

The role of IL-25 in rhinovirus-induced asthma exacerbations

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Abstract

Rhinovirus (RV) infections are the principal cause of asthma exacerbations. While Th2-mediated inflammation is clearly implicated in the asthmatic response, it is unknown how the immune response to RV infection interacts with Th2 immunity to enhance disease pathogenesis. The epithelial-derived cytokine, IL-25, has been identified as an initiator and regulator of Th2 immunity and plays a role in asthma pathogenesis. Based on the fact that RV infects bronchial epithelial cells, we hypothesized that RV induces IL-25 production providing a link between infection and Th2 driven allergic inflammation.

RV-induced IL-25 expression was measured in human bronchial epithelial cells (HBECs) obtained from bronchoscopic brushings from atopic asthmatics and healthy patients. Mouse models of RV infection and RV-induced allergic airways disease were also employed to examine IL-25 induction in response to RV infection and/or OVA sensitisation and challenge. Finally, to define a mechanistic role for RV-induced IL-25, signalling mediated by IL-25 was blocked in our model of RV-induced allergic airways disease by neutralising the IL-25 receptor.

RV-infected HBECs from asthmatics expressed significantly greater IL-25 gene and protein compared with cells from healthy controls. Furthermore, RV infection of mice induced IL-25 expression in the airway epithelium as well as in inflammatory cells in the airway lamina propria. Using a mouse model of RV-induced allergic disease, we demonstrated that RV enhanced allergen-driven IL-25 gene and protein expression which was associated with increased Th2 inflammation in the lung. Finally, by blocking IL-25 signalling in an RV-infected and OVA-sensitised and challenged mouse, several key features of the exacerbation phenotype were significantly reduced including airway leukocyte infiltration, BAL Th2 cytokines and chemokines and Th2 cells.

These novel findings indicate that RV-induced IL-25 plays an important role in enhancing Th2 inflammation associated with the exacerbation phenotype which is mediated by binding to the IL-25 receptor.

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Abbreviations

AA	atopic asthmatic
AHR	airway hyperreactivity
Alum	aluminium hydroxide
ANOVA	analysis of variance
APC	antigen presenting cell
ATCC	American type tissue culture collection
BAL	bronchial alveolar lavage
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
CNS	central nervous system
CPE	cytopathic effect
Cy	cyanine
DC	dendritic cell
dNTP	deoxyribonucleotide triphosphate
EAE	experimental autoimmune encephalomyelitis
EAR	early asthmatic/allergic reaction
ECP	eosinophil cationic protein
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPO	eosinophil peroxidase
FCS	foetal calf serum
FEV	forced expiratory volume
FITC	fluorescein isothiocyanate
FVC	forced vital capacity
g	gram
h	hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HBEC	human bronchial epithelial cell
HRP	horseradish peroxidase
HRV	human rhinovirus
ICAM-1	intracellular adhesion molecule 1
ICS	intracellular staining

IFN	interferon
Ig	immunoglobulin
Ih2	innate type 2 helper
IL	interleukin
ILC	innate lymphoid cell
i.n.	intranasal
i.p.	intraperitoneal
IP-10	interferon-gamma inducible protein 10kDa/CXCL10
ISO	isotype
ISH	<i>in situ</i> hybridisation
kB	kilobase (pairs)
KO	knockout
L	litre
LDL	low density lipoprotein
lin	lineage
LPS	lipopolysaccharide
LTR	lower respiratory tract infection
MAPK	mitogen-activated protein kinase
min	minute
MMP	matrix metalloproteinase
MPP ^{type2}	multi-potent progenitor type 2
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NaCl	sodium chloride
NANA	non atopic non asthmatic
NFATc1	nuclear factor of activated T-cell cytoplasmic
NH	natural helper
NF-κB	nuclear factor-κB
NK	natural killer
OVA	ovalbumin
PBS	phosphate buffer saline
PBMC	peripheral blood mononuclear cells
PC ₂₀	provacative concentration
PCR	polymerase chain reaction
PE	phycoerythrin
PEF	peak expiratory flow

PEG	poly ethylene glycol
PenH	enhanced pause
PerCP	peridin chlorophyll proteins
PMA	phorbol myristate acetate
PolyI:C	polyinosinic-polycytidylic acid
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
RANTES	regulated upon activation, normal T cell expressed and secreted
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	respiratory syncytial virus
RT PCR	reverse transcription polymerase chain reaction
RV	rhinovirus
SEM	standard error of the mean
ss	single stranded
T cell	T lymphocyte
TCID ₅₀	tissue culture infectious dose
TLR	toll like receptor
TMB	tetramethyl benzidine chromogen
TNF	tumour necrosis factor
TSLP	thymic stromal lymphopoietin
T2M	type 2 myeloid
URT	upper respiratory tract
UV	ultraviolet
w/v	weight per volume

Chapter 1: Introduction

1.1 Human Rhinoviruses

1.1.1. Disease Burden

First isolated and cultured in 1953 from subjects displaying cold symptoms, human rhinoviruses (RV) have since been identified as the singular greatest cause of the common cold, accounting for approximately two-thirds of all acute and upper respiratory tract (URT) infections^{1,2}. Non-influenza-related URT infections account for a total healthcare cost of \$40 billion per year in the US, while in the UK, children younger than 5 years of age with URT infections account for 59% of general practitioner visits^{3,4}. Therefore, the extrapolated economic burden of RV infections is substantial.

Diagnosis and detection of RVs has improved significantly since the application of polymerase chain reaction (PCR)-based techniques for RV screening which had previously relied on less sensitive cell or organ culture techniques⁵. These methods have not only allowed discovery of genetically divergent, novel RV strains, including the new species HRV-C, but have also enabled higher detection rates than earlier epidemiological studies^{6,7}. Notably, using reverse transcription (RT) PCR, investigators were able to confirm a theory long suspected by clinicians; namely, that respiratory viruses, most frequently RVs, are the principal trigger for asthma exacerbations in the vast majority of children and approximately two-thirds of adults^{8,9}. More recently, increasing evidence also indicates a causative role for rhinovirus in chronic obstructive pulmonary disease (COPD) exacerbations¹⁰.

While it was previously assumed that RV replication was restricted to the upper airways due to its ability to replicate optimally at 33-35°C, it has since become apparent that RVs have the ability to infect the lower airway¹¹. Increasing clinical and epidemiological evidence have linked RV infections to bronchitis, bronchiolitis and pneumonia in infants. Further to this, asthmatics, immunocompromised patients and the elderly have also been demonstrated to have increased susceptibility to lower respiratory tract (LRT) infections¹²⁻¹⁴. Studies in infants also indicate a role for RV in the progression of disease from allergic sensitisation to asthma^{15,16}.

RV infections, which were previously viewed as self-limiting conditions in the upper airways, have the potential to cause severe lower respiratory tract infections, exacerbations and potentially induce asthma in susceptible individuals^{8,15,17}. Mechanisms of how RVs interact with these underlying immune conditions to precipitate disease are currently unknown.

1.1.2 Circulation patterns and transmission

RV infections occur year-round but have a peak incidence in the early fall and to a lesser extent in the spring in temperate climates^{18,19}. Higher rates of infection in the young have been linked with return from school holidays^{19,20}. On average, pre-school children have 6 RV infections per year while the rate of symptomatic infection decreases with age as illustrated by a lower incidence of 2-3 infections per year in adults^{18,21}. Whether this is due to acquired immunity or lower transmission rates in adults is unknown^{19,22}. Introduction of virus into the eye or nose from contaminated fingertips is the predominant route of transmission, though aerosolised inhalation can occur^{23,24}.

1.1.3 Classification, structure and life cycle

RVs belong to the *Picornaviridae* family within the *Enterovirus* genus. Approximately 101 serotypes have been identified which encompass a major group (HRV-A) that makes up ~90% and a minor group (HRV-B) which accounts for the remaining 10%^{5,25}. These serotypes are classified based on the receptor used for host cell entry; HRV-A utilises the intercellular adhesion molecule 1 (ICAM-1) while HRV-B binds the low-density lipoprotein (LDL) receptor^{26,27}. More recently, a third species classified as HRV-C has been discovered by genomic sequencing although its receptor is currently unknown⁷.

RVs are small, nonenveloped viruses comprised of a single-stranded, positive sense RNA genome of ~7200 nucleotides. A single open frame encodes 4 structural capsid proteins (VP1-VP4) and 7 non-structural proteins. These non-structural components include an RNA polymerase, Vpg (needed for negative strand synthesis), the protease 2A (serves to shut down the translational machinery of the host cells) and the protease 3C/3CD (required for post-translational maturation)²⁸. The viral shell is composed of 60 copies of the capsid proteins folded into an icosahedron which create characteristic depressions or 'canyons'.

These 'canyons' contain ligands to the ICAM-1 receptor which, upon binding, cause destabilisation of the capsid²⁹. RV is internalised via receptor-mediated endocytosis and uncoating occurs in the endosome allowing transfer of genomic RNA into the cytosol³⁰. Due to the positive sense RNA orientation the genome is directly translated by the host cell. Utilising an internal ribosome-entry site (IRES) sequence that is located in the 5' untranslated region (5' UTR), RV is able to initiate a cap-independent mechanism of translation. This, together with RVs ability to inhibit the hosts mRNA translation, enables rapid propagation of viral replication and synthesis²⁸. In subjects experimentally infected with RV the growth cycle was reported to be approximately 5-7 hours (h) and progeny (released by cell lysis) were detectable within 11h post-infection (p.i.)³¹. The duration of onset of viral shedding was similar to conditions in cell culture³². Additional RV inoculation studies have demonstrated a peak in viral titres at 48h-72h p.i. while viral shedding can persist for up to 20 days p.i.^{31,33}.

1.1.4 Disease pathogenesis and the host immune response

RVs primarily enter and propagate within the upper respiratory tract, particularly the nasopharynx and nasal mucosa²⁰. In an otherwise healthy individual, the double-stranded viral RNA intermediate is recognised by the host pattern-recognition receptor (PRR), Toll like receptor (TLR) 3, which coordinates with melanoma differentiation associated gene-5 (MDA5) and retinoic acid inducible gene (RIG-I), to trigger a cascade of proinflammatory cytokines including the antiviral type I (IFN- β and IFN- α) and type III interferons (IFN- λ)^{34,35}. One of the most well characterised pathways is the induction of neutrophil-recruiting chemokine, CXCL8/IL-8, by RV-induced nuclear factor (NF)- κ B activation which results in nasal obstruction via increased neutrophilia^{36,37}. Congestive symptoms are related to nasal epithelial oedema, mucus hypersecretion and vascular permeability²⁰. In a healthy individual, proinflammatory mediators including IL-1, IL-6, CCL5/RANTES, IFN- γ and TNF- α have been demonstrated to be induced by RV infections. This is characteristic of a T helper (h) 1 response and has been shown to be associated with enhanced viral clearance and shortened symptoms^{38,39}. Cellular inflammation during RV infection involves neutrophil and T cell infiltration into the lung which in certain susceptible individuals (such as asthmatics) may be enhanced, worsening symptoms and affecting lung function. Bronchial CD4+ and

CD8+ T lymphocyte infiltration have also been observed at the height of cold symptoms in experimentally infected patients⁴⁰. Due to RV's limited cytotoxicity and damage of the mucosal epithelium, clinical symptoms of disease are largely thought to be caused by the host's inflammatory response⁴¹. For example, bradykinins are implicated in the induction of rhinorrhea, sore throat and nasal obstruction pathogenesis whilst prostaglandins D2 and F2 α can induce coughing and sneezing⁴². Mild fever may also present as a result of the release of pyrogens, including IL-1 and TNF. Symptom scores have most commonly been demonstrated to correlate with viral titre and neutrophil infiltration in the upper airways^{36,43}. Indications of disease can be exhibited as early as 10-12h p.i. but on average develop 1-2 days p.i. and peak around 2-3 days p.i. usually resolving in 7-10 days after inoculation^{32,44}.

1.1.5 Therapies

Due to the limited cross-reactivity of neutralizing antibodies to the ~101 RV serotypes generated in traditional vaccine strategies, RV-specific therapies have shifted to development of antiviral agents⁴⁵. Examples include compounds synthesized to prevent uncoating by stabilisation of the viral capsid, the most promising of which is the drug, pleconaril. Reported findings from phase II clinical trials demonstrated that pleconaril treatment reduced symptom duration by 3 days compared with placebo treatment. Although the drug was generally well tolerated, the authors highlighted the need for further characterisation of its potential for drug interactions⁴⁶. Another compound, rupintrivir, which is still in the preclinical stage, acts as an irreversible inhibitor of RV 3C protease and has been demonstrated to reduce cold symptoms, nasal shedding and viral titre compared with placebo⁴⁷. Two pharmaceutical companies have also generated truncated, soluble forms of ICAM-1 which effectively neutralizes the virus to prevent cell surface attachment. Although treatment significantly reduced disease symptoms, maintenance of drug concentration in the nasopharynx proved to be difficult due to mucociliary clearance, sneezing and nose-blowing^{48,49}.

Despite a number of promising results using antiviral compounds, RV-specific prophylactic or therapeutic treatments remain unlicensed. As a result, treatment of symptoms by non-

prescriptive medicines including nasal decongestants, analgesics and antitussives remains the most common approach⁴⁸.

1.2 Asthma

1.2.1 Epidemiology

Asthma is a disease of global importance as demonstrated by statistics relating to morbidity, mortality and associated healthcare expenditures⁵⁰. Approximately 300 million people worldwide suffer from asthma, with prevalence among people in most European countries increasing. This trend is also evident in less developed countries and has been attributed to urbanisation and implementation of Westernised lifestyles. Estimations predict that if this rising trend persists, an additional 100 million people will be afflicted with asthma by 2025⁵¹. In the UK, approximately 5.4 million people are currently receiving treatment for asthma, representing one of the highest prevalence in the world. Incidence is higher in children affecting approximately 1 in 10, making it the most common chronic childhood disease in the UK, while mortality statistics indicated ~1100 deaths attributed directly to asthma in 2009⁵². In addition to the UK, prevalence of clinically diagnosed asthma is high in Australia, New Zealand, and developed countries within North, Central, and South America⁵¹.

Economic costs including hospital admissions, physician visits and pharmacological expenditures associated with this condition rank as one of the highest among chronic diseases⁵³. The cost to the NHS for treatment and care of asthma sufferers is estimated at £1 billion a year. When this amount is extrapolated to include missed work/school days and lost productivity, an approximate £2.5 billion is spent on asthma-related expenditures per year in the UK⁵³.

1.2.2 Definition and diagnosis

Asthma is a heterogeneous syndrome that can broadly be defined as a disorder of the conducting airways resulting in variable degrees of airflow obstruction that is reversible or partly reversible⁵⁴. Airway inflammation is central in disease pathology and, together with alterations in airway smooth muscle growth and structural changes throughout the conducting airways, contribute to airway hyperresponsiveness (AHR), mucosal inflammation and airway remodelling⁵⁵. Pathology is aggravated by repeated episodes of airway inflammation and altered tissue repair and regeneration which can occur spontaneously or in response to environmental stimuli⁵⁴.

Diagnostic features of disease include recurrent wheezing, cough, shortness of breath (dyspnea) and chest tightness or any combination thereof and are reported in 90% of patients with asthma⁵⁶. However, these symptoms alone cannot confirm diagnosis and additional information about the subject's personal history such as cough or shortness of breath after exercise, worsened chest symptoms in the early morning or night waking as a result of aggravated symptoms can assist diagnosis. A family history of asthma and/or other allergic disease is also a useful diagnostic marker as asthma clusters in families, as well as worsening of symptoms in response to known triggers such as pets, house-dust mites, tobacco smoke or cold/humid air^{57,58}. Although these respiratory symptoms are indicative of disease, lung function tests are a main criterion for an objective diagnosis of asthma. Airflow limitation is indicated by the spirometric measurements, forced expiratory volume in one second (FEV₁) and slow or forced vital capacity (FVC). In milder asthmatics where there is an absence of fixed airway obstruction, airway flow limitation is usually reversible, either spontaneously or in response to treatment, as indicated by an increased FEV₁/FVC ratio. In addition, peak expiratory flow (PEF), which is associated with FEV₁ in most asthmatics, is useful for detection of variability in airway function. Bronchial provocation tests which indicate the concentration of histamine/methacholine required to produce a 20% fall (PC₂₀) in FEV₁ are also a useful measure of AHR⁵⁷.

1.2.3 Classification

Due to its heterogeneous nature, asthma has been grouped under various phenotypes; however classification on a clinical basis is broadly divided into two forms, namely allergic and non-allergic asthma⁵⁹. Allergic or atopic asthma is the most common form of disease and is predominantly characterised by the presence of allergen-specific IgE usually indicated by a positive skin-prick test (SPT) to a panel of common environmental allergens^{60,61}. By contrast, non-allergic asthma is not driven by allergen although sufferers can present with symptoms of allergic disease such as eosinophilic infiltration in the lung. Alternatively, a number of asthmatic sufferers present with a robust neutrophilic response in the airways which is indicative of a more severe, steroid-resistant asthma phenotype or viral-induced asthma exacerbation⁶². In accordance with these subsets, Woodruff *et al.* classified two unique molecular phenotypes based on the extent of Th2 inflammation; groups were defined as 'Th2-high' and 'Th2-low' with the latter subset characterised as poor responders to current asthma therapies⁶³.

The Global Initiative for Asthma (GINA) guidelines previously classified asthma based on disease severity including intermittent, mild persistent, moderate persistent and severe persistent subdivisions. This classification is based on lung function variability and airflow limitation as well as level of symptoms and frequency of disease⁵⁸. More recently, statistical cluster analyses of cross-sectional data have identified phenotypic subsets of asthmatic patients that differ based on age of disease onset, gender, atopic status, disease duration, medication use and lung function tests⁶⁴. A key purpose of these groupings is to tailor asthma management to specific phenotypes with the hope of creating more targeted and therefore more effective therapies⁶².

1.2.4 Disease models of allergen-driven asthma

Asthma pathophysiology is associated with airway inflammation, however multiple immune pathways are implicated in the activation of inflammatory components that bring about disease symptoms⁶⁵. The majority of research into the cellular components and mechanisms of asthma has been centred on allergic asthma induced by environmental allergens. These findings are largely derived from studies of allergen-challenged atopic patients and analyses

of biopsies, bronchial alveolar lavage (BAL), sputum and serum and *ex vivo* studies of clinical samples⁶⁶⁻⁶⁸.

In addition, mouse models of allergic airways inflammation have proven invaluable in investigating the role of cellular and molecular components *in vivo* using genetically modified mouse strains, monoclonal antibodies that block a singular component of the immune system or local gene overexpression. The most commonly employed mouse model of acute allergic airways inflammation entails systemic sensitisation with allergen combined with an adjuvant such as aluminium hydroxide (alum). This is followed by multiple allergen challenges to the airways over 1-2 weeks, although variations on this basic model have been described including the route of delivery of the sensitising allergen and the number of challenges administered⁶⁹. The most frequently used experimental allergen is the hen egg ovalbumin (OVA), which is utilised in this study. Other allergens that are commonly described include complex aeroallergens including house dust mite (HDM), cockroach extract, ragweed and *Aspergillus* species⁶⁹⁻⁷². To model more chronic aspects of asthmatic disease such as remodelling in the airways, prolonged exposure to low allergen levels in the airways for periods of up to 12 weeks in mice have also been employed⁷². Findings from these models and data from human studies have enabled characterisation of the early and late response to allergen challenge in the allergic asthmatic phenotype, which is briefly outlined below.

1.2.5 Allergen-driven asthma

In the asthmatic lung, inhaled allergen that is not removed by mucociliary clearance is taken up by antigen presenting cells (APC) that are distributed throughout the respiratory tract⁵⁹. Allergen is presented to naive T and B cells and, in the presence of high levels of IL-4 (the source of which is still unclear) and low levels of IL-12, T cells differentiate towards the Th2 phenotype⁵⁴. Isotype switching of B cells to IgE synthesis requires IL-4 and IL-13 provided by Th2 cells resulting in allergen-specific IgE synthesis and secretion which is followed by maturation into high-secreting IgE plasma cells. Binding of IgE to the α -chain of high-affinity Fc ϵ RI on mast cells and basophils represents the process of sensitisation⁷³. Subsequent allergen re-exposure results in cross-linking of the receptor-bound IgE and triggers

degranulation of cells and the release of preformed mediators (including histamine), newly generated mediators (including leukotrienes and prostaglandins) and an array of chemokines and cytokines⁵⁴. These mediators are responsible for the induction of the early-phase asthmatic response characterised by constriction of airway smooth muscle cells, increased AHR, vascular leakage, mucus secretion and recruitment of additional inflammatory cells. Via the release of factors including IL-5, CCL5/RANTES and CCL11/Eotaxin-1, eosinophils infiltrate the asthmatic lung and are one of the principal cellular features of the late-phase asthmatic reaction. Furthermore, mast cells contribute to inflammatory damage on airway endothelial cells, extracellular matrix and neurons through the release of granule proteins including eosinophil cationic protein (ECP), major basic protein (MBP) and eosinophil peroxidase (EPO)^{73,74}. Th2 cells, in addition to playing a role in allergen sensitisation, are central in the late-phase asthmatic response due to their ability to augment inflammation through the release of various chemokines and cytokines. The most commonly characterised Th2 cytokines in the allergic response include IL-4, IL-5, IL-9 and IL-13. In addition to the functions described for IL-4 and IL-5, IL-13 plays an integral role in regulating mucus hypersecretion and bronchioconstriction by acting on smooth muscle cells while IL-9 has been demonstrated to promote differentiation and recruitment of mast cells to the airways (Figure 1.1)⁶².

