrate a clear induction of the Th2 chemokines TARC/CCL17 and MDC/CCL22 by rhinovirus in asthma in-vivo, together with IL-33, the Th2 cytokines IL-4, -5, and -13 as well as the demonstration of eosinophilia in the asthmatic lung following infection, thus this study
has shown the induction of type 2 immunity by rhinovirus at multiple points along the immune pathway. The absence of these findings in the vast majority of healthy subjects in this study further implicates augmented type 2 immune responses in the pathogenesis of virus induced asthma exacerbations.

It has been hypothesised that a relatively strong Th2 bias at the time of a viral infection might impair Th1 responses to the viral infection and thereby increase the severity of outcomes. Such an imbalance was first described by Legg and colleagues in a study of 28 infants experiencing natural RSV infection. The 9/28 infants who developed signs of acute bronchiolitis had an elevated IL-4/IFN-γ ratio compared with infants with upper respiratory tract infection alone, implicating excess type 2 and/or deficient type 1 immune responses in the pathogenesis of RSV bronchiolitis.

A clear deficiency of the type 1 response to infection (as opposed to simply an increased type 2 response and therefore increased ratio) in asthma has not been shown in-vivo. The results presented in the current study also fail to demonstrate an obviously deficient response. The only evidence of one was with IL-12, as it could be argued that the levels in asthma (which were non-significantly lower on each day of infection than in healthy subjects) were low relative to the increased virus loads and symptoms seen in asthma.

This finding aside, a significant induction of IL-12, IL-15, IFN-γ, IP-10/CXCL10, and I-TAC/CXCL11 in the upper airway of both healthy and asthmatic individuals was seen, with additional induction in the asthmatic but not healthy lung during rhinovirus infection. Levels in asthma related to virus load as well as symptom severity, but in contrast to IL-5 and IL-13 had no relationship to airway hyperresponsiveness.

Due to the fact that relationships with symptom scores were seen for both type 1 and type 2 mediators, the relative contribution of each relationship is unclear and cannot be addressed in this study. However it is interesting to note that despite being non-atopic, a few healthy subjects also
demonstrated a degree of type 2 induction in their upper airway during infection and that these subjects were the ones reporting the greatest upper respiratory symptom scores.

**Future work**

**Airway mucosal sampling**

A number of outstanding questions and uncertainties regarding airway mucosal sampling remain and require future studies to adequately address them. These include:

1. Does the site of bronchosorption (either a more proximal or distal bronchus) affect the cytokine measurements significantly? Is there an ideal site for sampling?

2. Is there an optimal length of time to sample the nasal / bronchial mucosa in order to maximise cytokine recovery?

3. Does the lignocaine used to anaesthetise the lower airway prior to instrumentation affect cytokine recovery in any way?

4. Does prior sampling with bronchial brushings or bronchial biopsies lead to a localised inflammatory response that could affect bronchosorption findings in a neighbouring airway?

5. Is there any scope for increasing the surface area of the Leukosorb tip to improve mediator concentration through proportionately less dilution by buffer?

6. Is there a superior alternative to Leukosorb for this purpose?

7. Can the concentration of Triton X or other constituents of the buffer be altered to improve cytokine recovery?

Many of these questions can be addressed in a fairly straightforward and safe manner by repeating a number of bronchosorptions in different parts of the bronchial tree in the same individual during a single bronchoscopy. By administering lignocaine just distal to the carina on one side only, one could directly compare of its effects following both right and left lung sampling.
Placement of the sampling tip for 30, 60, 90, 120, and possibly 240 seconds would permit an improved understanding of the optimal length to sample for. It is noteworthy that the original description by Alam of a nasal sampling technique using filter paper sampled the nose for 10 min at a time. The strips were then air-dried, and stored before being washed with small volumes of Hepes buffer containing 0.3% human serum albumin. Nasal sampling in the current study was performed for 2 minutes at a time. Whether any advantage can be gained by longer placement needs to be evaluated. Following the findings by Scadding, a direct comparison between Leukosorb and the polyurethane sponge needs to be undertaken. Assessment of the sponge with and without a subsequent wash step with a buffer containing Triton X is also required.

The usefulness of nasosorption as a biomarker test

A simple, easy to perform, cheap, and relatively non-invasive biomarker test to inform physicians of the Th2 status of patients is needed to guide therapy. The results presented in this thesis identify nasosorption as potentially such a tool. However, these findings need to be repeated and expanded in larger studies.