More recently, attention has shifted to the asthmatic airway epithelium and secreted factors have been demonstrated to play a role in the initiation and amplification of Th2 inflammation⁷⁵. Specifically, there has been considerable interest in the epithelial-derived cytokines, thymic stromal lymphopoietin (TSLP) and IL-33 as well as IL-25, which forms the focus of this research. Importantly, IL-25 and/or IL-33 have been demonstrated to promote Th2 inflammation by activating a subset of newly identified type 2 innate cells that secrete Th2 cytokines upon binding^{71,76-78}. In doing so, these innate cell populations create an environment at mucosal sites that is permissive for the development and activation of CD4+ Th2 cells and augmented Th2 inflammation⁷⁹. These cell populations have been reported to be recruited in response to IL-25 and/or IL-33, helminth infection and most recently, allergen^{76-78,80}. Of relevance is the presence of a member of the newly grouped innate lymphoid cells (ILC) 2 termed nuocytes in the lungs of allergen-sensitised and challenged

mice, suggesting a potential role in the asthmatic lung^{76,81}. This novel subset as well as additional IL-25-responsive innate populations are described in section 1.5.4.1 and summarised in table 1.3.

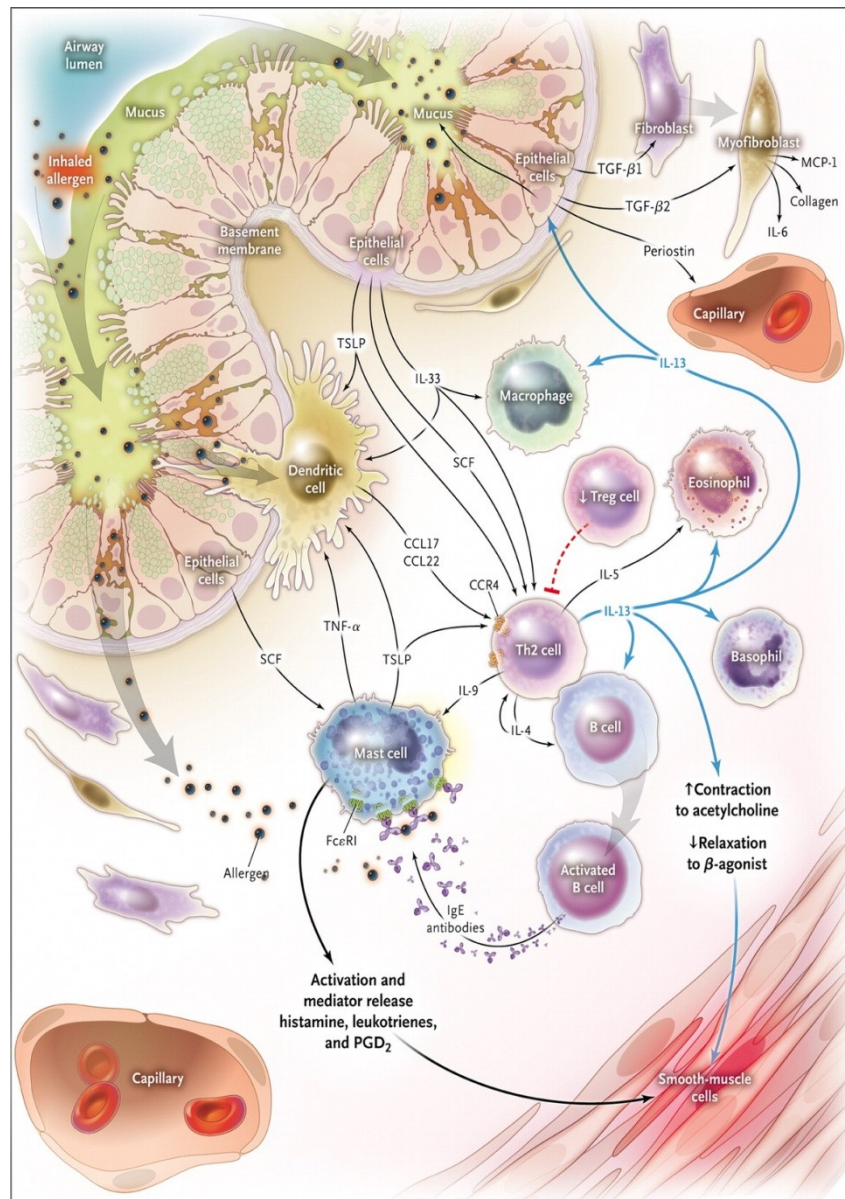


Figure 1.1 Representation of the Th2 immune response in the allergic pulmonary environment. Inhaled allergen is taken up by DCs, which then recruit and activate Th2 cells via binding of CCL17/TARC and CCL22/MDC to CCR4 expressed on Th2 cells. Binding of IgE to FcεRI on mast cells and basophils represents the process of sensitisation. Subsequent allergen re-exposure results in degranulation of cells and the release of histamine, leukotrienes and prostaglandins and an array of chemokines and cytokines (Diagram taken from Kraft, M. *et al.*⁸²).

1.2.6 Asthma therapies

There is currently no cure for asthma and therapeutic approaches are based on reducing the day-to-day variability of symptoms to improve quality of life. These therapies vary according to disease severity and symptoms⁵⁸. For example, mild, infrequent asthma is most widely treated by administration of a quick-acting bronchodilator such as a short-acting beta agonist (SABA) (e.g. salbutamol) which reverses smooth muscle constriction. For more persistent asthma, treatment is aimed at symptom prevention and better control by suppression of airway inflammation using an inhaled corticosteroid 1-2 times daily. Alternatively, a leukotriene-receptor antagonist can be used instead of corticosteroids to control mild asthma which works by blocking the effects of leukotrienes. For severe, persistent asthma, a combinative therapy of long-acting beta agonist and inhaled corticosteroids is effective due to their ability to reduce both bronchoconstriction and airway inflammation. Furthermore, an anti-IgE blocking monoclonal antibody (omalizumab) has been demonstrated to block circulating IgE and may be an attractive option for treatment of refractory allergic asthma⁵⁶.

The therapeutic approaches listed above are largely based on treating allergen-induced airway responses. Although these therapies have been demonstrated to increase stability of disease and improve the quality of life of patients, acute exacerbations of asthma still occur⁸³, which are discussed in detail below.

1.3 Asthma Exacerbations

1.3.1 Epidemiology

Loss of control of symptoms can result in an exaggerated lower airway response to environmental triggers termed an acute exacerbation⁸⁴. Allergens, environmental pollutants, medication, cold/humid air, exercise and occupational irritants have been identified as triggers, however virus infections account for the large majority of severe episodes⁵⁵. RVs are the most frequently detected viruses during exacerbations however other respiratory viruses such as respiratory syncytial virus (RSV), coronavirus, adenovirus, bocavirus, influenza and parainfluenza have also been observed^{8,9}. Severe exacerbations have been broadly classified as those that require an emergency department visit, hospital

admission or unscheduled visits to the doctor, and account for the majority of asthma-related expenses as well as the greatest morbidity and mortality in sufferers^{65,85}.

Johnston *et al.* demonstrated prevalence of respiratory viruses in asthma exacerbations in a seminal longitudinal study of 108 asthmatic children between 9 and 11 years of age. For 13 months nasal aspirate samples were collected and tested using RT-PCR as a viral diagnostic at the onset of infection or when asthma symptoms worsened. Viruses were detected in 80% of children with a reduced PEF and episodes of wheeze and in 85% of those with a combination of URT infections, wheeze, cough and a fall in PEF. Nearly two-thirds of detected infections were due to RV while coronavirus (15%), influenza and parainfluenza (both 8%) and RSV (5%) accounted for the remaining cases⁸. Similar associations have been recorded in asthmatic adults where cold symptoms were associated with the majority of asthma exacerbations with RVs accounting for 61% of the viruses detected⁹. A more recent study examined a mixed population of children and adolescents (aged 7-16 years old) admitted to hospital for acute exacerbations and reported that respiratory viruses were detected in 78% of subjects. Similar to previous findings, RV infections comprised 83% of the viruses found while influenza (15%), enterovirus (4%), and respiratory syncytial virus (2%) were also identified⁸⁶. A causal relationship for RV-induced exacerbations was also implicated by the fact that hospitalisations due to acute exacerbations corresponded closely with patterns of increased infectious rates of RV in the early fall in the northern hemisphere. This phenomenon was reported in asthmatic children aged 5-15 years who presented to the emergency department during 3 weeks in September with acute exacerbations. Respiratory viruses were detected in 62% of afflicted children, of which 52% were caused by human picornaviruses⁸⁷.

1.3.2 Pathogenesis and treatment

The variations in airway inflammation between chronic asthma and acute exacerbations have been demonstrated by studies of induced sputum samples. These cellular profiles indicate a heterogeneous mixture of eosinophils and neutrophils which was associated with a significant increase in IL-8, IL-5 and ECP in acute asthma⁸⁸. Increased T cell activation has also been reported in severe cases with an increased population of both Th1 and Th2 cells in

the peripheral blood and in induced sputum⁸⁹. In addition, increased activated CD8+ cells have been measured in tissue from fatal asthma exacerbations. Mucus plugging of the airways, which results in hyperinflation due to air trapping, has also been recorded in fatal cases. These reports are associated with increased neutrophilia in the submucosal gland and augmented degranulation of mast cells^{65,83}. Although multifactorial, acute asthma is predominantly characterised by increased airway inflammation and an augmented number of granulocytes particularly in the lower airways. Inflammatory profiles can vary depending on the stimulus, with virus-induced exacerbations associated with greater neutrophilia while allergen-induced episodes may present with more eosinophilic inflammation (Figure 1.2)⁸³.

The limited therapies available during an acute exacerbation indicate the diverse immunopathogenesis of exacerbations and highlight a need to investigate mechanisms of disease to improve treatment⁶⁵. Although conventional asthma therapy can reduce the frequency of exacerbation, the characteristic neutrophilic inflammatory response in a virus-induced attack has been demonstrated to be more resistant to corticosteroid treatment^{83,90}. In fact, based on findings that patients experimentally infected with RV and treated with oral corticosteroids had an increased nasal RV titre, therapy in some instances may be more detrimental⁹¹.

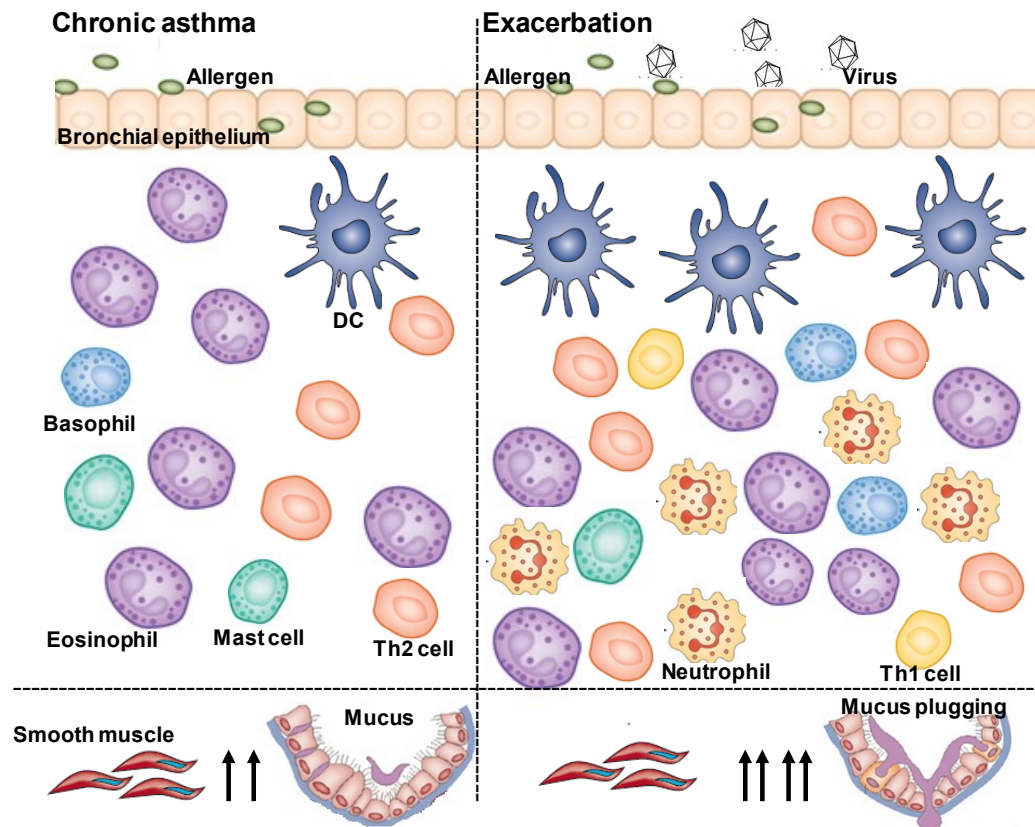


Figure 1.2 Variations in cellular profiles between chronic asthma and an acute exacerbation. The latter is characterised by a heterogeneous mixture of granulocytes (eosinophils and neutrophils), CD4+ T cells (Th1 and Th2 cells) and a greater influx of inflammatory cells (macrophages and dendritic cells). Mucus plugging of the airways and increased smooth muscle constriction have also been reported in severe exacerbations.

1.3.3 Interaction between infection and allergic asthma

A fundamental question to address is why RV infections that usually manifest as a self-limiting mild disease of upper airways in healthy individuals (cold) cause severe lower respiratory tract infections and precipitate asthma exacerbations in asthmatic individuals⁹²⁻⁹⁴. Studies have highlighted a strong association between atopy, allergen exposure and respiratory viral infections with an increased risk of exacerbations of asthma in children and adults^{92,95}. In addition, research indicates that atopic sensitisation and respiratory viral infections in early life may interact to increase the risk of asthma inception and/or development^{15,16}. Notably, Kusel *et al.* evaluated a community-based cohort of 198 children with a familial history of asthma and compared acute respiratory illnesses in the first year of

birth to respiratory outcomes at age 5. The presence of asthma at age 5 was significantly associated with children who were sensitised early (by 2 years of age) to common aeroallergens and those with virus-induced LRT infections in the first year of life¹⁵. In a mixed population of children aged 2 months to 16 years, the strongest likelihood for wheezing in children older than 2 years old were those who tested positive for RV (via RT-PCR) in combination with either increased allergen-specific IgE, nasal eosinophilia or ECP⁹⁶. Heymann *et al.* also demonstrated that RV was the predominant respiratory virus detected in children aged 3-18 years admitted to hospital with wheezing. In accordance with previous findings, serum IgE values were higher in wheezing children while 84% of those with wheeze were sensitised to at least one aeroallergen⁹⁷.

In adults, RV infection of atopic (as indicated by allergen-specific IgE) asthmatics resulted in increased duration of disease as well as increased severity of lower respiratory symptoms compared with infected cohabitating partners who were non-atopic non-asthmatics⁹⁸. This study indicated that intrinsic differences may exist that potentially affect the way viral infections interact with the asthmatic pulmonary environment.

Two seminal studies assessed whether allergen exposure increased the risk of acute asthma in conjunction with viral infection in sensitised asthmatics. Green *et al.* investigated 60 patients aged 17-50 admitted with acute exacerbations who were assessed for the presence of respiratory infection as well as total and allergen-specific IgE. The combination of sensitisation, exposure to high levels of common household and environmental allergens and viral infection was strongly associated with the risk of hospital admittance with acute asthma⁹². Similar findings in children (aged 3-17 years) were also reported by Murray *et al.* indicating a synergism between sensitisation, allergen exposure and viral infections in inducing asthma exacerbations⁹⁵.

1.3.4 Mechanisms of RV-induced asthma exacerbations

Experimental RV infection of volunteers in the presence or absence of allergic inflammation (most commonly in the form of atopic asthma and/or allergic rhinitis) has proven invaluable in investigating the interaction between virus and allergy. Clinical samples including induced sputum, BAL, tissue biopsies, serum as well as *ex vivo* studies of human bronchial epithelial

cells (HBEC) and peripheral blood mononuclear cells (PBMCs) from patients have been used to investigate mechanisms of RV-induced exacerbation⁹². These results are discussed below.

1.3.4.1 RV-modulated ICAM-1 expression

The major RV serotype has been demonstrated to upregulate expression of its own receptor, ICAM-1, in a Th2 environment. *In vitro* studies demonstrated that RV infection enhanced receptor expression on nasal epithelial cells from atopic asthmatics compared with non-atopic healthy controls⁹⁹. Grunberg *et al.* similarly reported ICAM-1 was upregulated in bronchial biopsies in asthmatic subjects experimentally infected with RV-16¹⁰⁰. Furthermore, Th2 cytokine pre-treatment of a nasal epithelial cell line resulted in enhanced ICAM-1 expression which was also associated with increased viral titre¹⁰¹. Based on these findings, it was theorised that elevation of ICAM-1 by Th2-associated factors and RV infection may contribute to increased symptom severity due to increased viral load. In addition, due to its role in the recruitment of inflammatory cells, it has been hypothesised that ICAM-1 may exacerbate disease by augmenting inflammation⁸³.

1.3.4.2 Deficient type I and type III IFNs

An abnormal innate antiviral response has also been implicated as a potential mechanism in HBECs from atopic asthmatics. Specifically, cultured primary HBECs obtained from atopic asthmatics and non-atopic, healthy controls were infected with RV-16 *in vitro*. Asthmatic HBECs produced less type I IFN- β , exhibited enhanced viral replication and decreased cell apoptosis compared with HBECs from healthy controls. Exogenous treatment of HBECs with IFN- β reversed pathogenic effects by reducing viral release indicating that 1.) the IFN- β pathway was intact and the lack of response in asthmatics cells was due to deficient production and 2.) IFN- β may prove a useful therapeutic by restoring the deficient antiviral response in asthma¹⁰².

Further evidence of the importance of an effective antiviral response was demonstrated in BAL cells from asthmatic subjects that were found to have reduced levels of type III IFN- λ . RV-infected HBECs and macrophages from asthmatics were also deficient in IFN- λ which inversely correlated with asthma symptom scores, BAL viral load and markers of inflammation in the BAL and sputum of RV-infected asthmatics¹⁰³. These findings indicate

that increased RV-induced cytotoxicity and reduced apoptosis due to decreased primary IFN production may result in enhanced release of inflammatory products and viral shedding resulting in exacerbation of disease.

1.3.4.3 Imbalanced Th1/Th2 responses

In addition to deficient innate immune responses, an imbalanced adaptive response has also been reported in RV-infected asthmatics. While a robust Th1 response is associated with an effective antiviral response, RV-infected asthmatics have been reported to have a defective type I response and/or a heightened Th2 response. PBMCs from atopic asthmatics infected with RV produced significantly less IFN- γ and IL-12 compared with cells from healthy controls. In addition, IL-4 expression was induced exclusively in asthmatic cells while the IFN- γ /IL-4 ratio was approximately 3 times lower compared with controls¹⁰⁴.

A lower IFN- γ /IL-5 mRNA ratio was also detected in sputum from RV-infected atopic patients which was inversely related to peak cold symptoms and the length it took to clear the virus³⁹. Message *et al.* also measured blood and BAL IFN- γ and IL-10 in RV-infected atopic asthmatics and healthy controls and demonstrated that higher levels were associated with less severe drops in lung function, lower RV load and diminished symptoms. Conversely, Th2 cytokines including IL-4, IL-5, and IL-13 were related to increased pathogenesis of virus-induced asthma symptoms⁹³.

1.3.4.4 Innate Fc ϵ RI-expressing cells

Studies have demonstrated that allergic rhinitis patients infected with RV and treated with segmental allergen challenge one month before, during and after infection, had significantly greater BAL histamine levels compared with non-allergic controls. These increases, which were observed during acute infection and one month after infection, were absent in non-allergic and individuals challenged with allergen alone. Proposed mechanisms for this enhancement included RV-induced enhanced mediator release from pulmonary mast cells or circulating basophils¹⁰⁵. Although yet to be investigated in asthmatics, *in vitro* studies have reported that RV-infected cell lines from human mast cells and basophils can enhance IgE/anti-IgE-induced histamine release in both cell lines as well as IL-4 and IL-6 protein in basophil supernatant¹⁰⁶. Furthermore, RV infection increased total serum IgE in asthmatic patients which correlated with reduced lung function while RV-infected allergic rhinitis

patients displayed elevated total serum IgE and histamine compared with infected non-allergic controls^{107,108}.

1.3.4.5 Aberrant epithelial cell structure

The asthmatic epithelium has been reported to have a deficient physical barrier function with patchy disruption of tight junctions⁷⁵. Newly identified asthma susceptibility genes identified in genome-wide association studies indicated that the majority were predominantly expressed in the epithelium^{109,110}. These polymorphisms may contribute to enhanced susceptibility to environmental stimuli as indicated by weakened barrier function of asthmatic epithelium to oxidant-induced apoptosis and protease allergens^{111,112}. Furthermore, undifferentiated primary HBECs were reported to be more susceptible to RV infection with approximately 30 to 130 times more viral RNA measured compared with differentiated epithelium¹¹³; similarly, RV replication was also demonstrated to be greater in damaged epithelium than intact epithelium¹¹⁴. Due to the fact the major precipitants of asthma exacerbations, namely viruses and allergens, both modulate the epithelium, it may be that both factors work synergistically to further weaken barrier function in the intrinsically impaired asthmatic epithelium; allergens, particularly of a proteolytic nature, may damage the airway epithelium promoting greater RV replication while the damage caused by RV may enhance absorption of allergen. Both outcomes have the potential to enhance airway inflammation as a result of an increased release of proinflammatory mediators¹¹⁵. Furthermore, by weakening the barrier function via proteolytic activity or by increasing epithelium permeability, some allergens have the potential to facilitate access to DCs and may promote Th2 sensitisation and enhance activation of allergen-specific Th2 cells⁵⁹.