A future study should take place in two stages. In the first instance, it is vital to establish the stability of the Th2 (or other) phenotype and address the question of whether basal levels of Th2 cytokines vary over time in the non-exacerbating asthmatic. If so, a one-off measurement of these cytokines by nasosorption may not provide useful information about the likelihood of success with mAb therapies or other drugs. However, if the phenotype appears relatively constant over time, then IL-5 or IL-13 measurement via nasosorption could be identified as a superior test to those currently available (eosinophils, IgE, periostin) as it allows the direct measurement of the cytokine in question. A study over a period of 12 months with monthly nasosorption performed on a cohort of in excess of 100 asthmatics with additional sampling at the time of an exacerbation should address these initial questions adequately.
The second stage of the study would involve randomising those asthmatics found to have significant Th2 inflammation to either mepolizumab or placebo prior to experimental inoculation with rhinovirus. An additional arm could incorporate a low Th2 group. The hypothesis would be that mepolizumab would reduce exacerbation severity in Th2-high asthmatics only. Fortunately, due to recent large trials of mepolizumab, its safety is not a significant concern.\textsuperscript{143}

**Evidence for virus-induced remodelling in asthma**

Our understanding of virus-induced airway remodelling is in its infancy and largely limited to mouse models of disease. The idea that the relationship between poor baseline lung function and clinical outcome is linked through an increased predisposition for virus-induced remodelling is intriguing and deserves future work. A study could specifically look for induction of a variety of potential remodelling factors following experimental infection with rhinovirus in a group of asthmatics with normal lung function compared to those with evidence of airway remodelling / significant airflow limitation. An additional 6 week bronchoscopy would permit assessment of any failure of inflammatory resolution following infection that might be specific to the more severe group. Production of remodelling factors following RV infection of cultured epithelial cells would be performed in parallel, and would allow the inclusion of asthmatics too severe to inoculate experimentally. A comparison of remodelling factors from bronchial epithelial cells of asthmatics known to have normal lung function would follow.

**The role of atopy**

A future human experimental infection study could aim to exclude asthmatics with evidence of atopy altogether and simply recruit non-atopic asthmatics. However, such asthmatics are far less common than their atopic counterparts (no more than 5% of all asthmatics screened in this study failed to respond to at least one aeroallergen on skin prick testing) and are frequently more severe
making recruitment to a study involving experimental infection both difficult and in some cases unsafe. However, recruiting a third arm of subjects with evidence of atopy but an absence of asthma may be significantly easier to complete and offer similar insights. Another possibility is ensuring that asthmatics with evidence of a perennial allergen such as house dust mite are excluded (even if sensitisation is subclinical), and instead only recruiting asthmatics sensitised to seasonal aeroallergens and ensuring they are 'out of season' e.g. an asthmatic mono-allergic to grass pollen being inoculated with rhinovirus in November.

**Studying the effect of improved asthma control on the outcome of rhinovirus infections in asthma**

It would seem reasonable that a reduction in virus-induced morbidity through better disease control should be readily demonstrable using the experimental exacerbation model. A study in which asthmatics with poor baseline asthma control were matched for age, sex, atopy, asthma severity, and baseline ACQ and then randomised to either immediate challenge with rhinovirus or a period of intensive treatment to gain adequate control and subsequently challenged with virus would allow such an assessment - and is a study that now demands to be undertaken.

**The role of IL-33 and other epithelial-derived inducers of Th2 inflammation in virus-induced exacerbations of asthma**

IL-33 induction by rhinovirus and its relationship to virus load, exacerbation severity, other type 2 cytokines, as well as its upstream position in the type 2 pathway highlights IL-33 as one likely to play a critical role in virus-induced type 2 inflammation in asthma. The precise nature of this role now needs to be investigated further. In the absence of a safe, humanised monoclonal antibody directed against IL-33 (currently in production by a number of pharmaceutical companies), initial work using the mouse model of rhinovirus-induced exacerbation of allergic airway inflammation along with IL-33 blocking antibodies could be attempted. *In-vitro* human work addressing the role of IL-33 using
rhinovirus infected bronchial epithelial cells from asthmatics is currently in progress within the Johnston group at Imperial College and elsewhere in the world but to date there are no published observations.

TSLP is increasingly regarded as a key factor in the development of atopic asthma and is expressed at increased levels in the lungs of both human asthmatics and mice with allergic airway inflammation. A strong link has been demonstrated between TSLP expression and the production of IL-4, -5, and -13, whilst the opposite association has been shown between TSLP and Th1 responses. Infection of BECs from asthmatic children with RSV led to significantly more TSLP production than following infection of BECs from healthy children. In addition, RSV-infected TSLPR-deficient mice have reduced immunopathology as defined by mucous secretion and AHR, suggesting a role for TSLP in the pathogenesis of virus-induced asthma exacerbations.

To date there is no published data demonstrating a role for TSLP during rhinovirus-induced asthma exacerbations and although measurement of this mediator was attempted during this study, problems were encountered with the TSLP assay resulting in unreliable data and levels mostly below the lower limit of detection. However, in view of the clear induction of the Th2 pathway demonstrated in this study, the In-vivo measurement of TSLP along with the other upstream epithelial-derived Th2 inducer IL-25 is of great importance and should be attempted in the next study using this exacerbation model and sampling techniques.
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**Table A1** Individual virus load and serum rhinovirus-16 antibody titres. (Mod = moderate severity)

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Table A2  Bronchial hyperresponsiveness measured by histamine PC$_{20}$.

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Table A3. Changes in BAL inflammatory cell proportions during RV infection. Data shown as median percentages (interquartile range).

 (+, P<0.05; ++, P<0.01; +++ , P<0.001 compared to healthy subjects; *, P<0.05 day 4 compared to baseline).

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