1.3.4.6 Induction of epithelial-derived mediators

The airway epithelium has been reported to express a wide array of inflammatory mediators in response to RV infection which play an important role in the recruitment of innate and adaptive effector cells. Namely, RV-infected HBECs have been shown to secrete IL-1 α and β , IL-6, CXCL8/IL-8, CCL5/RANTES, CCL11/Eotaxin-1, CCL24/Eotaxin-2, TNF- α and CXCL10/interferon-inducible protein 10 (IP-10) in HBECs *in vitro*^{12,116,117}. Analyses of human clinical samples have reported an increase in CXCL8/IL-8 in the nasal lavage of RV-infected asthmatic children which correlated with increased neutrophilia and symptom severity¹¹⁸. Similarly, eosinophil granule proteins have been detected in RV-infected asthmatic children, which were associated with significantly increased levels of the eosinophilic chemoattractant, CCL5/RANTES¹¹⁹. The T cell chemoattractant, CXCL10/IP-10, has also been demonstrated to be increased in the serum of patients with acute asthma which was also predictive of virus-induced asthma exacerbations and increased disease severity¹²⁰.

The possibility exists that RV-induced epithelial-derived cytokines are capable of directly initiating and amplifying Th2 immunity. For example, RV infection has been reported to stimulate TLR-3-dependant secretion of the Th2 cytokine, TSLP, which has been demonstrated to induce Th2 cell differentiation. Furthermore, the finding that IL-4, together with RV infection, synergistically enhanced TSLP provides a mechanism whereby RV could exacerbate pre-existing allergic inflammation in the asthmatic lung¹²¹. Of interest, IL-33, also epithelial-derived, has been demonstrated to be induced by Influenza A virus in the human epithelial cell line, A549, as well as in mouse respiratory epithelial cells (MLE-15)¹²².

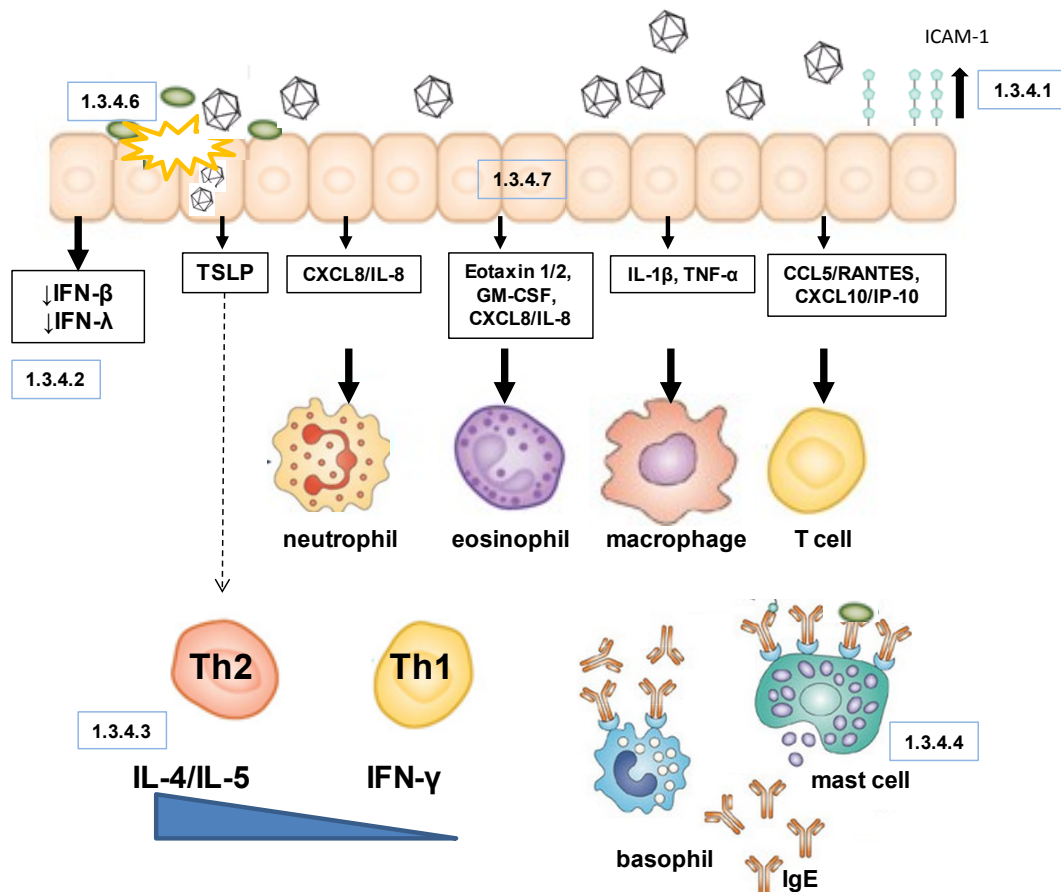


Figure 1.3 Potential mechanisms of RV-induced asthma exacerbations including RV-modulated ICAM-1 expression (1.3.4.1), deficient type I and type III IFNs (1.3.4.2), imbalanced Th1/Th2 responses (1.3.4.3), increased innate FcεRI-expressing cells and IgE (1.3.4.4), aberrant epithelial cell structure (1.3.4.5) and increased induction of epithelial-derived mediators (1.3.4.6)

1.4 TSLP and IL-33

Both TSLP and IL-33 have primarily been identified to play a role in Th2 immunity in clinical and experimental findings. Epithelial cells from asthmatic subjects have been shown to express more TSLP compared with healthy controls while enhanced levels of IL-33 have been reported in epithelial cells from bronchial biopsies and in the BAL fluid from moderate and severe asthmatics compared with healthy controls¹²³⁻¹²⁵. In mice, transgenic overexpression of TSLP in the airways was reported to induce Th2 inflammation while naive mice administered IL-33 displayed eosinophilia in the airways, splenomegaly, enhanced serum IgE and increased IL-5 and IL-13 levels, clearly indicating a role for both cytokines in type 2 immunity¹²⁶.

TSLP signals through a heterodimeric receptor complex composed of the IL-7R α (CD127) chain and a γ_c -like chain specific for TSLP (TSLPR) which is located on a number of cell types (Table 1.1). Studies indicate that TSLP binding promotes Th2 skewing; Wang *et al.* reported that TSLP-treated DCs upregulated the OX40L cell surface expression priming naive T cells to become Th2 cells. This OX40L-OX40 interaction and the suppression of IL-12 production by DCs (induced by TSLP) was theorised to contribute to Th2 cell differentiation¹²⁷.

As a member of the IL-1 family, IL-33 signals through an IL-1R like (IL-1RL1) subunit, also known as ST2 or type 1 ST2 (T1/ST2), and associates with the IL-1R accessory protein (IL-1RAcP)¹²⁸⁻¹³⁰. Together with IL-3, IL-33 has been demonstrated to act directly on human basophils to induce IL-4 production^{131,132}. In addition to the induction of T1/ST2-expressing innate lymphoid cells reported in helminth infected and allergen-challenged mice, infection with influenza induced an innate T1/ST2+ population termed natural helper (NH) cells which responded to IL-33 by producing IL-13 and were required for virus-induced AHR (Table 1.1)¹³³. These findings indicate that, like TSLP, type 2-inducing agents as well as virus are capable of inducing IL-33.

	Cell sources	Inducing stimuli	Cell targets
TSLP	Epithelial cells, basophils, mast cells and keratinocytes ^{121,134-136}	Allergen, helminth infection, RV and/or IL-4, IL-13, RSV ^{134 137 121}	DCs, mast cells, monocytes, macrophages, B cells, T cells, basophils, eosinophils and epithelial cells ^{130,138-141}
IL-33	Epithelial cells, endothelial cells, bronchial smooth muscle cells, keratinocytes, fibroblasts and adipocytes ^{122,126,142,143}	Allergen, helminth infection, Influenza A virus ^{67,122,144}	Endothelial cells, eosinophils, basophils, mast cells, NK cells, natural killer T (NKT) cells, B cells, differentiated Th2 cells, NH cells, ^{78,133,145-151}

Table 1.1 Cellular sources, targets and inducing stimuli for TSLP and IL-33. Abbreviations: DC=dendritic cell; NK=natural killer; NKT=natural killer T; NH=natural helper

1.5 IL-25

1.5.1 Structure and regulation

IL-25, originally designated IL-17E, was first identified in 2000 by homology based cloning of the IL-17 family members¹⁵². In contrast to other members of the IL-17 family members, which exhibit roles in autoimmune disease and pathogen defence, IL-25 has been shown to play a role in the Th2 immune response¹⁵³.

Encoded by the *il25* gene located at locus 14q11.2 on chromosome 14, pre-mRNA splicing events produce two IL-25 isoforms. One isoform forms the canonical sequence while the second differs by the exclusion of 16 amino acids (a.a.) in the signal peptide as a result of removal of one intron, making it 161 a.a. long. The precursor protein undergoes proteolytic processing, and in the case of the canonical sequence, forms a 32 residue signal peptide attached to a 145 amino acid chain (177 a.a. in total). The amino acid chain is glycosylated at the 136th amino acid and contains 2 disulphide bonds which facilitate folding and processing into its mature form which is then secreted¹⁵⁴. The calculated mature molecular weight is ~16.7kDa although recent studies suggest that this form is cleaved further by matrix metalloproteinase (MMP)7 to form a more active form with an enhanced binding affinity to its receptor¹⁵⁵. The encoded human protein possesses approximately 16-20% sequence homology to the IL-17 family members, IL-17A, IL-17B and IL-17C. The greatest sequence homology lies within the C-terminal where 4 conserved cysteine residues, which form the disulphide bonds, are located while the greatest sequence divergence is observed at the N-terminal¹⁵². Comparison of the human canonical sequence with the singular encoded mouse sequence (~169 a.a.) reveals approximately 80% homology¹⁵³.

Although regulatory gene expression has been characterised for the majority of the IL-17 family, very little is known about regulation of IL-25 gene expression. Analysis of the gene sequence upstream of the IL-25 encoding region indicates potential binding sites for signal transducer and activator transcription 6 (STAT6), GATA-3 and NF- κ B¹⁵⁶. However, whether these transcription factors are functionally required for IL-25 induction is currently unknown.

1.5.2 Cellular expression

Original RT-PCR analysis of IL-25 expression indicated the presence of low levels of mRNA in several tissues, including brain, kidney, lung, prostate, testis, spinal cord, adrenal glands and trachea¹⁵². Further analysis by Fort *et al.* on the cellular level demonstrated enhanced mRNA expression in highly polarized Th2 cells from mice cultured *in vitro*¹⁵³. Since then, a multitude of cell types have been reported as IL-25 producers, predominantly in response to classical Th2 inducing stimuli including allergen, pollutants and/or IgE cross-linking. These findings are summarised in table 1.2. Importantly, IL-25 has been reported to be produced by lung epithelial cells *in vitro* and *in vivo* and has therefore been theorised to play a role in the initiation of Th2 inflammation^{70,157}. For example, Angkasekwinai *et al.* reported direct induction of IL-25 gene expression from the human alveolar cell line, A549, and primary mouse lung epithelial cells in response to the allergens, *Aspergillus oryzae* and ragweed⁷⁰.

Human samples				
Cell type	Sample source	Inducing stimuli	IL-25 product	Ref.
Eosinophils	Cultured from PBMCs from normal and atopics	IL-5 and GM-CSF or IL-3 and GM-CSF added to media	mRNA (RT-PCR) and protein (ELISA)	127
	Biopsy of bronchial mucosa and skin sections from asthmatics/atopics	SPT-positive allergen	Protein (IHC)	158
	Serum and whole blood	Churg-Strauss Syndrome	Protein (ELISA and FC)	159
Basophils	Cultured from PBMCs from normal and allergic subjects	IgE cross-linking	mRNA (RT-PCR) and protein (ELISA)	127
Mast cells	Cultured from PBMCs from normal and allergic subjects	PMA/Ionomycin	mRNA (RT-PCR)	127
	Biopsy of bronchial mucosa and skin sections from asthmatics/atopics	SPT-positive allergen	Protein (IHC)	158
Keratinocytes	Cultured from normal subjects	Ragweed extract	mRNA (RT-PCR)	127
	Biopsies from patients with atopic dermatitis and normal subjects	Atopic dermatitis	mRNA (RT-PCR) and protein (IHC)	
Epithelial cells	Primary nasal epithelial cells cultured from normal subjects	Polyriboinosinic: polyribocytidilic acid (poly I:C)	mRNA (RT-PCR) and protein (ELISA)	160
Epithelial cell line	Human alveolar adenocarcinoma cell line (A549)	<i>Aspergillus oryzae</i> and ragweed	mRNA (RT-PCR)	70
Dendritic cells	Biopsies from patients with atopic dermatitis and normal subjects	Atopic dermatitis	Protein (confocal microscopy)	161
		LPS	Protein (FC)	

Murine samples				
Cell type	Sample source	Inducing stimuli	IL-25 product	Ref.
Mast cells	Cultured bone marrow-derived mast cell cultures	IgE cross-linking	mRNA (RT-PCR) and protein (immunoblotting)	¹⁶²
Th2-polarised T cells	Naive CD4+ T cells isolated from BALB/c, B6 and 129 SvEv mice	Polarisation with IL-4/anti-IL-12/anti IFN- γ mAb	mRNA (RT-PCR)	¹⁵³
CD4+ and CD8+ T cells	Cecal patch of naive BALB/c mice		mRNA (RT-PCR)	¹⁶³
Airway epithelial cells	Lung sections from NK-cell depleted mice	Depletion of NK cell/IFN- γ	Protein (IHC)	¹⁵⁷
	Primary type II alveolar epithelial cells cultured from BL6 mice	<i>Aspergillus oryzae</i> and ragweed	mRNA (RT-PCR)	⁷⁰
Airway epithelial cell line	Cultured mouse lung epithelial (MLE) cell line	<i>Aspergillus oryzae</i> and ragweed	mRNA (RT-PCR)	⁷⁰
Intestinal epithelial cells	Isolation of intestinal epithelial cells	Commensal bacteria in large intestine	mRNA (RT-PCR)	¹⁶⁴
Alveolar macrophages	Lung sections and tissue samples from a rat model of titanium dioxide-induced inflammation	Titanium dioxide	mRNA (RT-PCR) and protein (IHC)	¹⁶⁵
	Cultured alveolar macrophages from the BAL cells of rats stimulated with titanium dioxide		mRNA (RT-PCR) and protein (immunoblotting)	
Microglia	Digested brain and spinal cord homogenate from a mouse model of EAE and WT controls	EAE	mRNA (RT-PCR)	¹⁶⁶

Table 1.2 Summary of cellular producers of IL-25 in human and murine studies. Abbreviations: FC=flow cytometry; B6=Black6; LPS=Lipopolysaccharide; mAb=monoclonal antibody; IHC=immunohistochemistry; NK=natural killer; EAE=experimental autoimmune encephalomyelitis; WT=wild type

1.5.3 Role of IL-25 in the immune system

1.5.3.1 Type 2 immunity

Shortly after its initial discovery, Fort *et al.* carried out preliminary characterisation of IL-25 by injecting mice intraperitoneally with IL-25 and observed a Th2-like response. This was marked by production of IL-4, IL-5 and IL-13, blood eosinophilia, increased serum IgE, IgG₁ and IgA as well as increased mucus production, eosinophilia and epithelial cell hyperplasia in the lungs and digestive tract. Interestingly, IL-25 induction of eosinophilia and histopathological changes were also present in recombination-activating gene (*Rag*)-knockout mice indicating a T cell-independent source of Th2 inflammation¹⁵³. In accordance with this data, it was demonstrated that transgenic overexpression of IL-25 in mice resulted in increased circulating eosinophils, IL-4, IL-5, CCL11/Eotaxin-1, and IgE levels^{167,168}. By overexpressing IL-25 in the lung epithelium, transgenic mice also displayed increased CCL11/eotaxin-1, CCL24/eotaxin-2, CCL22/MDC and MUC5ac gene expression in lung tissue as well as increased epithelial hyperplasia and eosinophils in the lung parenchyma⁷⁰. Clinical studies have since shown increased gene expression in IL-25 and its receptor, IL-17RB, in tissue from patients with asthma and atopic dermatitis as well as increased IL-25 protein from the nasal lavage of allergic rhinitis patients^{127,160,161}.

Tamachi *et al.* further demonstrated induction of IL-25 by allergen in a mouse model of allergic airways inflammation showing that gene expression was upregulated in response to OVA inhalation. However, in contrast with previous studies, they reported that overexpression of IL-25 alone was not sufficient to initiate allergic inflammation but instead enhanced OVA-induced eosinophil and CD4 T cell recruitment¹⁶⁹. In addition, IL-25 mRNA was induced in response to intranasal and intratracheal challenge with cockroach allergen in a mouse model of chronic allergic asthma⁷¹.

A functional role for IL-25 in allergic inflammation was confirmed by inhibiting IL-25 in a mouse model of allergic airways disease. By blocking IL-25, using a neutralising antibody before OVA sensitisation and 4 hours prior to OVA challenge, AHR was prevented and airway eosinophila, goblet cell hyperplasia, serum IgE as well as BAL IL-5 and IL-13 protein were decreased. Strikingly, administration of blocking antibody before OVA challenge in

sensitised mice prevented AHR and reduced IL-5 and IL-13 expression in the airways. IL-25 also caused airway changes independently of Th2 cytokines; mice lacking IL-4, IL-5, IL-9 and IL-13 exhibited increased AHR following IL-25 treatment¹⁷⁰. Using a different IL-25 blocking antibody clone, similar findings were demonstrated in a model of allergic airways inflammation using a combination of *Aspergillus oryzae* and OVA; inhibiting IL-25 before each allergen challenge resulted in decreased airway eosinophilia and reduced BAL IL-4, IL-5 and IL-13 production⁷⁰. Using a soluble form of the IL-25 receptor to neutralise endogenous IL-25, OVA-sensitised and challenged mice displayed decreased OVA-induced eosinophilia and CD4+ T cell recruitment in the lung¹⁶⁹.

A role for IL-25 in driving airway remodelling has also been reported in mice overexpressing the TGF- β family signalling molecule, Smad2, in the airway epithelium. In addition to promoting an airway remodelling phenotype, these mice were also demonstrated to produce elevated IL-25. By administering an IL-25 blocking antibody in mice exposed to HDM extract, airway smooth muscle hyperplasia, collagen deposition, and AHR were attenuated. IL-25 neutralisation also blocked recruitment of endothelial progenitor cells to the lung as well as completely inhibiting IL-33 and TSLP expression. Gregory *et al.* theorised that IL-25 may drive the production of IL-33 and TSLP *in vivo* and thus play a key role in orchestrating airway remodelling²⁰¹.

IL-25 deficient mice have also been employed to demonstrate the ability of IL-25 to induce a protective type 2 protective immune response to parasitic infections^{80,163}. Specifically, *Il-25*^{-/-} mice displayed inefficient expulsion of the nematode, *Nippostrongylus brasiliensis*, which correlated with delayed cytokine production by Th2 cells and a reduction in an IL-4, IL-5 and IL-13-producing non-B non-T (NBNT) cell population⁸⁰. Conversely, IL-25 treatment resulted in rapid worm expulsion which was proposed to be driven by IL-25-induced expansion of this newly identified cell population. Similarly, knock-out mice failed to clear a *Trichuris* infection which was associated with reduced IL-4 and IL-13 levels and reciprocally increased amounts of IFN- γ and IL-17A. Treatment of these knock-out mice with exogenous IL-25 promoted immunity and Th2 cytokine responses in the form of antigen-specific IL-4 and lymphocyte-dependant goblet cell hyperplasia¹⁶³. Using oxazolone to induce a type 2

model of colitis, IL-25 was also demonstrated to have a proinflammatory role in the mucosa by inducing IL-13-producing NKT cells and nuocytes which enhanced intestinal inflammation. By blocking the IL-25 receptor using the antibody clone D9.2, symptoms of disease were lessened and mucosal infiltration of NKT cells and nuocytes was reduced. This was associated with decreased IL-13 levels, blood eosinophilia and IgE and correlated with reduced IL-4, IL-5 and IL-13 production by cultured mesenteric lymph node (MLN) cells¹⁷¹.

1.5.3.2 Autoimmunity and anti-inflammatory effects

In contrast with its abilities to augment Th2 inflammation, IL-25 has been demonstrated to limit Th17-driven autoimmune inflammation. For example, IL-25 deficient mice have been reported to be highly susceptible to experimental autoimmune encephalomyelitis (EAE), a Th17-driven autoimmune inflammatory disease of the central nervous system (CNS) that serves as a model of human multiple sclerosis (MS). This increased susceptibility was associated with enhanced levels of IL-17A, IFN- γ and TNF- α -producing T cells in the CNS. The cause of disease was demonstrated to be directly linked to a resultant lack of IL-13 as *IL-13*^{-/-} mice failed to protect against EAE. Due to inhibition of IL-23, IL-1 and IL-6 expression, which are required for Th17 differentiation and function, it was suggested that IL-25 may play a role in the regulation of Th17 development¹⁶⁶. This study was corroborated by recent findings that IL-25 acts as a critical regulator of the IL-13/IL17A axis during AHR in a model of allergic airways disease. By inhibiting IL-25 during allergen challenge, IL-13 levels were decreased significantly with a reciprocal increase in IL-17A that was shown to be integral for protection against AHR. This regulation of IL-17A was once again demonstrated to be directly associated with IL-13¹⁷².

Unlike findings that demonstrated a proinflammatory effect in a type 2 model of colitis, IL-25 has been reported to have protective, anti-inflammatory properties in a Th1-mediated colitis model of gut inflammation in mice. Reports indicated that these effects may be mediated by downregulated IL-12 and IL-23 level^{173,174} or via IL-25-induced alternatively activated macrophages¹⁷⁵. IL-25 was also shown to downregulate lipopolysaccharide (LPS)-induced IL-6, TNF- α and IL-1 β expression by human CD14⁺ cells. Further studies in mice

showed that IL-25 was also protective against LPS-induced lethal endotoxemia by inhibiting monocyte-derived proinflammatory cytokines¹⁷⁶.

1.5.3.3 Viral infection and IL-25

Whilst numerous studies have demonstrated the importance of IL-25 in allergy and parasite expulsion, comparatively little is known about IL-25 during viral infection. Siegle *et al.* demonstrated increased IL-25 gene expression in response to pneumonia virus of mice (PVM). Neonatal mice were infected with virus to provide a species-specific model of RSV disease and then sensitised and challenged with low amounts of OVA to mimic chronic asthma. A moderate dose of allergen was then administered to induce exacerbation of disease in an attempt to investigate pathogenic mechanisms of childhood asthma. Induction of IL-25 by virus was theorised as a possible driver of Th2 inflammation that was noted to be upregulated by PVM infection 14 days p.i.¹⁷⁷. However, an additional study by the same group demonstrated that blocking IL-25 had limited effects on preventing induction of Th2 inflammation but did suppress subepithelial fibrosis and epithelial cell hypertrophy, again highlighting a role in airway remodelling. By blocking IL-4 and IL-25, Th2 inflammation was abrogated¹⁷⁸.

One other study involving RSV-infected NK/IFN- γ -depleted mice demonstrated development of viral-specific Th2 effector cells and the induction of Th2 inflammation in the airways. In the absence of IFN- γ , lung epithelial-derived IL-25, as measured by IHC, was upregulated in response to RSV infection. By administering an IL-25 blocking antibody, IL-25 was shown to play an integral part in the differentiation of Th2 cells. This was demonstrated to be partly due to upregulation on DCs of Jagged1 expression, a costimulatory molecule that has been reported to polarise naive CD4⁺ T cells toward a Th2 phenotype¹⁵⁷.

1.5.4 Signalling and the IL-25 receptor

Bioactivity of IL-25 is dependent on non-covalent binding to its principal receptor, IL-17RB, which forms a heterodimer with IL-17RA^{152,179}. IL-17RB, also designated IL-17Rh1, Evi27 and IL7BR, is a 506 amino-acid single transmembrane protein that also acts as a receptor to IL-17B but binds with much lower affinity compared with IL-25^{152,180}. Notably, polymorphisms

in the IL-17RB gene in humans have also been linked with asthma¹⁸¹. Although IL-17RA does not bind directly to IL-25 and instead interacts with the IL-17RB-IL-25 complex, it is integral for IL-25 signal transduction^{179,180}. This has been demonstrated in *Il-17rb*^{-/-} and *Il-17ra*^{-/-} mice that failed to respond to intranasal administration of IL-25 indicated by an absence of Th2 inflammation. Furthermore, *in vitro* studies have shown that blocking IL-17RA resulted in a decrease in IL-5 by IL-25-stimulated human PBMCs¹⁷⁹. Like the rest of the IL-17 family, IL-17RB contains a SEF/IL-17R (SEFIR) subdomain in its cytoplasmic tail which has been demonstrated to bind Act1 in a SEFIR-dependant manner following IL-25 binding¹⁸². Accordingly, mice deficient in Act1 and administered IL-25 displayed decreased allergen-induced airway eosinophilia while Act1 deficiencies in epithelial cells resulted in reduced Th2 lung inflammation¹⁸². Similarly, an Act1 deficiency in T cells resulted in decreased eosinophilia and abrogated AHR in a model of allergic airways inflammation¹⁸³. The cytoplasmic domain of IL-17RB also contains a TNFR-associated factor (TRAF)6-binding motif which directly associates with TRAF6 upon receptor ligation. TRAF6 then activates NF-κB which has been demonstrated by blocking this pathway using a double-negative form of TRAF6¹⁸⁴.

Other studies of IL-25-IL-17RB signal transduction have investigated T cell signalling reporting activation of GATA-3, Nuclear factor of activated T-cells cytoplasmic (NFATc) 1, JunB, c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) and STAT6 pathways^{70,127,169}. IL-25-induced activation of these pathways mediates a range of effects including induction of Th2 cytokines, cell differentiation and IL-17RB receptor upregulation which will be discussed in greater detail in the next section.

1.5.5 IL-25 regulation and IL-25-responsive cells

IL-17RB gene expression has been detected in the kidney, liver, lung and gastrointestinal tract amongst others. While IL-25 has been shown to act on peripheral blood and intestinal CD14⁺ cells to reduce inflammatory cytokine responses¹⁷⁶, the majority of IL-17RB-expressing cells induce a Th2 response upon IL-25 binding. These populations include invariant NKT (iNKT) cell^{185,186}, Th2 cells^{70,127}, Th9 cells¹⁸⁷, eosinophils¹⁸⁸, epithelial cells¹⁸², airway smooth muscle cells¹⁸⁹, lung fibroblasts¹⁹⁰, endothelial cells¹⁹¹ and several newly

identified innate immune cells including multi-potent progenitor type 2 cells (MPP^{type2}) cells⁷⁷, innate type2 helper (Ih2) cells¹⁹², NH cells¹⁹³, human innate lymphoid cells (ILC)⁶⁸, nuocytes⁷⁸ and type 2 myeloid (T2M) cells⁷¹.

1.5.5.1 IL-25-mediated immunity

i. Innate immune cells

Recently, several different groups have identified populations of innate cell populations that express Th2 cytokines in response to allergen challenge or helminth infections^{68,71,78,193}. Aside from the shared ability to express Th2 cytokines, these populations vary with regard to surface marker expression, anatomical location and expression of transcription factors (Table 1.3)¹⁹⁴. Recently, type 2 innate cells formed from lymphoid precursors have been grouped together under the heading innate lymphoid cells (ILC) 2 which include NH cells, Ih2 cells and nuocytes. Nuocytes represents one of the most well characterised ILC2 subsets. First identified as an IL-25-induced lineage negative (lin-) (i.e. absence of T cell, B cell, macrophage, DC, neutrophil, eosinophil, mast cell, basophil, NKT cell or NK cell markers) NBNT cell population, these cell were demonstrated to be required for eradication of *N. brasiliensis* infection⁸⁰. Neill *et al.* further characterised these cells as major producers of IL-13 and therefore designated them nuocytes after the 13th letter of the greek alphabet, *nu*. These cells have since been defined as lin-ICOS+CD44+CD45+Sca1+CD90.2+CD117+(IL-7R α /CD127)+MHCclass II+T1/ST2+IL-17RB+ and were shown to expand in the MLN and spleen in response to intraperitoneal injection of IL-25 and IL-33. In addition to producing IL-5, nuocytes represented the major IL-13-producing cell population in mice infected with *N. brasiliensis* at day 5 after infection and were demonstrated to be crucial for efficient worm expulsion⁷⁸. Importantly, nuocytes have recently been reported to infiltrate the lung in response to OVA challenge representing the dominant innate source of IL-13 in a mouse model of allergic airways disease. Using a 12 day and 25 day OVA-induced lung allergy model, nuocytes were not only detected at the start of the allergic response but also persisted through the adaptive response with greater numbers detected at day 25 post-sensitisation. Notably, intranasal administration of IL-25 or IL-33 induced nuocytes in the airways while adoptive transfer was able to restore AHR and eosinophilia in IL-25-dosed *IL13*^{-/-}

γ -deficient mice. These results highlight a role for IL-25-induced nuocytes in the initiation of Th2 cytokine production in the lung that is independent of CD4⁺ Th2 cells⁷⁶.

An additional group of innate myeloid IL-25-responsive cells that have been reported to accumulate in the lung following allergen exposure are the recently termed T2M cells⁷¹. This population was first identified as a CD11b+GR1+Ly6C+/-CD117-CD4-CD11c-MHC class II^{low} cell population that produced IL-4 in the lung after chronic allergen challenge¹⁹⁵. Petersen *et al.* demonstrated that an IL-17RB+CD11b+Gr-1^{mid} population represented the major source of IL-4 and IL-13 in a model of chronic allergic asthma and, in contrast with Neill *et al.*, reported a small number of lin-cKit+Sca-1+IL-17RB+ NBNT cells after allergen challenge. Using *Il-17rb*^{-/-} mice, lung inflammation, airway eosinophilia and mucus production was demonstrated to decrease after chronic allergen exposure which was associated with reduced Th2 cytokine production by T2M and CD4⁺ T cells. Similarly, airway administration of IL-25 significantly increased IL-4 and IL-13-producing IL-25-responsive CD11b+Gr-1^{mid} infiltrates in the lung while adoptive transfer of T2M cells together with IL-25 in *Il-17rb*^{-/-} mice induced mucus production and inflammation marked by an increase in IL-13 and Muc5ac mRNA.

Of clinical importance is an innate lymphoid population described as lin-(IL-7R α /CD127)+ which express the Th2 marker chemoattractant receptor homologous molecule expressed on Th2 lymphocytes (CRTh2) and have been identified in the human lung, gut and nasal tissue and peripheral blood. These cells responded to IL-25 and IL-33 by producing IL-5 and IL-13 and were found in higher proportions in nasal polyps of patients with rhinosinusitis. Findings suggest that these cells may represent the human equivalent of ILC2s previously identified in mouse studies^{68,81}.

Additional IL-25-responsive ILC2s that have been identified in other anatomical locations aside from the lung include NH and Ih2 cells. NH cells were identified in fat associated lymphoid clusters (FALC) in mice, which are located along the gut mesentery surrounded by adipose tissue. Characterised as a lin-CD117+Sca-1+CD25+(IL-7R α /CD127)+T1/ST2+ population, these cells were reported to express IL-4, IL-5, IL-6, and IL-13 mRNA constitutively, which when stimulated with IL-25 and IL-2 or IL-33 produced large amounts

of IL-5 and IL-13. Importantly, the existence of these cells in human FALC was demonstrated. Ih2 cells have been reported to be a major source of IL-13 in the MLN, spleen, liver and bone marrow in mice following infection with *N. brasiliensis*. These lin-ICOS+CD117+Sca-1-CD90.1+CD44+CD45+ cells expand robustly in response to intraperitoneal treatment with IL-25 or IL-33 and adoptive cell transfer, together with IL-25, is sufficient for clearance of nematode infections.

Lastly, MPP^{type2} cells have been identified in the gut associated lymphoid tissue (GALT) of mice administered IL-25. Uniquely, this population has the ability to differentiate into multiple cell lineages in culture, including macrophages, basophils and mast cells. Defined as lin-Sca-1+CD117^{int}, MPP^{type2} cells were reported to confer immunity to *T. muris* infection when adoptively transferred to *Il-25*^{-/-} mice and were associated with the production of IL-4, IL-5 and IL-13.

	Mouse					Human
	T2M cells	MMP ^{type2}	Nuocytes (ILC2)	Ih2 cells (ILC2)	NH cells (ILC2)	CRT ^{h2+} type 2
Anatomical location	Lung, BM, PB	MLN, GALT	MLN, spleen, peritoneum, lung	MLN, spleen, liver, BM	FALC	Gut, lung, PB
Lin-CD117+Sca1+	No (CD11b+Gr-1 ^{mid})	Yes	Yes	Lin-CD117+Sca1-	Yes	Lin-CD117+
T1/ST2+	No	No	Yes	Unknown	Yes	IL1R1 expression
(IL-7Ra+/CD127)	No	No	Yes	Unknown	Yes	Yes
CD44+	No	No	Yes	Yes	Yes	Unknown
CD90.1+	No	No	Yes	Yes	Yes	Unknown
Transcription factor expression	<i>Unknown</i>		<i>RORA</i>	<i>Gata-3, Stat6</i>	<i>Maf, Gata-3, JunB, Stat6, Id2, Tbx21</i>	<i>Id2</i>
Cytokine responsiveness	IL-25	IL-25	IL-25, IL-33	IL-25, IL-33	IL-25, IL-33	IL-25, IL-33
Physiological significance	Secrete IL-4 and IL-13; increased mucus	Upregulate IL-4, IL-5 and IL-13 mRNA; increased mucus	Secrete IL-5 and IL-13; increased mucus	Secrete IL-5 and IL-13; increased eosinophilia	Secrete IL-5 and IL-13; increased mucus	Secrete IL-5 and IL-13

Table 1.3 A summary of characterised innate cell populations. Abbreviations: MLN=mesenteric lymph nodes; FALC=fat associated lymphoid clusters; BM=bone marrow; GALT=gut associated lymphoid tissue; PB=peripheral blood. Table was adapted from Hams *et al.*¹⁹⁴

ii. iNKT cells

A population of iNKT cells, defined as an innate T cell subset that recognise glycolipid ligands on target cell instead of peptides, have also been demonstrated to express IL-17RB together with CD4^{185,186,196}. By stimulating with IL-25, this population produced IL-13 and IL-4 *in vitro*¹⁹⁶. In addition, intranasal administration of IL-25 induced AHR in wild-type mice which was absent in iNKT cell-deficient mice. Furthermore, adoptive transfer of IL-17RB+CD4+iNKT cells reconstituted AHR in iNKT-deficient mice, strongly indicating a role for this cell population in asthma pathogenesis¹⁸⁵. Recently, this IL-17RB+ iNKT cell subset has been further characterised into CD4+ and CD4- expressing populations; IL-17RB+CD4- cells secrete Th17 cytokines in response to IL-23 while IL-17RB+CD4+ cells secrete Th2, Th9 and Th17 cytokines in response to IL-25. The latter subset has been demonstrated to produce IL-9, -10, -17A and -22 in addition to IL-4 and IL-13 in response to IL-25 which is dependent on the transcription factor, E4BP4 (E4 promoter-binding protein 4). Notably, IL-17RB+ iNKT cells were demonstrated to play a role in the pathogenesis of virus-induced AHR. Specifically, mice were infected intranasally with RSV four times at 10 day intervals and were treated with the immunogen, recombinant G of RSV (rec Gs) 4 days after the first infection. After the last dosing with RSV, mice were exposed to rec Gs and AHR measured 24 hours later. AHR was observed only in wild-type mice but was absent in iNKT cell-deficient and *Il-17rb*^{-/-} mice. Accordingly, adoptive transfer of IL-17RB+ iNKT cells but not an IL-17RB-iNKT subset induced AHR in iNKT cell-deficient mice¹⁸⁶.

iii. Th2 cells

Th2 cells represent key mediators of IL-25-driven allergic inflammation in the adaptive response. Subsequent to early studies that reported enhanced expression of IL-17RB on human Th2 memory cells, Angkasekwinai *et al.* demonstrated a role for IL-25 in promoting Th2 cell differentiation in an IL-4 and STAT6-dependant manner. Specifically, IL-25 was shown to induce early IL-4 expression in naive CD4+ T cells from mice via upregulation of NFATc1 and JunB which resulted in increased GATA3 expression and Th2 cell differentiation *in vitro*. Furthermore, IL-25 treatment of cultured naive T cells induced IL-4, IL-5 and IL-13 levels which was inhibited by anti-IL-25 antibody treatment⁷⁰.

In contrast with these findings, Wang *et al.* reported that IL-25 promoted proliferation in activated human Th2 memory cells while resting Th2 memory cells were unable to respond

to IL-25 due to low surface IL-17RB expression. However, activation of DCs via stimulation with TSLP induced robust enhancement of IL-17RB expression on cultured human Th2 memory cells. Stimulation with IL-25 enhanced proliferation and induced activated Th2 memory cells to acquire an effector-memory phenotype by CCR7 and CD27 downregulation while maintaining CCRTh2 and CD62L/L-selectin surface expression. Further findings suggested that these effects may be due to sustained GATA3 expression by IL-25 which further promotes c-MAF and JunB expression in an IL-4-independent manner¹²⁷.

IL-25 has also been demonstrated to act indirectly to induce Th2 polarisation. Kaiko *et al.* reported that IL-25 initiated RSV-induced Th2 cell responses in an NK-depleted mouse model via upregulation of Jagged1. This was demonstrated by culturing lymph node cells from mice with anti-Jagged1 antibody which reduced levels of IL-13 by approximately 3-fold indicating a role for Jagged1 in Th2 induction. Similarly, by inhibiting IL-25 in cultured DCs, Jagged1 expression was significantly reduced supporting the hypothesis that IL-25- and Jagged1-mediated Th2 responses may be linked¹⁵⁷.

In a mouse model of allergic airways disease, IL-25 was demonstrated to enhance Th2 inflammation via an OVA-activated CD4⁺ Th2 cell-dependent pathway. Transgenic expression of IL-25 in the lung enhanced OVA-induced CD4⁺ T cell and eosinophil airway recruitment. In this model, IL-25-driven eosinophilia was dependent on CD4⁺ T cells. Furthermore, *Stat6*^{-/-} mice displayed an absence of IL-25-induced enhancement in eosinophil and CD4⁺T cell recruitment into the airways suggesting that this process is STAT6 dependent¹⁶⁹.

iv. Th9 cells

Recent findings suggest that IL-9-producing T cells are divergent from the conventional Th2 lineage. The presence of IL-4 alone cannot induce IL-9 production during naive T cell differentiation and requires TGF- β which greatly enhances IL-9 expression and inhibits IL-4, IL-5 and IL-13 production^{197,198}. As a result, these cells have been defined as Th9 cells and were reported by Angkasekwina *et al.* to express IL-17RB. It was also reported that naive T cells from mice were polarised to Th9 cells by stimulating with IL-4 and TGF- β . Subsequent treatment of differentiated cells with IL-25 induced significantly enhanced IL-9 and IL-10 production which was dependent on IL-17RA and IL-17RB signalling. However IL-25 alone was not sufficient to initiate Th9 polarisation. These results were substantiated in a mouse

model of allergic disease using *Aspergillus oryzae* and OVA which demonstrated that mice overexpressing IL-17RB on CD4+ T cells displayed increased IL-9 production¹⁸⁷.

v. Eosinophils

Stimulation of human eosinophils with IL-25 showed increased expression of IL-6, CXCL8/IL-8, CCL2/MCP-1 and CCL3/MIP-1 α *in vitro*¹⁸⁸. IL-25 was also shown to increase eosinophil viability as determined by flow cytometry analysis as well as upregulate surface expression of ICAM-1 while suppressing ICAM-3 and CD62L/L-selectin expression. This IL-25-dependent production of mediators as well as upregulated eosinophil surface markers was mediated via JNK, p38 MAPK, and NF- κ B pathways^{188,199}.

vi. Structural cells

In addition to the lung epithelium acting as a source of IL-25, treatment of the mouse lung epithelial cell line (MLE12) with IL-25 strongly induced expression of CCL11/Eotaxin-1, CCL5/RANTES, CCL22/macrophage-derived chemokine (MDC), TSLP and MUC5ac gene expression⁷⁰. Additional structural cells in the lung that have been reported to be responsive to IL-25 stimulation include airway smooth muscle cells¹⁸⁹, lung fibroblasts¹⁹⁰ and human vascular endothelial cells (HUVEC)¹⁹¹. Specifically, IL-25-treated HUVECs displayed angiogenesis which was associated with induced expression of vascular endothelial growth factor (VEGF) expression and upregulation of its receptors¹⁹¹. These cells were also recently reported to produce basic fibroblast growth factor (BFGF) in response to IL-25 stimulation²⁰⁰.

1.5.6 IL-25-mediated positive feedback loop and a potential role in asthma exacerbations

Numerous IL-25-responsive Th2 cytokine-producing T and non-T cell populations have been identified^{70,78,187,199}. The finding that IL-25 is produced by the respiratory epithelium directly in response to allergen suggests that it acts upstream of Th2 inflammation, possibly acting as an initiating factor⁷⁰. Based on recent findings, it is possible this early source of IL-25 acts on IL-25-responsive innate cell populations such as lineage negative cells, releasing Th2 cytokines in a T cell independent manner^{76,78}. Further to this, bioactive IL-25 produced by eosinophils has been reported to act directly on activated Th2 memory cells stimulating IL-5

production¹²⁷. Based on the findings that IL-25 increases eosinophil viability and Th2 cell-derived IL-5 promotes longevity as well as activation of eosinophils, a positive IL-25-mediated feedback loop between innate (non-T) Th2-cytokine producing cells and effector memory CD4+ Th2 cells has been theorised^{127,199}.

Furthermore, preliminary data demonstrate that, in addition to allergen, virus may play a role in the induction of IL-25^{157,177}. The findings that the associated epithelial-derived cytokines, IL-33 and TSLP, have been reported to be induced by influenza A and RV respectively is suggestive that IL-25 may also be upregulated in response to virus¹²². Given that bronchial epithelial cells are the primary site of RV infection, it may be hypothesised that RV infection of the airway epithelium induces the pro-Th2 cytokine, IL-25. The implications of RV-induced IL-25 by the airway epithelium suggest that in a Th2 pulmonary environment, formed by exposure to environmental allergens, IL-25 has the potential to activate innate and adaptive Th2 effector cells enhancing allergic inflammation^{76,127}.

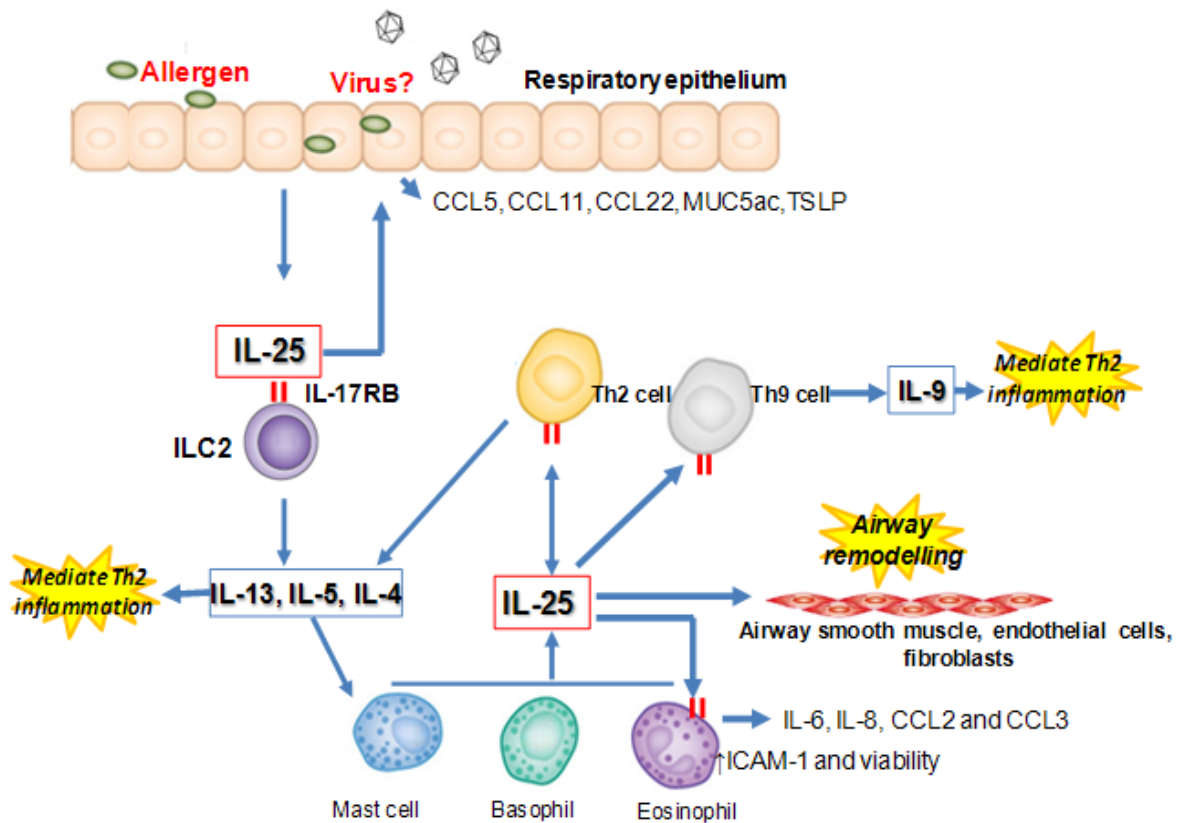


Figure 1.4 Cellular producers of IL-25 and IL-25-responsive cells that form a potential IL-25-mediated positive feedback loop between innate and adaptive immunity

1.5.7 Investigation of IL-25 using mouse models of RV infection and RV-induced allergic airways disease

The induction of IL-25 by RV, with or without exposure to allergen, has not been investigated. Therefore, in this study we assessed RV-induced IL-25 expression in human airway epithelial cells lines as well as HBECS from atopic asthmatics and healthy controls *in vitro*. We then investigated IL-25 induction in a mouse model of RV infection which was developed by Bartlett *et al.* within our laboratory²⁰². This mouse model can be employed for investigating minor group RV infections in BALB/c mice as well as major group RV infections in transgenic BALB/c mice expressing a mouse-human ICAM-1 chimera. Characteristic features of human RV infection have been demonstrated as displayed by airway neutrophilia, expression of proinflammatory cytokines and chemokines and mucus production in both major and minor group infected mice. Furthermore, *in situ* hybridisation detection of negative-strand replicative viral RNA in lung tissue confirmed RV replication in mice²⁰².

In addition to examining RV-induced IL-25 *in vitro* and *in vivo*, the primary aim of this study was to elucidate interplay between allergen exposure and RV infection by investigating the role of IL-25 in RV-induced asthma exacerbations. To achieve these aims, we employed a mouse model of RV-induced allergic airways disease which was also developed within our laboratory. This model incorporates the extensively characterised OVA-induced allergic airways disease model, together with concomitant exposure to minor group RV infection administered with the final airways allergen challenge, thus modelling exacerbation of ongoing atopic airways inflammation by RV infection²⁰². Using this model, we investigated the effects of infection and allergen on IL-25 and Th2-associated mediators as well as IL-17RB-expressing cell populations. Finally, to define a mechanistic role for RV-induced IL-25, we blocked IL-25-mediated signalling in our model of RV-induced allergic airways disease by neutralising IL-17RB and assessed effects on Th2 responses associated with disease pathogenesis to define a role for IL-25 in RV-induced asthma exacerbations.

Chapter 2: Methods and Materials

2.1 Virological techniques

2.1.1 Rhinovirus propagation: preparation of inoculum

RV-1B stocks were obtained from the American Type Culture Collection (ATCC) and propagated in H1 HeLa cells (human epithelial carcinoma cell line) (ATCC). To generate laboratory working stocks, monolayer cultures of HeLa cells were infected for 24 hours (h) to attain 100% cytopathic effect (CPE). The cell/virus suspension was harvested and cells were lysed by freezing and thawing twice before pelleting the debris by centrifugation. The supernatant was collected and aliquots of inoculums were stored at -80°C and subsequently used for viral propagation for *in vivo* studies and infection of bronchial epithelial cells for *in vitro* studies. Serotype specificity was confirmed by neutralisation with specific antiserum (ATCC).

2.1.2 Rhinovirus propagation for *in vivo* work

H1 HeLa cells were grown to 90% confluency in 25 T175 flasks (Nunc) in HeLa growth media (Table 2.7) at 37°C with 5% CO₂. A 30ml working stock of RV-1B (procedure described above) was added to 500ml of HeLa infection media (Table 2.7) and a volume of 20ml was aliquoted per flask and incubated at room temperature (RT) for 1h with constant shaking. To attain approximately 90% cytopathic effect (CPE), cells were incubated at 37°C for 20h. Cells containing virus were pelleted by centrifugation and resuspended in 15ml phosphate buffered saline (PBS) (Table 2.6). Centrifugation and resuspension was repeated to remove residual media before resuspending cells in 36ml of PBS and freeze thawing twice to lyse cells to mediate viral release. Cell debris was removed by centrifugation and supernatant was filtered through a 0.2µm syringe filter. Virus was precipitated by adding polyethylene glycol and 0.5M sodium chloride (Table 2.6) and the solution was placed on ice for 1h.

The precipitate was collected by centrifugation and the resultant pellet resuspended in PBS and concentrated to a volume of ~2.5ml using an Amicon Ultra-15 centrifugal filtration tube (Millipore). Concentrated virus stock was aliquoted and stored at -80 °C until use.

2.1.3 Rhinovirus inactivation

RV-1B stock was exposed to 1200 $\mu\text{J}/\text{cm}^2$ UV light for 30 minutes (min) under sterile conditions and used as a negative control for *in vivo* and *in vitro* experiments.

2.1.4 Rhinovirus titration

Ohio HeLa cells (ATCC) were seeded in 96-well cell culture plates (Costar Corning) at 1×10^5 cells/ml in HeLa infection media (Table 2.7) and exposed to serial 10-fold dilutions of viral stocks. Specifically, 50 μl of concentrated virus was added to 150 μl of cells and diluted 1:10 in quadruplicate wells. Cells were incubated at 37°C for 96h and CPE was assessed and scored by observation using a light microscope. As a comparative measure, a stock of RV-1B of a known titre was tested alongside virus samples. The Spearman-Kärber formula was used to calculate the tissue culture infectious dose (TCID₅₀)²⁰³.

2.2 Mouse models

2.2.1 Mice

6-8 week old, female BALB/c mice were obtained from Charles River UK and maintained within the CBS facility at St Mary's Campus, Imperial College London, under specific pathogen free (SPF) conditions.

2.2.2 General Anaesthesia

Mice were administered the inhalant anaesthetic, isoflurane (Table 2.6), prior to intranasal (i.n.) challenges. For terminal anaesthesia, mice were injected intraperitoneally (i.p.) with 200 μl of pentobarbitone solution (Table 2.6).

2.2.3 RV-1B infection model

BALB/c mice (n=4 or n=5) were infected i.n. with 50 μ l of RV-1B or UV-inactivated RV-1B (UV-RV-1B) at a TCID₅₀ of approximately 5x10⁶. In addition to treating with inactivated virus as a negative control, one group of mice were mock infected i.n. with PBS of the same volume. Terminal anaesthesia was carried out at 10h, day (d) 1, d2, d4 and d7 post-infection (p.i.). (Figure 2.1).

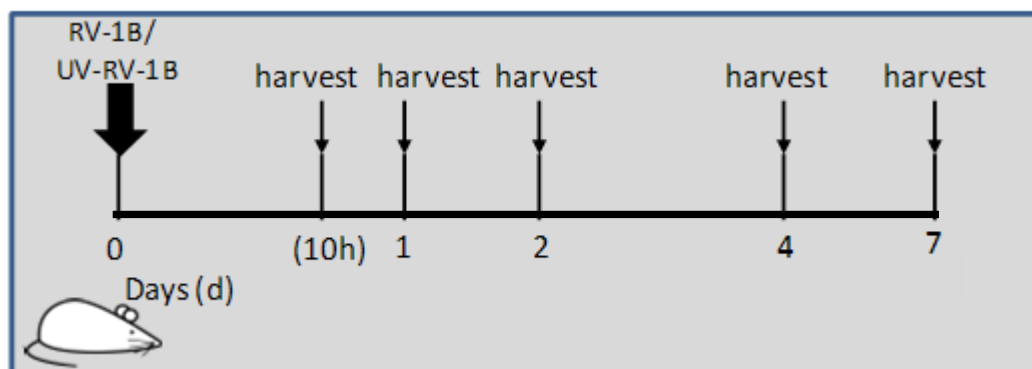


Figure 2.1 Mouse model of RV infection

2.2.4 RV-induced allergic airways disease model

Mice were sensitised i.p. with 50 μ g hen egg ovalbumin (OVA) (Table 2.6) and 2mg of Aluminium Hydroxide (Alum) (Table 2.6) in 200 μ l of PBS on d-13. Mice were challenged i.n. with 30 μ g OVA in 30 μ l of PBS, or PBS alone, on d-2, d-1 and d0. Mice were then infected i.n. with 50 μ l 2 x 10⁶ TCID₅₀ RV-1B or UV-RV-1B immediately after the third OVA challenge on d0 (Table 2.1). Animals were culled at various times p.i. (Figure 2.2).

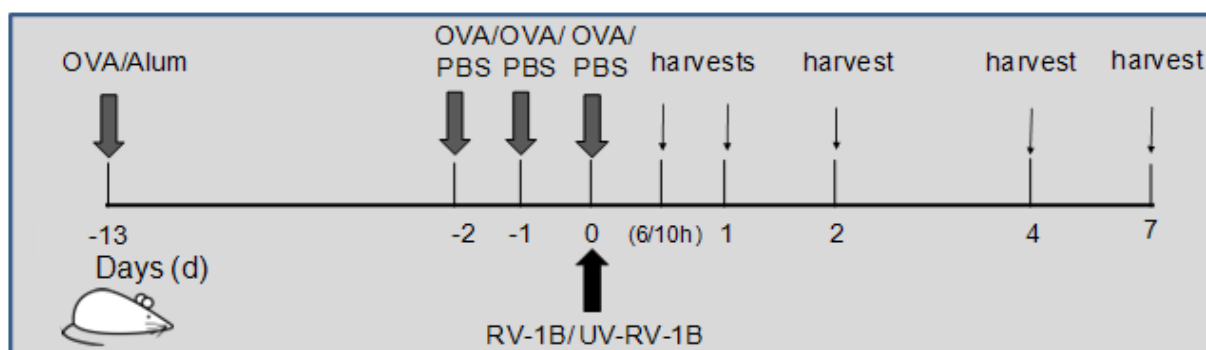


Figure 2.2 Model of RV-induced allergic airways disease.

Experimental groups	Sensitisation (OVA/Alum i.p.)	Challenge (3x OVA i.n)	RV-1B infection (RV-1B i.n./UV-RV-1B control)
RV OVA	Yes	Yes	Yes
UV OVA	Yes	Yes	No (UV-RV-1B control)
RV PBS	Yes	No (PBS control)	Yes
UV PBS	Yes	No (PBS control)	No (UV-RV-1B control)

Table 2.1 Summarised nomenclature of treatment groups in model of RV-induced allergic airways disease. Abbreviations: OVA=ovalbumin; Alum=aluminium hydroxide

2.2.5 Blocking of IL-25 signalling in a model of RV-induced allergic airways disease

Mice were sensitised, challenged and infected as described above in section 2.2.4 with the exclusion of the RV PBS control group. To block RV-induced IL-25 signalling, 0.5mg of anti-IL-17RB antibody (clone D9.2) (provided by Prof. Andrew McKenzie, MRC Laboratory of Molecular Biology, Cambridge) was administered i.p. in 200µl sterile PBS to all treatment groups 4h before infection as well as on d3 and d5 p.i. In addition, identically treated groups were administered 0.5 mg of anti-c-myc mouse IgG1 isotype control antibody (Clone 9e10.2) (also provided by Prof. Andrew McKenzie, MRC Laboratory of Molecular Biology, Cambridge) under the same treatment regime. Animals were culled at the time-points represented in figure 2.3 while nomenclatures of all treated groups are summarized in table 2.2 below for clarification.

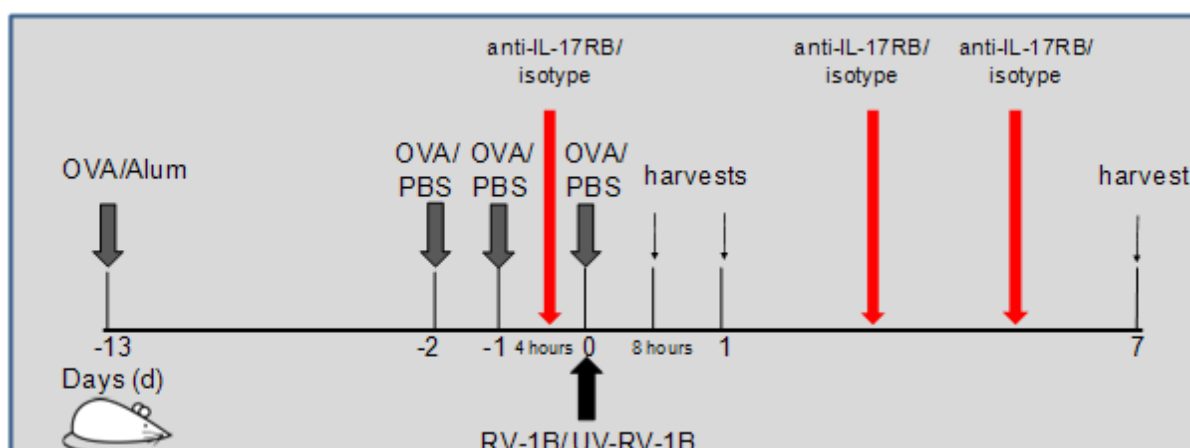


Figure 2.3 Blocking of IL-25 signalling in a model of RV-induced allergic airways disease. Abbreviations: OVA=ovalbumin; Alum=aluminium hydroxide.

Experimental groups	Sensitisation (OVA/Alum i.p.)	Challenge (OVA i.n)	RV-1B infection (RV-1B i.n./UV-RV-1B)	Antibody treatment
RV OVA (anti -IL-17RB)	Yes	Yes	Yes	Anti-IL-17RB
UV OVA (anti-IL-17RB)	Yes	Yes	No (UV-RV-1B control)	Anti-IL-17RB
UV PBS (anti-IL-17RB)	Yes	No (PBS control)	No (UV-RV-1B control)	Anti-IL-17RB
RV OVA (Isotype)	Yes	Yes	Yes	Isotype control
UV OVA (Isotype)	Yes	Yes	No (UV-RV-1B control)	Isotype control
UV PBS (Isotype)	Yes	No (PBS control)	No (UV-RV-1B control)	Isotype control

Table 2.2 Summarised nomenclature of treatment groups of mice administered anti-IL-17RB and isotype control antibody in a model of RV-induced allergic airways disease

2.3 Sample recovery and processing from *in vivo* studies

2.3.1 Bronchoalveolar lavage (BAL)

Mice were intubated and lungs were lavaged by syringing 1.5ml of BAL fluid (Table 2.6) in and out approximately 5 times. BAL fluid cells were pelleted by centrifugation to separate cells and supernatant. Supernatant was collected, snap-frozen and stored at -80°C for cytokine and chemokine analysis by ELISA while cells were re-suspended in 100µl ACK (Table 2.6) for 1min. The solution was neutralised with 1ml of RPMI media containing 10% foetal bovine serum (FBS) (Table 2.6) and cells were centrifuged, resuspended in 1ml of the same media and counted before processing the BAL cytopins. After completion of cytopins, the

remaining BAL cell suspension was centrifuged and the pellet resuspended in 350µl of Buffer RLT containing 1% β-mercaptoethanol (Table 2.6) for RNA purification.

2.3.2 BAL cytopins and differential counts

Using the Cytospin3 system (Shandon), BAL cells resuspended in media were centrifuged onto cytoslides (Tharmac). Slides were air-dried and fixed and stained using the REASTAIN Quick-Diff Kit (Table 2.8). Groups were blinded and differential cell counts were carried out on a total of 200 cells per slide.

2.3.3 Processing of lungs for RNA extraction

The right apical lung lobe was excised and transferred immediately into 600µl of 'RNA Later' buffer (Table 2.6). Samples were stored at -80°C until use. Lung tissue was homogenised mechanically in 600µl Buffer RLT containing 1% β-mercaptoethanol and centrifuged to attain the supernatant which was then processed as described in section 2.9.1.

2.3.4 Processing of lung homogenate for ELISA

The remaining lobes of the right lung (excluding the apical lobe) were harvested and rinsed with PBS, snap-frozen and stored at -80°C. Lobes were homogenised in 600µl of PBS containing dissolved Protease Inhibitor Cocktail (Table 2.6) and lung homogenate was centrifuged to attain the supernatant which was snap-frozen and stored at -80°C until use.

2.3.5 Processing of lungs for flow cytometry

The harvested left lung lobe was crudely chopped in gentleMACS™ C Tubes (Miltenyi Biotech) containing 5ml of lung digestion buffer (Table 2.6) using a gentleMACS™ Dissociator (Miltenyi Biotech). After incubating samples for 45min at 37°C, a second round of mechanical dissociation was performed on lung samples and cells were pelleted by centrifugation. Red blood cells were lysed in 5ml of ACK (Table 2.6) before neutralising the solution with RPMI media containing 10% FBS and attaining a single cell suspension via

filtration through a 100µm cell strainer (BD Biosciences). Cells were pelleted and resuspended in 2ml of RPMI media containing 10% FBS for the purpose of calculating total live cell counts using trypan blue exclusion as determined by light microscopy.

2.3.6 Processing of lungs for histology

Mice were subjected to cardiac perfusion with PBS prior to inflating whole mouse lungs with 4% paraformaldehyde fixative (Table 2.6). Inflated lungs were arranged on labelled cassettes and placed in 4% paraformaldehyde fixative for staining with anti-mouse/human IL-25 antibody (Table 2.9).

2.3.7 Processing of serum

Blood was collected from the carotid artery and processed in microtainer serum separation tubes (BD Biosciences). Blood was left to coagulate at RT for 1h and serum was separated by centrifugation prior to storing samples at -80°C.

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

2.4.1 Cytokines and chemokines in lung homogenate and BAL

Chemokine and cytokines were measured using Mouse DuoSet ELISA Development kits. In accordance with the protocol supplied with kits, 96 well plates (Nunc Maxisorp, Thermo Scientific) were coated with 100µl of capture antibody diluted in PBS and left at RT overnight. Plates were washed 3X with ELISA wash buffer (Table 2.6) and blocked with 200µl of ELISA blocking solution (Table 2.6) for 2h at RT. After a repeated wash step, 100µl of samples and recombinant protein standards were added and incubated for 2h at RT. For analysis of IL-4, IL-5, IL-13 and IL-33 from lung homogenate, samples were diluted 1:10 in ELISA reagent diluent (Table 2.6) or used 1:2 for IL-25 and TSLP analysis. BAL samples were diluted 1:2 for chemokine and IL-4, IL-5 and IL-13 analyses and undiluted for remaining analytes. Standard curves were prepared using 2-fold dilutions of recombinant protein in ELISA reagent diluent with the recommended high standard. Plates were washed and 100µl secondary biotinylated antibodies diluted in ELISA reagent diluent were incubated for 1h.

After a repeated wash step, streptavidin-HRP (Table 2.6), diluted 1:200 in ELISA reagent diluent, was added to plates and incubated for 20min at RT in the dark. Subsequent to washing, 100µl TMB substrate (Table 2.6) was added to plates and the colour reaction was stopped after approximately 15min with 50µl ELISA stop solution (Table 2.6). The optical density of each well was measured using a microplate reader (Spectramax plus384) set to 450nm and quantified by comparison to an 8 point standard curve. Analyses of results were carried out using Softmax Pro 3.1.2 software (Molecular Devices).

2.4.2 IL-25 from cell supernatant

IL-25 in cell supernatant from cultured human airway epithelial cell lines and HBECs from patients was measured using an ultrasensitive human IL-25 in-house ELISA developed by Novartis. Assays were performed by Matt Edwards and Betty Shamji from Novartis, Horsham Research Centre, UK.

2.4.3 MUC5ac and MUC5b

BAL samples were diluted 1:10 in PBS and serial dilutions of supernatant from PMA and ionomycin stimulated-NCI-H292 cells (for MUC5ac) and BAL supernatants from OVA-sensitised and challenge mice (for MUC5b) were used to generate an 8 point standard curve. Samples and standards were added to 96 well plates and incubated at 37°C overnight. For detection of MUC5ac, plates were washed and blocked with PBS/2% BSA before adding an anti-MUC5ac biotinylated antibody (Table 2.9) diluted to a final concentration of 400ng/ml and incubated for 2h at RT. For detection of MUC5b, a mouse anti-MUC5b (clone EU-MUC5b) antibody (attained from Prof. Dallas M. Swallow) was used as a secondary antibody and bound antibody was detected with peroxidase conjugated goat anti-mouse IgG (Table 2.9) Plates were washed and streptavidin-HRP was added and incubated for 20min at RT in the dark. ELISAs were developed and read as described above.

2.4.4 IgE

2.4.4.1 OVA-specific IgE

96 well plates were coated with 2.5µg/ml OVA in PBS and incubated overnight at 4°C. Plates were washed and blocked with ELISA blocking solution and left for 2h at RT. After a repeated wash step, 100µl of mouse serum diluted 1:80 in ELISA reagent diluent was added to plates. Standards were prepared using two-fold dilutions of a mouse anti-OVA IgE antibody (Table 2.9) in ELISA reagent diluent with a high standard of 0.075µg/ml. After 2h incubation at RT, plates were washed and a rat anti-mouse IgE detection antibody (Table 2.9), diluted 1:1000 in ELISA reagent diluents, was added and incubated for 2h at RT. Plates were processed as described above and samples compared to an 8 point standard curve.

2.4.4.2 Total IgE

Total IgE was measured using BD OptEIA™ Mouse IgE Set (See Table 2.9). In accordance with the supplied protocol, 96 well plates were coated with 100µl of capture antibody (anti-mouse IgE) diluted 1:250 in PBS and left at RT overnight. Plates were washed 3X with ELISA wash buffer and blocked with 200µl of PBS with 10% FBS for 2h at RT. After a repeated wash step, 100µl of mouse serum diluted 1:100 in PBS with 10% FBS and purified mouse IgE standard were added and incubated for 2h at RT. Plates were washed and 100µl secondary biotinylated antibody (anti-mouse IgE) diluted 1:500 in PBS with 10% FBS were incubated for 1h. After a total of 5 washes, plates were processed as described above and samples compared to an 8 point standard curve.

2.5 Flow cytometry analysis

2.5.1 Surface staining of lung and BAL leukocytes

Approximately $1-10 \times 10^5$ BAL cells and 3×10^6 lung cells were incubated in 100µl FACS buffer (Table 2.6) containing 5µg/ml Fc Block (Table 2.10) for 20min at 4°C. Fluorochrome-conjugated antibodies specific for cell surface markers (Table 2.10) were added in a volume of 100µl of FACS buffer to cells and incubated for 30min at 4°C in the dark. Cells were washed with PBS and stained with a viability marker (LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit, Invitrogen) for 20min at 4°C in the dark. After washing again with PBS, cells were

fixed using BD Cytofix buffer (Table 2.6) and, if intracellular staining was performed, samples were permeabilised by washing cells with BD Cytoperm buffer (Table 2.6). Cells were resuspended in FACS buffer and analysed within 24h.

2.5.2 Intracellular staining of lung and BAL leukocytes

To detect IL-4 production, 1×10^6 cells/ml of stimulation media (Table 2.7) was incubated at 37°C for 4h. Cells were then stained for surface markers and fixed and permeabilised as described above. Fluorochrome-conjugated IL-4 antibody (Table 2.10) was added to cells in a volume of 200µl of BD Cytoperm buffer and incubated for 30min at 4°C in the dark. After washing with BD Cytoperm buffer, cells were resuspended in FACS buffer and analysed within 24h.

2.5.3 Acquisition and analysis of flow cytometry data

Flow cytometry was performed using a LSRII Fortessa flow cytometer (BD Biosciences) and BD FACS Diva software was used to acquire data. Data analysis was performed using FlowJo analysis software (Version 7.6.1)(Tree Star Inc). Representative flow plots illustrating gating strategies implemented as well as Fluorescence Minus One (FMO) controls which were used to set the gates are represented in Figures 2.4.

2.6 Airway hyperreactivity measurements

Airway hyperreactivity was measured by inhalation of methacholine aerosol (Table 2.6) in unanaesthetised, spontaneously breathing mice. Specific airway resistance (sRaw) was measured in a double chamber plethysmograph (Electromedsystems) while enhanced pause (Penh) was quantified using an unrestrained whole body plethysmography system (Electromedsystems). Both techniques were performed by Dr Ross Walton, Imperial College London. sRaw was measured over 5min by exposing mice to increasing aerosolised concentrations of 5mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 30 mg/ml and each dose was administered for 1min. Quantification of Penh was assessed over 5min periods after 1min aerosolisation challenge after exposure to 3 mg/ml, 10 mg/ml, 15 mg/ml, 30 mg/ml and 100

mg/ml of methacholine. For both plethysmograph experiments, aerosolised water was used as a control and used as baseline values. Data was analysed using eDacq v1.8 software (Electromedsystems) and the following equations applied to calculate Penh and sRaw respectively.

$$\text{Penh} = [(T_E/RT)-1] \times (PEF/PIF)$$

T_E = Duration of expiration

RT = Relaxation Time (Time needed to exhale the first 64% of the tidal volume)

PEF = Peak Expiratory Flow (ml/s)

PIF = Peak Inspiratory Flow (ml/s)

$$\text{sRaw} = [(T_I+T_E)/(2 \times \pi) \times (P_{atm}-47) \times 1.36 \times 2 \times \pi \times dT/(T_I+T_E)]$$

T_I = Duration of inspiration

T_E = Duration of expiration

P_{atm} = atmospheric pressure

dT = delay between nasal and thoracoabdominal flow

2.7 Immunohistochemistry

Formalin-fixed, paraffin-embedded lung tissues were deparaffinised and antigen unmasking was carried out by immersing sections in citrate buffer (10mM at pH6.0). Sections were then covered with 3% hydrogen peroxidase to inhibit endogenous peroxidase activity and left for 5min before washing with PBS. Cell membranes were permeabilised adding 0.1% saponin in PBS and non-specific staining was blocked by incubating with 5% rabbit serum for 20min at RT. Sections were incubated with 60µg/ml rat anti-mouse IL-25 antibody or a rat IgG isotype control (Table 2.9) at 4°C overnight. Sections were then washed and incubated with 1:100 dilution of HRP labelled rabbit anti-rat secondary antibody for 1h. After a further wash with PBS, sections were incubated with ABC reagent (Vector ABC Kit) and the reaction visualised with chromogen-fast diaminobenzidine5 (DAB) as a chromogenic substrate. Slides were counterstained with haematoxylin to provide nuclear and morphological detail, and mounted in DPX Mountant (Sigma-Aldrich).

For quantification of IL-25+ epithelial cells, immunostaining intensity for IL-25 protein was scored semi-quantitatively between 0 to 1.5 (0 = negative; 0.5 =weak staining, 1 = moderate

staining, and 1.5 =strong staining). Infiltrating IL-25+ inflammatory cells in the airway lamina propria were quantified and expressed as the number of positive inflammatory cells per millimetre length of the reticular basement membrane. All counting of histology slides were performed by one investigator and slides were blinded to experimental treatments. Staining and scoring was carried out by Dr Jie Zhu (Royal Brompton Hospital, Imperial College London).

2.8 *In vitro* work

2.8.1 Cell maintenance

The human bronchial epithelial cell line (BEAS-2B) (ATCC) were cultured in BEAS-2B growth media (Table 2.7) in T175 flasks while commercially purchased primary HBECS (Lonza) and primary HBECS from patients were cultured in HBEC BEGM complete media (Table 2.7) in T75 flasks (Nunc) at 37°C with 5% CO₂. For maintenance of HeLa cell lines (including Ohio and H1 sublines), cells were cultured in HeLa growth media (Table 2.7) in T175 flasks under identical conditions. Respective media was changed every 2-3 days and cells passaged when 80-90% confluent.

2.8.2 Subculture procedure

2.8.2.1 BEAS-2B and HeLa cell lines

BEAS-2B or HeLa cells were washed twice with PBS and 2.5ml of trypsin-EDTA (TE) (10X) (Table 2.7) diluted to 1X in PBS was added to cells and incubated for 2-3min at 37°C. Trypsin was neutralised by adding 7.5ml of respective growth media to BEAS-2B or HeLa cells and 2.5ml of the cell suspension was added to T175 flasks containing 28ml of growth media for propagation.

2.8.2.2 Commercially purchased primary HBECS (Lonza)

Primary HBECS obtained from Lonza were sourced from 4 individuals that were non-asthmatic, non-smoking and non-alcoholic (Table 2.3). For subculturing, primary cells were washed with 4ml of HEPES Buffered saline solution (Table 2.7) and 2ml trypsin-EDTA (1X) (Table 2.7) was added to cells for ~2min at 37°C with intermittent banging of the flask to ensure detachment of cells. The cells were centrifuged after adding 4ml of trypsin neutralizing solution (TNS) (Table 2.7). Pelleted cells were resuspended in 10ml HBEC BEGM complete media and a third of the total volume was propagated into a new T75 flask containing 12ml of complete media.

HBECs source number	Age (years)	Sex	Race	Smoking and alcohol consumption
4F0174	18	Female	C	No
4F0872	34	Male	AA	No
4F1604	27	Male	AA	No
6F4181	19	Male	C	No

Table 2.3 Primary HBECS (Lonza). Abbreviations: C=Caucasian; AA=African American

2.8.3 Cell quantification

Cells to be quantified were added to 0.1% trypan blue (Table 2.6) at a 1:1 ratio. A Neubauer haemocytometer was used to count cells in a volume of 10µl and observed using a light microscope. Viable cells were counted in three squares (each square measuring 1mmx1mm in dimension and composed of 9 sub-squares) bordered by double lines and the average taken and multiplied by the dilution factor to give cell number X10⁴/ml.

2.8.4 *Ex vivo* human bronchial epithelial cells from bronchial brushings

2.8.4.1 Bronchoscopy

Bronchoscopies were carried out in the Endoscopy Unit at St. Marys Hospital, Paddington, in accordance with British Thoracic Society guidelines. Lung function (FEV₁) was recorded before and after bronchoscopy and all participants received nebulised 0.5mg Salbutamol and 0.5mg Ipratropium bromide prior to the procedure.

2.8.4.2 Processing of human bronchial epithelial cells from bronchial brushings

Bronchial epithelial cells were obtained by scraping a subsection of the patient's bronchial wall 5-10 times with a sterile 5mm sheathed endobronchial brush (Olympus BC-202D-5010). Sheathed brushes were stirred vigorously in a sterile tube containing 10ml of prewarmed HBEC BEGM complete media. Both brushes and sheaths were detached into the media and immediately transported to the laboratory for processing while maintaining samples at 37°C.

Media containing sheathed brushes were gently vortexed and brushes and sheaths were flushed with fresh media to facilitate detachment of cells into solution. In addition, brushes were passed through a pipette tip into a small amount of media to ensure complete removal of adherent cells. All media containing detached cells was pooled and centrifuged at 10000g for 6min. Supernatant was discarded and the cell pellet was resuspended in 10ml complete media and cell counted as described in section 2.8.3. The cell suspension was seeded into T25 (Nunc) flasks pre-coated with collagen coating solution (Table 2.7) that had been incubated at 37°C /5% CO₂ overnight. Cells were then left undisturbed for 48h to allow maximum adherence to the flask, after which the media was discarded and replaced with 10ml fresh complete media containing 100µl of Ultrosor G (Table 2.7). Complete media supplemented with Ultrosor G was replaced every two days for a week, after which only complete media was used. Cells were cultured until 70–80% confluent and subcultured into larger flasks as described above for HBECS (Lonza). At passage 2-3, cells were seeded into 12 well plates.

2.8.5 RV infection of human bronchial epithelial cells

BEAS-2B cell lines and all primary HBECs were grown to 80% confluency in 12 well plates. Approximately 16h before infection, cells were serum starved by adding BEGM serum free media (Table 2.7) to primary HBECs or BEAS-2B serum free media (Table 2.7) to BEAS-2B cell lines. After removal of media, cells were incubated with 200µl RV-1B working stock diluted in PBS for 1h at RT with constant shaking. A dose-dependent response to a range of RV titres was carried out prior to experiments to attain a desirable MOI. A titre of $\sim 5 \times 10^6$ was used for infection of BEAS-2B cells while HBECs were infected with a titre of $\sim 2.5 \times 10^6$. Cells

were exposed to virus in parallel with UV-inactivated RV-1B and/or PBS as negative controls. After infection, inoculum was replaced with BEAS-2B infection media (Table 2.7) and BEGM serum free media for BEAS-2B and HBECs respectively. Supernatant was collected and cells lysed by adding Buffer RLT and 1% β -mercaptoethanol to attain RNA at various hours after infection.

2.9 Molecular Biology Techniques

2.9.1 RNA extraction and cDNA synthesis

Cells were lysed with Buffer RLT and 1% β -mercaptoethanol prior to RNA extraction. Total extractions were carried out using the Qiagen Rneasy Mini Kit (Table 2.8) according to manufacturers' instruction which included the optional on-column DNase I digestion step (Table 2.8). Resultant RNA was resuspended in 30 μ l of Rnase/Dnase-free water. The quantity (in ng/ μ l) of the RNA was analysed using a ND-1000 Spectrophotometer system (Thermo Scientific). Approximately 15 μ g of RNA/sample of mouse lung tissue and ~5 μ g of RNA/sample from cell culture experiments was used to prepare cDNA. Synthesis of cDNA was carried out using random hexamer primers (Table 2.6) and reagents from the Omniscript Reverse Transcription Kit (Table 2.8). The volumes of reagents added per reaction are listed in table 2.4 and cDNA reactions were performed at 37°C for 1h.

Reagent	Volume/reaction
10x Buffer RT	4 μ l
dNTP mix (each dNTP 5mM)	4 μ l
Random primers (Promega)	2 μ l
Omniscript RT	2 μ l
Rnase/Dnase free water	8 μ l
RNA Template	20 μ l

Table 2.4 Composition of cDNA reactions

2.9.2 Primer/probe design

Primers and probes for mouse and human IL-25 were designed for this study while additional genes, listed in table 2.5, were previously established within the laboratory. Genomic and mRNA sequences of genes of interest were obtained using NCBI PubMed and compared to create primers/probes that crossed exon junctions and had a low penalty score using Clone Manager (Sci-Ed) and Primer Express v3.0 Software (Applied Biosystems). Forward/reverse primers were optimised for use by testing concentrations of 50nM, 300nM and 900nM while probes were used at a fixed concentration of 175nM per reaction.

PRIMER NAME	Sequence (5'-3')	Concentration (nM)
<i>Murine specific</i>		
IL-25 forward	CACACCCACCACGCAGAAT	300
IL-25 reverse	CAACTCATAGCTCCAAGGAGAGATG	300
IL-25 probe	FAM-CCAGCAAGGATGGCCCCCTCA-TAMRA	100
<i>Human specific</i>		
IL-25 forward	GAGATATGAGTTGGACAGAGACTTGAA	300
IL-25 reverse	CCATGTGGGAGCCTGTCTGTA	300
IL-25 probe	FAM-CTCCCCCAGGACCTGTACCACGC-TAMRA	100
RV forward	GTGAAGAGCCSRTGTGCT	50
RV reverse	GCTSCAGGGTTAAGGTTAGCC	300
RV probe	FAM-TGAGTCCTCCGGCCCCCTGAATG-TAMRA	100
<i>Murine and human</i>		
18S forward	CGCCGCTAGAGGTGAAATTCT	300
18S reverse	CATTCTTGGCAAATGCTTTCG	300
18S probe	FAM-ACCGGCGCAAGACGGACCAGA-TAMRA	100

Table 2.5 Sequences and concentrations for primer and probes used in q-PCR

2.9.3 Taqman q-PCR

Quantitative (q) PCR was carried out on 1µl of cDNA by adding Quantitect Probe PCR Mastermix (Table 2.4), Rnase/Dnase free water and gene specific primers and probes (Table 2.5). Reactions were run on an ABI 7500 Real-Time PCR System (Applied Biosystems) under the following cycling conditions: 50°C for 2min; 95°C for 1min; 45 cycles at 95°C for 15sec and 60°C for 1min. Using cDNA diluted 1:100, 18S ribosomal RNA (rRNA) quantification was carried out using specific primers and probes (Table 2.5) for normalising qPCR expression analysis. All samples were analysed in duplicates. Data was analysed using ABI Prism 7500

SDS Software Version 4.0 (Applied Biosystems). Messenger RNA copy number was quantified by comparison to a 10-fold dilution series of plasmid containing PCR product and normalised for 18S rRNA.

2.9.4 Cloning of standards

Products from qPCR were analysed on a 1% (w/v) agarose tris-acetate-EDTA (TAE) gel (Table 2.6) together with a DNA ladder (Table 2.6) to indicate product size. PCR products were excised from the gel, purified using a QIAquick PCR purification kit (Table 2.8) and inserted into a pCR2.1 TOPO TA vector (Table 2.8). Vector and insert were transformed into chemically competent TOP10 *E.coli* cells (Table 2.8) by heat shock treatment at 42°C for 30sec. Cells were plated onto lysogeny broth (LB) agar plates (Table 2.7) containing 100µg/ml of ampicillin (Table 2.6) and incubated at 37°C overnight. Correct insert ligation was assessed by restriction enzyme digest with EcoRI endonuclease and analysed on a gel and by amplification of insert b qPCR. Positive clones were grown up in LB broth (Table 2.7) containing 100 µg/ml of ampicillin and purification of plasmid DNA was carried out using Qiagen Plasmid Midi and Maxi Kits (Table 2.8).

2.10 Statistical analysis

All graphical data is expressed as mean $\pm$ SEM (standard error of the mean). A minimum of 4 animals per group were used for all *in vivo* experiments. In addition, a representative of at least 2 independent experiments or combined experiments is depicted. For *in vitro* experiments, at least 4 independent experiments were performed in duplicate or triplicate wells for studies in BEAS-2B and HBECs (Lonza). Experiments performed on HBECs from patients' bronchial brushings were performed in duplicate wells but only carried out once due to limitation of cells.

Where groups were greater than 2 and/or multiple comparisons were analysed, results were analyzed by ANOVA and differences between groups identified using Bonferroni's post-test with 95% confidence using GraphPad Prism4 software. When only two groups were analysed and one condition was variable a 2 tailed unpaired t test was used to

compare groups. Clinical data was analysed by one-way ANOVA using the Kruskal-Wallis test with a 95% confidence interval with Dunn's multiple comparison test. Correlations were assessed with linear regression and Spearman's coefficient (r) value. P values are represented throughout as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

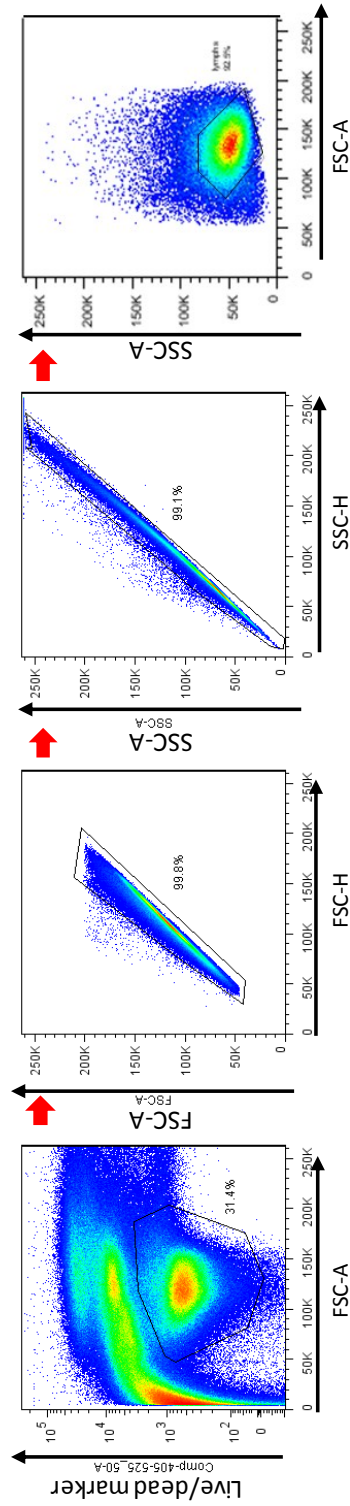


Figure 2.4.1 Representative flow cytometry gating for live, single cells Lung cells in a model of RV-induced allergic airways disease were gated to include live, single lymphocytes. Numbers indicated on the plots reflect the percentage of cells that are present within the gated areas. Abbreviations: FSC-A=forward scatter-area; FSC-H=forward scatter-height; SSC-A=side scatter-area; SSC-H=side scatter-height

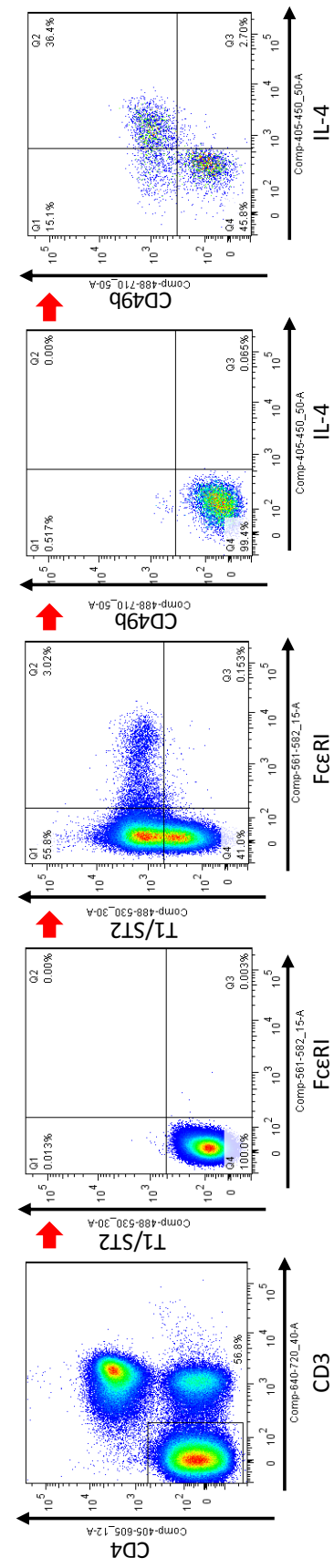


Figure 2.4.2 Representative flow cytometry gating for basophils Basophils in a model of RV-induced allergic airways disease were identified by gating CD3-/CD4- cells while lung cells stained with all markers with the exclusion of T1/ST2 and FcεRI were used as a control to set gates to identify CD49b+/FcεRI+ cells. Similarly, lung cells stained with all markers with the exclusion of CD49b and IL-4 were used as a control to set gates for measurement of CD49b+/IL-4+ cells.

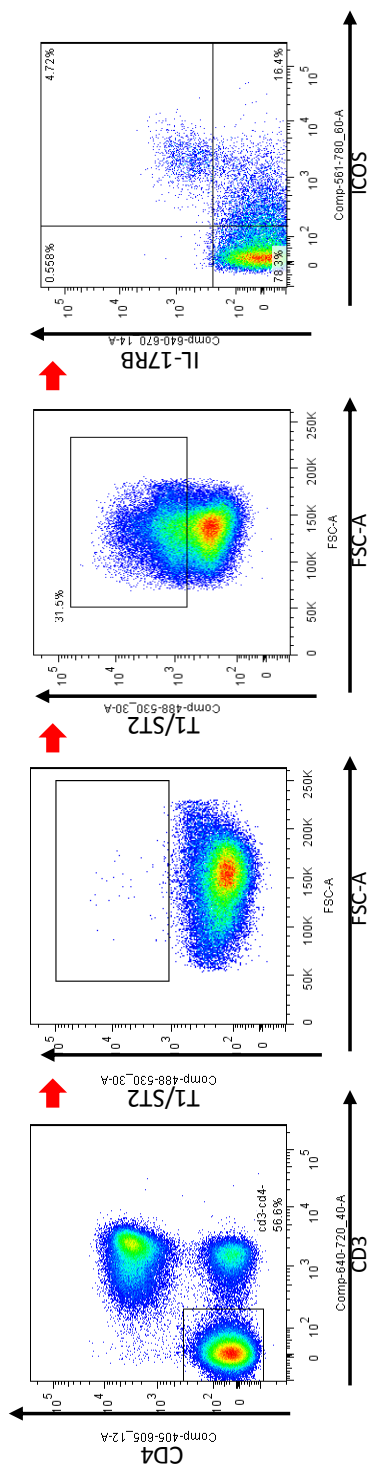


Figure 2.4.3 Representative flow cytometry gating for innate lymphoid cells (ILC) 2. Cells that were negative for FcεRI, IL-4 and CD49b were sorted further to exclude CD3 and CD4 expressing cells. To define T1/ST2+ cells, samples were stained with all markers with the exclusion of T1/ST2 which were then gated to identify IL-17RB+/ICOS+ cells.

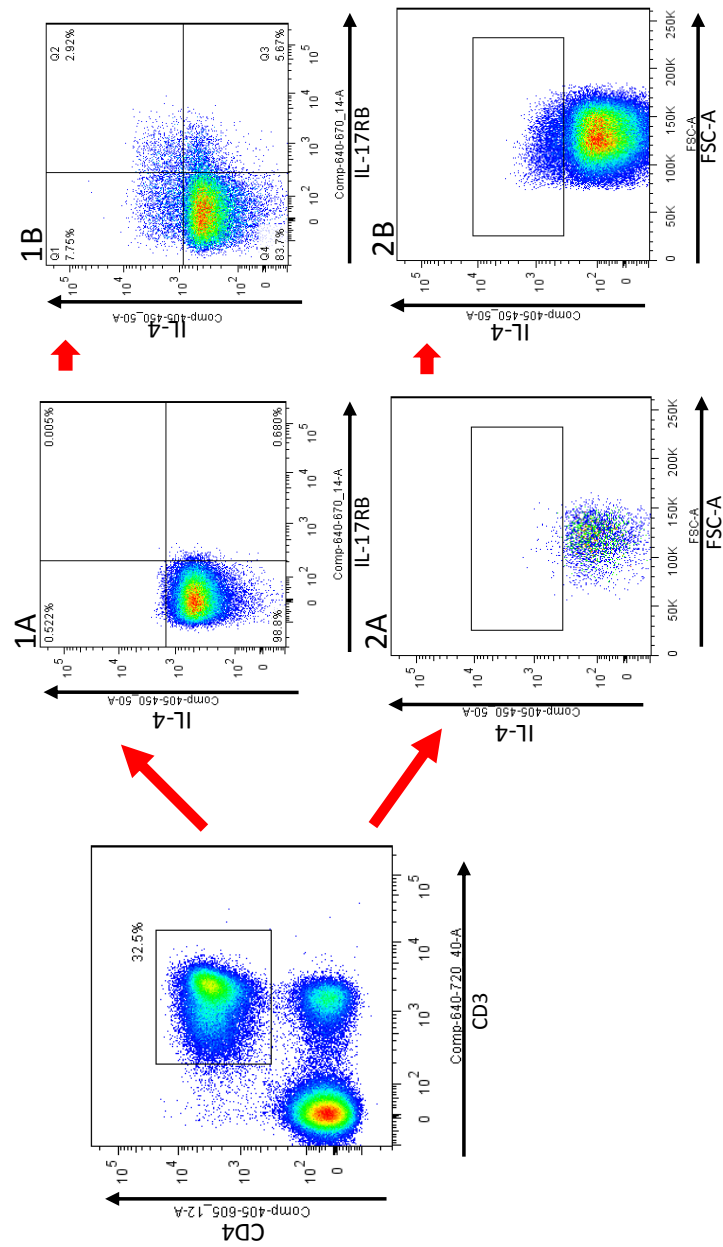


Figure 2.4.4 Representative flow cytometry gating for IL-17RB+ IL-4+ CD4+ T cells and IL-4+ CD4+ T cells CD4+/CD3+ cells were stained with all markers with the exclusion of IL-4 and IL-17RB (1A) as well as IL-4 (2A) which were used as controls to set gates and define IL-4+/IL-17RB+ (1B) and IL-4+ CD4+ T cells (2B).

Table 2.6 Reagents and buffers

Name	Composition	Application	Supplier
ACK lysis buffer	0.15M Ammonium Chloride, 1mM Potassium Hydrogen Carbonate, 0.1mM Disodium EDTA in 500ml dH ₂ O. Sterile filtered	Depletion of red blood cells	All reagents from Sigma-Aldrich
Agarose	Agarose molecular grade powder	Agarose gel electrophoresis	Sigma-Aldrich
Aluminium Hydroxide (Alum)	Aluminium Hydroxide (Al)OH ₃ Hydrate	Sensitisation of mice	Sigma-Aldrich
Ampicillin (100mg/ml)	Supplied in 70%(v/v) ethanol	<i>E. coli</i> culture for plasmid cloning	Sigma-Aldrich
β-mercaptoethanol (β-ME)	14.3M C ₂ H ₆ O ₅	Isolation of RNA	Sigma-Aldrich
BAL fluid	Earle's Balanced Salt Solution 55mM Disodium EDTA 12mM lidocaine hydrochloride monohydrate	Lavage of mouse lung	Gibco Gibco Sigma-Aldrich
Buffer RLT		Lysis of mouse tissue/cells and human cells	Qiagen
Cytofix/Cytoperm™ solution	1% Paraformaldehyde Saponin	Simultaneous fixation and permeabilization of cells for flow cytometry	BD biosciences
DNA ladder	1kB Plus DNA ladder 100bp-12kB DNA standard (1µg/µl)	Agarose gel electrophoresis	Invitrogen
EcoRI restriction enzyme	Buffer H 10X Buffer, BSA, 5,000u EcoRI	Cloning for TaqMan	Promega
ELISA reagent diluent/blocking solution	1% BSA in Dulbecco's Phosphate-Buffered Saline (DPBS)	Sample and standard dilution buffer and blocking solution	Sigma-Aldrich Gibco
ELISA wash buffer	PBS and 0.05% tween20	ELISA plate washing	Sigma-Aldrich
ELISA stop solution	2M H ₂ SO ₄	Stopping of ELISA reaction	Sigma-Aldrich
FACS buffer	1% (w/v) BSA and 0.01% (w/v) Sodium Azide in DPBS. Sterile filtered	Processing of cells for staining in flow cytometry	Sigma-Aldrich
Isoflurane	'Isoflurane-Vet' 100% w/w	Anaesthesia in mice	Merial
Lung digestion buffer	10% (v/v) FCS, 2% (v/v) HEPES, 1% (v/v) sodium bicarbonate in RPMI, 1mg/ml collagenase Type XI and 80units/ml Bovine Pancreatic Dnase Type IV		Sigma-Aldrich Sigma-Aldrich
Methacholine	Acetyl-β-methyl-choline chloride, dessicate	Assessment of airway hyperreactivity (AHR) in mice	Sigma-Aldrich
Ovalbumin	Chicken egg ovalbumin, 5X crystalline	Mouse sensitisation and challenge	Calbiochem
Paraformaldehyde fixative (4%)	4% paraformaldehyde (w/v) heated to 60°C in PBS, pH increased by 1 N NaOH to increase solubility and adjusted to pH 7.4	Fixative of mouse lung cells	Sigma
Pentobarbitone	Pentoject Pentobarbitone Sodium, 20% w/v	Terminal anaesthesia in mice	AnimalCare Ltd.
Phosphate buffer saline (PBS) (1X)	Dulbecco's PBS (1x) without calcium and magnesium	General use	A&E Scientific (PAA)
PBS (1X)	0.014M KH ₂ PO ₄ , 0.008M Na ₂ HPO ₄ -7H ₂ O, 0.0026M KCl, 0.137M NaCl	Cell culture	Invitrogen
Polyethylene glycol (PEG)	Polyethylene glycol 6000, 7% w/v	Precipitation of virus for RV1B propagation	Fluka
Protease Inhibitor Cocktail Tablets	Composed of several protease inhibitors for the inhibition of serine and cysteine proteases (one tablet dissolved in 50ml PBS)	Prevent break down of proteins in solution for lung homogenate ELISAs	Roche
Quantitect Probe PCR	HotStarTaq DNA Polymerase,	Taqman RT PCR	(Qiagen, USA)

Mastermix	QuantiTect Probe RT-PCR Buffer, dNTP mix, ROX dye		
Quantitative PCR probes	Random hexadeoxynucleotides (0.5µg/ml)	cDNA synthesis	Eurofins
Random primers	Random hexadeoxynucleotides (0.5µg/ml)	cDNA synthesis	Invitrogen
RNA Later buffer	RNA stabilisation buffer	Stabilisation of RNA from mouse tissue and human cells	Qiagen
Streptavidin-HRP (Horse Radish Peroxidase)	ELISA grade Streptavidin-HRP conjugate	ELISA	R and D Systems
T4 DNA ligase	100 units T4 DNA ligase in storage buffer	Ligation	Promega
T4 DNA ligase (10X) reaction buffer	300mM Tris-HCl, 100mM MgCl ₂ , 100mM DTT, 10mM ATP	Ligation	Promega
TE buffer (1X), Molecular grade	10mM Tris-HCl containing 1mM EDTA. Na ₂	Plasmid, primer/probe resuspension	Promega
Tetramethyl benzidine (TMB) substrate	3,3',5,5'- Tetramethyl benzidine liquid substrate	Substrate for horseradish peroxidase in ELISA	Invitrogen
Tris-acetate-EDTA buffer (TAE)	2M tris-acetate solution containing 50mM EDTA	Agarose gel electrophoresis	Invitrogen
Trypan blue	0.4% trypan blue in 0.85% saline solution	Assessment of cell viability	Invitrogen

Table 2.7 Media and media supplements

Name	Composition	Application	Supplier
Bronchial epithelium basal medium (BEBM)	Refer to manufacturer's information on media composition	HBEC culture	Lonza
BEGM single quot additives	Refer to manufacturer's composition	HBEC culture	Lonza
HBEC BEGM serum free media	HBEC BEGM complete growth media without 13g/L bovine pituitary extract	Serum starving for HBEC infection	Lonza
Collagen coating solution	10% BSA (sterile filtered), 1% Recombinant human like collagen 1% fibronectin in BEGM	Seeding of HBECs from bronchial brushings	Sigma-Aldrich Biovision Invitrogen
HBEC BEGM complete growth media	BEBM with BEGM single quot additives	HBEC culture	As stated above
Dulbeccos modified eagles medium (DMEM)	Refer to manufacturer's media composition	HeLa culture	PAA Laboratories
RPMI 1640 media	Refer to manufacturer's media composition	BEAS-2B culture	PAA Laboratories
Foetal Bovine Serum	Heat inactivated foetal bovine serum	HeLa and BEAS-2B culture	Invitrogen
HEPES buffered saline solution	30mM HEPES	HBEC culture	Lonza
HEPES buffered solution	238.3 g/L prepared in distilled water. pH: 7.2 to 7.5	HeLa and BEAS-2B culture	Invitrogen
Sodium Bicarbonate	7.5% sodium bicarbonate in solution	HeLa and BEAS-2B culture	Invitrogen
Penicillin-Streptomycin	10,000U/ml penicillin G sodium and 10,000µg/ml Streptomycin sulphate in 0.85% saline	HBEC culture	Invitrogen
HeLa growth media	10% (v/v) FCS, 2% (v/v) HEPES, 1% (v/v) sodium bicarbonate in DMEM	HeLa culture	Invitrogen
HeLa serum free media	2% (v/v) HEPES, 1% (v/v) sodium bicarbonate in DMEM	HeLa culture	Invitrogen
HeLa infection media	2% (v/v) FCS, 2% (v/v) HEPES, 1% (v/v) sodium bicarbonate in DMEM	HeLa culture	Invitrogen
BEAS-2B growth media	10% (v/v) FCS, 2% (v/v) HEPES, 1% (v/v) sodium bicarbonate in RPMI	BEAS-2B culture	Invitrogen
BEAS-2B serum free media	2% (v/v) HEPES, 1% (v/v) sodium	BEAS-2B culture	Invitrogen

	bicarbonate in RPMI		
BEAS-2B infection media	2% (v/v) FCS, 2% (v/v) HEPES, 1% (v/v) sodium bicarbonate in RPMI	BEAS-2B culture	Invitrogen
Trypsin-EDTA (1X)	0.025% trypsin, 0.01% EDTA	HBEC sub-culture	Lonza
Trypsin-EDTA (10X)	0.5% trypsin, 5.3mM EDTA	HeLa and BEAS-2B sub-culture	Invitrogen
Trypsin Neutralising solution (TNS)	Refer to manufacturer's media composition	HBEC sub-culture	Lonza
Luria-Bertani (LB) media	10g tryptone, 5g yeast extract, 5g NaCl, in 1L distilled water	<i>E. coli</i> culture for plasmid cloning	Sigma-Aldrich
Stimulation media	10% (v/v) FCS, 2% (v/v) HEPES, 1% (v/v) sodium bicarbonate in RPMI 50ng/ml phorbol myristate acetate (PMA) 500ng/ml ionomycin 1µl/ml GolgiStop containing monensin		Sigma-Aldrich Sigma-Aldrich BD Biosciences
LB agar	14g of agarose in 1L LB media	<i>E. coli</i> culture for plasmid cloning	Sigma-Aldrich
Ultroser-G BioSpera	Serum substitute lyophilized Ultroser G	Supplement medium for HBECs from bronchial brushings	Pall Life Sciences, UK

Table 2.8 Commercially available kits

Name	Components	Application	Supplier
Mouse DuoSet ELISA Development kit (IL-4, -5, -25, 33, CCL11, CCL24, CCL5, CCL17)	Capture Antibody, Detection Antibody, Standard, Streptavidin-HRP	Measurement of cytokines and chemokines in mice	R and D Systems
Mouse IL-13 Quantikine Immunoassay	2x Mouse IL-13 Microplates, IL-13 Conjugate, IL-13 Standard, IL-13 Control, Assay Diluent RD1-14, Calibrator Diluent RD5T, Wash Buffer Concentrate, Colour Reagent A and B, Stop Solution	Measurement of IL-13 in mice	R and D Systems
Mouse Serum Total IgE ELISA Set	Anti-mouse IgE monoclonal antibody capture antibody, Biotinylated anti-mouse IgE monoclonal antibody detection antibody, Streptavidin-horseradish peroxidase conjugate (SAv-HRP), Purified mouse IgE standard	Measurement of total IgE in mice	BD OptEIA
Omniscript RT (Reverse Transcription) Kit	dATP, dCTP, dGTP, dTTP (10mM) in water, 10X Buffer RT, Omniscript reverse transcriptase, RNase-free water	cDNA synthesis	Promega
One Shot® TOP10 Competent Cells	Components used in study: One Shot® TOP10 Chemically Competent <i>E. Coli</i> , S.O.C Medium	Cloning of products for TaqMan	Invitrogen
Plasmid Midiprep Kit	Buffer P1, Buffer P2, Buffer P3, Buffer QBT, Buffer QC, Buffer QF, Qiagen-tip 100	Medium-scale plasmid isolation	Qiagen
Plasmid Maxiprep kit	Buffer P1, Buffer P2, Buffer P3, Buffer QBT, Buffer QC, Buffer QF, Qiagen-tip 500	Large-scale plasmid isolation	Qiagen
QIAquick PCR Purification Kit	QIAquick Spin Columns, Buffers, Collection Tubes	PCR purification for cloning	Qiagen
Quantitect Probe PCR Mastermix	HotStarTaq DNA Polymerase, QuantiTect Probe RT-PCR Buffer, dNTP mix, ROX dye	Taqman RT PCR	(Qiagen, USA)
REASTAIN Quick-Diff Kit	Reastain Quick-Diff Blue containing,	Differential staining of	REAGENA Ltd

	Azur II 0,09 % Glycerol 5 %, Sodiumazide <0,1 % Reastain Quick-Diff Fix containing Methanol 100 %, Methyleneblue <0,01 % Reastain Quick-Diff Red containing Eosin Y 0,12 %, Sodiumazide <0,1 %	cytopins	
RNase-free DNase set	1500 units RNase-free DNase I, RNase-free buffer RDD	DNase digestion in total RNA isolation	Qiagen
RNeasy Mini Kit	Buffer RLT, Buffer RW1, Buffer RPE, RNase-free water, RNeasy mini spin columns, collection tubes	Total RNA isolation	Qiagen
TOPO TA cloning kit (with pCR 2.1-TOPO vector)	Components used in study: pCR 2.1-TOPO (10ng/μl), salt solution (1.2M NaCl, 0.06M MgCl ₂)	Cloning of products for TaqMan	Invitrogen

Table 2.9 Antibodies used in IHC and ELISA

Name	Clone	Application	Working conc.	Supplier
Mouse anti-MUC5AC (biotinylated)	45-M1	ELISA	0.4μg/ml	ThermoScientific
Peroxidase-conjugated goat anti-mouse IgG (Fc specific)		ELISA	5μg/ml	Sigma-Aldrich
Mouse anti-OVA IgE	2C6	ELISA	1μg/ml	Genetex
Rat Anti-Mouse IgE (ε chain specific) (biotinylated)	23G3	ELISA	0.5μg/ml	Southern Biotech
Purified anti-mouse/human IL-25 (IL-17E)	35B	IHC	60μg/ml	Biolegend
Rat IgG control antibody		IHC	60μg/ml	Sigma-Aldrich
Peroxidase-conjugated rabbit anti-rat IgG-biotin y		IHC	1:100	Dako

Table 2.10 Antibodies used in flow cytometry

Name	Clone	Conjugate	Working conc.	Supplier
Hamster anti-mouse CD3e	500A2	Alexa Fluor 700	2.5μg/ml	eBiosciences
Rat anti-mouse CD4	RM4-5	Brilliant Violet 605	2.5 μg/ml	Biolegend
Rat anti-mouse CD49b	DX5	PerCP-Cy5.5	2.5 μg/ml	Biolegend
Hamster anti-mouse ICOS (CD278)	C398.4A	Pe-Cy7	2.5 μg/ml	Biolegend
Hamster anti-Mouse FcεR1	MAR-1	PE	2.5 μg/ml	eBiosciences
T1/ST2	DJ8	FITC	5 μg/ml	MD Biosciences
Anti-mouse IL-17RB	D9.2	Alexa Fluor 647	5 μg/ml	Obtained from Dr David Cousins (Kings College, London)
Rat anti-mouse IL-4	11B11	Brilliant violet 421	2.5 μg/ml	Biolegend
Fc Block (anti-mouse CD16/CD32)	2.4G2		5μg/ml	BD Biosciences

Chapter 3: Results

Characterisation of IL-25 induction in response to RV infection *in vitro* and *in vivo*

3.1 Introduction

The airway epithelium is the first point of contact for all inhaled stimuli in the asthmatic lung and plays a pivotal role in shaping subsequent immune responses via the release of mediators⁷⁵. Importantly, the major triggers of asthma exacerbations, namely RV and allergen, are both capable of modulating the lung epithelium and induce epithelial-derived chemokines and cytokines that initiate recruitment and activation of immune cells^{26,67}. Notably, IL-25 is released by adenocarcinomic human alveolar basal epithelial cells (A549) and the mouse lung epithelial cell line, MLE12, when stimulated with the allergens, *Aspergillus oryzae* and ragweed⁷⁰. Furthermore, stimulation of human nasal epithelial cells (HNEC) with poly I:C, a synthetic analog of dsRNA used to mimic viral infection, induced IL-25¹⁶⁰. Given that bronchial epithelial cells are the primary site of RV infection we hypothesized that RV infection of the airway epithelium induces the cytokine, IL-25. Therefore, we initially investigated *in vitro* expression of IL-25 in response to the human RV-1B (serotype 1B) in human airway epithelial cell lines and bronchial epithelial cells obtained from bronchoscopic brushings from atopic asthmatics and healthy patients.

Based on these findings, we then sought to characterise IL-25 induction in a mouse model of RV infection. The majority of studies in mice have reported IL-25 induction in response to established Th2 inducers including allergen and models of nematode infections^{70,80,170}. Though two recent studies have demonstrated enhanced IL-25 expression in response to RSV infection in NK cell depleted mice and to PVM infection in unmodified BALB/c mice^{157,177}, the induction of IL-25 by RV, with or without concomitant exposure to allergen, has not been investigated. The finding that the pro-Th2 cytokine, IL-25, is induced by RV could provide an association between the antiviral response and Th2 driven inflammation and implicate IL-25 as a potential initiator of RV-induced exacerbations. In addition, confirmation of IL-25 induction in response to RV in the airways of BALB/c mice would verify suitability of this model to investigate our primary aim, namely to investigate the role of IL-25 in exacerbation of Th2 inflammation in a model of RV-induced allergic airway disease.

3.2 Hypotheses and aims

3.2.1 Hypotheses

- i.) RV-1B infection induces IL-25 expression in human bronchial epithelial cells (HBECs) *in vitro*
- ii.) RV-1B infection induces IL-25 expression in the airways of mice *in vivo*

3.2.2 Aims

To characterise RV-1B-induced IL-25 expression *in vitro* (Part 1) and *in vivo* (Part 2)

Part 1: To investigate IL-25 expression in RV-1B-infected human airway epithelial cell lines and HBECs from atopic asthmatics (AAs) and non-atopic non-asthmatic (NANAs) patients

- i.) To use quantitative (q) PCR to determine the magnitude and kinetics of IL-25 gene expression in RV-infected BEAS-2Bs, A549s, H292s and commercially purchased primary HBECs
- ii.) To measure IL-25 gene expression by qPCR and IL-25 protein by ELISA in RV-infected cultured HBECs from AA and NANA subjects and assess if increased RV-induced IL-25 correlates with asthma relevant clinical parameters in patients

Part 2: To investigate IL-25 expression in the lungs of RV-1B-infected BALB/c mice.

- i.) To confirm successful RV-1B infection by measuring:
 - RV-1B RNA copies in the lung using qPCR
 - Inflammatory airway leukocyte infiltration in the bronchial alveolar lavage (BAL) via differential cell counts
 - Viral-associated proinflammatory cytokines and chemokines in the BAL by ELISA.
- ii.) To determine the magnitude, kinetics and cellular source/s of IL-25 in the airway and lung tissue by qPCR, ELISA and immunohistochemistry (IHC).

3.1 Results: Part 1

3.3.1 RV-induced IL-25 in human airway epithelial cell lines

3.3.1.1 BEAS-2B cells

The human bronchial epithelial cell line, BEAS-2B, was infected with RV-1B and IL-25 expression was measured at the indicated time-points post-infection (p.i.). Gene expression was quantified by qPCR while protein in cell supernatants was measured using an in-house ELISA developed by Novartis.

Using a multiplicity of infection (MOI) of 2 (TCID₅₀ of 5×10^6), RV-1B induced IL-25 mRNA which was significantly upregulated compared with media ($P < 0.05$) and UV-RV-1B-treated controls ($P < 0.05$) at 24h p.i. (Figure 3.1 A). This titre was selected based on previous dose-dependent assays that demonstrated using a MOI of 2 induced the highest amounts of IL-25 at 24h p.i. (Figure 3.1 B).

Previous reports have demonstrated that the proinflammatory cytokine, IL-6, is induced by RV infection and replication in BEAS-2B cells therefore levels were measured in cell supernatants by ELISA to confirm that cells were successfully infected^{116,204}. RV-1B infection induced significantly higher levels of IL-6 protein compared with UV-inactivated virus ($P < 0.05$) at 24h p.i. (Figure 3.1 C) indicating that IL-25 gene expression was RV replication-dependant.

IL-25 protein was not detectable in cell supernatant from RV-1B-infected or control-treated cell cultures (data not shown).

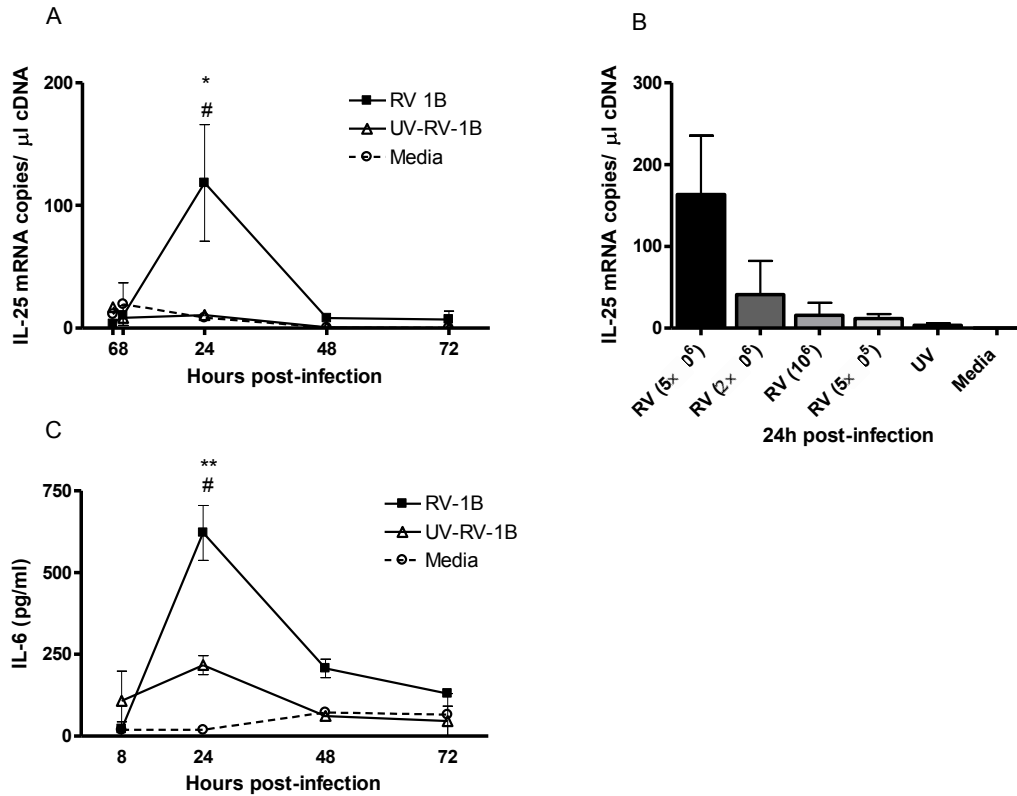


Figure 3.1 Measurement of IL-25 and IL-6 expression in RV-infected BEAS-2B cell lines BEAS-2B cells were exposed to RV-1B in parallel with UV-inactivated RV-1B (UV-RV-1B) and PBS (media) as negative controls. Cells were harvested at the indicated time-points p.i. and IL-25 mRNA (A) was measured after optimising the RV-1B viral titre for infection (B). IL-6 protein expression (C) was measured to confirm RV infection and replication. For detection of IL-25 gene, cellular RNA was extracted and cDNA synthesized by qPCR. Data is expressed as the number of mRNA copies per μl of cDNA added per qPCR reaction. IL-6 protein was detected in cell supernatant by ELISA. Data is represented as the mean ($\pm$ SEM) of triplicate wells per treatment condition and is representative of at least 4 independent experiments.* RV-1B vs UV-RV-1B; # RV-1B vs media (*/# $P < 0.05$) (** $P < 0.01$)

3.3.1.2 Commercially purchased primary HBECs (Lonza) and additional human airway epithelial cell lines

IL-25 gene expression was also measured in the human alveolar adenocarcinoma cell line, A549, the mucoepidermoid carcinoma cell line, NCI-H292, as well as commercially available

primary HBECs (Lonza). All cells were infected with RV-1B and IL-25 mRNA was measured by qPCR at 6h, 8h, 24h, 48h and 72h p.i.

IL-25 mRNA and protein was undetectable in all cell lines and primary HBECs. RV-1B replication was confirmed by significantly higher levels of IL-6 protein in cell supernatants as measured by ELISA in RV-1B-infected cells and control-treated cells (data not shown).

3.3.2 Analysis of RV-induced IL-25 in HBECs from atopic asthmatics (AA) and non-atopic non-asthmatic (NANA) patients

IL-25 gene and protein expression was measured in cDNA samples and in supernatants from RV-1B-infected and media-treated cells from 10 atopic asthmatics (AAs) and 10 non-atopic non-asthmatics (NANAs). These samples were obtained as part of a human clinical study carried out by Dr Jonathan Macintyre.

3.3.2.1 Clinical characteristics of subjects

HBECs were obtained from bronchoscopic brushings from moderate AAs (according to the BTS and GINA guidelines^{50,58}) with cells from NANAs as controls. Inclusion criteria for asthmatic patients was an age range of 18-65 years old, a clinical diagnosis of asthma, symptom questionnaires positive for asthma, a skin prick test (SPT) positive for at least one allergen, a histamine provocation concentration of <8mg/ml producing a 20% fall (PC₂₀) in FEV₁, an absence of other chronic respiratory or systemic disease and a status of either non-smoker or ex-smoker (with a history of <5 packs/year) (Table 3.1).

Healthy control subjects were aged between 18-65 years, were negative for asthma in symptom questionnaires, negative for allergen-specific SPT, had a PC₂₀ of >16mg/ml, possessed <80IU/ml of IgE, had an absence of chronic respiratory or systemic disease and were non-smokers or ex-smokers (with a history of <5 packs/year). Subjects were excluded if they had contracted a respiratory tract infection within the last 6 weeks before the study or possessed any other abnormalities in lung function (Table 3.1).

All subjects gave written informed consent and the study was approved by the St. Mary's Hospital Ethics Committee.

3.3.2.2 Statistical summary of clinical characteristics of subjects

All characteristics relating to clinical diagnosis of asthma, indications of lung function and atopic read-outs were significantly different in AAs compared with NANAs as indicated by represented P values in Table 3.1.

Clinical characteristics	Atopic Asthmatics	Non-atopic non-asthmatics	P value
Sex	70% M 30% F	30% M 70% F	
Age	34.5 (± 2.252)	28 (± 2.408)	0.1011
ACQ	1.054 (± 0.259)	0	0.0002
ICS	80% (± 0.133)	0	0.0021
ICS and LABA (Salbutamol)	80% (± 0.133)	0	0.0021
Exacerbations/year	1.4 (± 0.306)	0	0.025
PEF % predicted	93.3 (± 2.103)	106.286 (± 1.748)	0.002
FEV ₁ % predicted	89.667 (± 2.739)	106.857 (± 1.575)	0.0001
FEV1/FVC ratio	78.788 (± 2.107)	88.333 (± 2.092)	0.0046
P20 (mg/ml)	3.096 (± 0.764)	16 or >16	0.0001
IgE (units/ml)	218.87 (± 63.024)	9.286 (± 2.119)	0.0001
SPT (number)	3.4 (± 0.400)	0	0.0001

Table 3.1 Clinical characteristics of subjects from whom HBECs were attained. Values are presented as the mean for each group, namely atopic asthmatics (n=10) and non-atopic non-asthmatics (n=10), with the standard error of the mean (SEM) represented in brackets. Gender and asthmatic subjects on medication (inhaled corticosteroids and/or long acting beta agonists), are represented as percentages. Abbreviations: ACQ=asthma control questionnaire; ICS=inhaled corticosteroids; LABA=long acting beta agonist; PEF=peak expiratory flow rate; FEV₁=forced expiratory volume in 1 second; PC₂₀= provocative concentration of histamine causing a 20% drop in FEV₁; FVC=forced expiratory vital capacity, SPT= skin prick test

3.3.2.3 Quantification of IL-25 gene expression in HBEs

IL-25 mRNA was quantified in RV-1B-infected and media-treated cells from AAs and NANAs by qPCR. RV-1B infection induced significantly greater levels of IL-25 gene expression in cells from AAs at 8h p.i. compared with media-treated cells from AAs ($P<0.05$) and NANAs ($P<0.01$) harvested at the same time-point (Figure 3.2 A). No significant differences or trends in IL-25 expression were observed in any treatment groups at 24h p.i. (Figure 3.2 B).

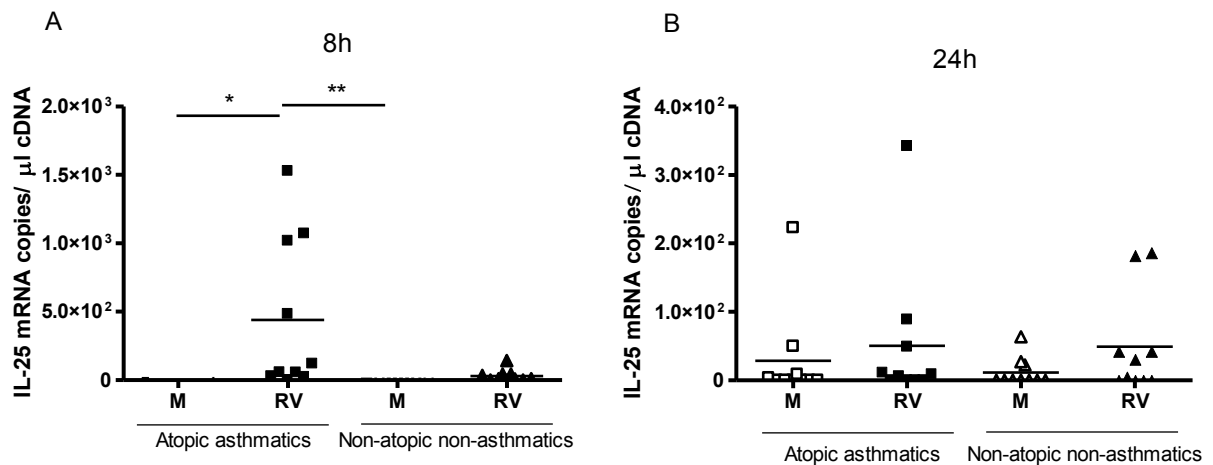


Figure 3.2 Measurement of IL-25 gene expression in RV-infected cells from AAs and NANAs Cells from 10 AAs and 10 NANAs were exposed to RV-1B (RV) in parallel with PBS (media [M]) as a negative control and were harvested to measure IL-25 gene expression at 8h (A) and 24h (B) p.i. For IL-25 gene quantification, cellular RNA was extracted and cDNA synthesized by qPCR. Data is expressed as the number of mRNA copies per μ l of cDNA added per qPCR reaction. Data is represented as the mean (+/-SEM) of triplicate wells per treatment condition and is representative of 1 independent experiment (* $P<0.05$; ** $P<0.01$).

3.3.2.4 Quantification of IL-25 protein in HBEs

IL-25 protein was detected in cell supernatants from RV-1B-infected and media-treated cells from AAs and NANAs using an in-house ELISA developed by Novartis. Though no significant differences were observed between treated cells at 8h p.i. (Figure 3.3 A), IL-25 levels were significantly elevated in supernatants from RV-1B-infected cells from AAs compared with supernatants from media-treated cells from NANAs ($P<0.01$) at 24h p.i (Figure 3.3 B).

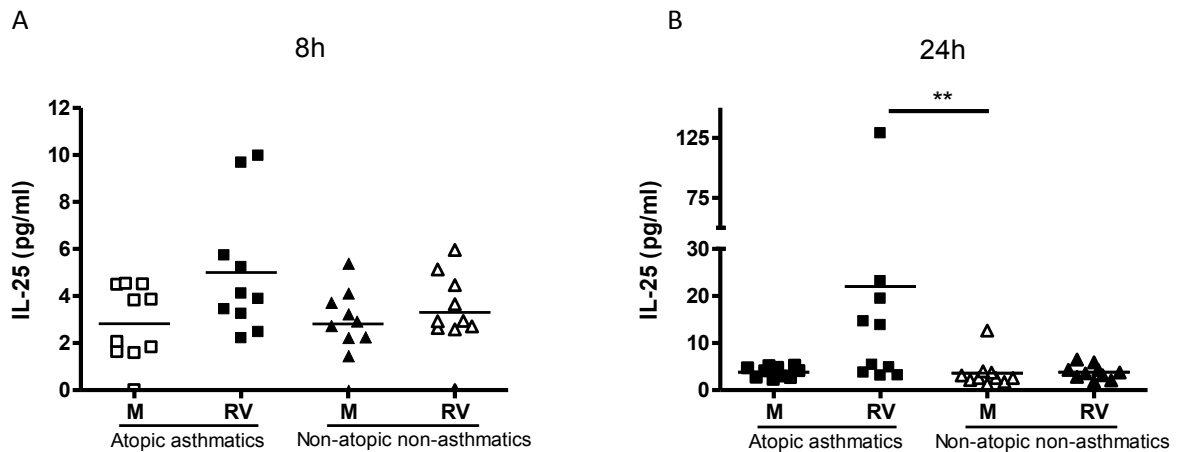


Figure 3.3 Measurement of IL-25 protein expression in RV-infected cells from AAs and NANAs Cells from 10 AAs and 10 NANAs were exposed to RV-1B (RV) in parallel with PBS (media [M]) as a negative control and were harvested to measure IL-25 protein expression at 8h (A) and 24h (B) p.i. IL-25 protein was measured in cell supernatants from cultured cells using an in-house ELISA developed at Novartis Horsham Research Centre, West Sussex. Data is represented as the mean (+/- SEM) of triplicate wells per treatment condition and is representative of 1 independent experiment (**P<0.01).

3.3.2.5 Correlations between IL-25 protein expression levels and clinical characteristics

To assess if increased IL-25 was related to clinical parameters, protein levels were correlated with clinical measurements in AAs and NANAs. IL-25 measurements at 8h and 24h p.i. in RV-1B-infected and media-treated controls were compared against clinical read-outs of atopy (IgE levels and SPT), indications of lung function (FEV₁/FVC ratio), AHR (PC₂₀ measurement) as well as the number of recorded AE/year and ACQ results. Correlations were assessed with linear regression and Spearman's coefficient (r) value which, together with summarised P values, are represented for IL-25 levels in cells from AAs at 24h p.i. vs clinical parameters in Table 3.2. No significant correlations between IL-25 levels and clinical parameters were observed in cells from NANAs at any time-point p.i. or in cells from AAs at 8h p.i. and media-treated cells at 24h p.i. However, increased IL-25 in RV-1B-infected cells from AAs at 24h p.i. was significantly associated with increased positive SPT scores ($r=0.8245$; $p=0.0047$) (Figure 3.4 A). A trend for increased IL-25 and an increased number of recorded AE/year was also evident at 24h p.i. ($r=0.6097$; $p=0.0667$) though this correlation was not significant (Figure 3.4 B).

IL-25 protein		ACQ	AE/ year	FEV1/FVC ratio	PC ₂₀	IgE	SPT	
Atopic Asthmatics 24h	RV-1B	Spearman r	-0.5260	0.6097	-0.08511	0.1622	-0.2000	0.8245
		p value	0.1231	0.0667	0.8113	0.6567	0.5837	0.0047
		p value summary	ns	ns	ns	ns	ns	**
	Media	Spearman r	0.6055	-0.2557	-0.0121	0.03892	-0.2727	0.2203
		p value	0.0667	0.4697	0.9730	0.9184	0.4483	0.5367
		p value summary	ns	ns	ns	ns	ns	Ns

Table 3.2 Summary of correlations between IL-25 protein levels in RV-1B-infected/media-treated controls from AAs (n=10) and clinical parameters. Spearman's coefficient (r) value and summarised p values are represented.

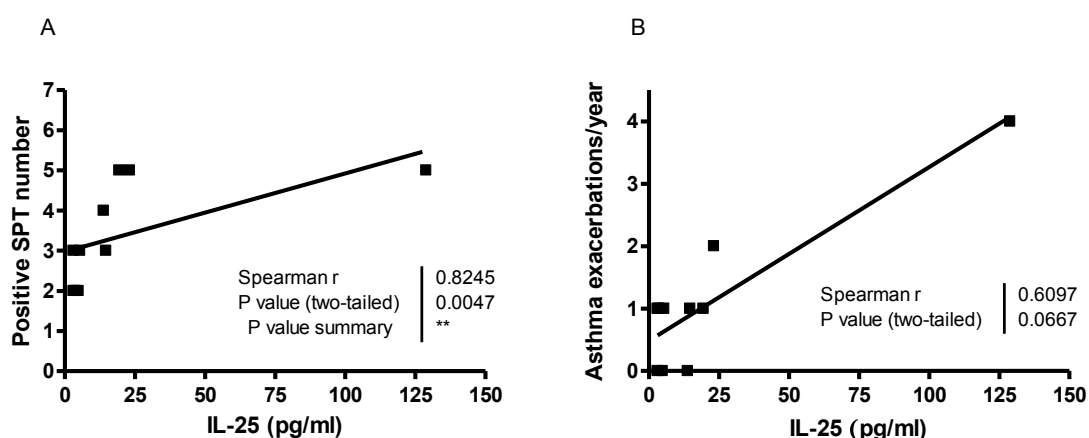


Figure 3.4 Correlations between IL-25 protein expression levels in RV-infected cells from AAs at 24h p.i. and clinical characteristics RV-1B-induced IL-25 protein levels in cells from AAs (n=10) were correlated with positive number of skin-prick tests (SPT) (A) and recorded asthma exacerbations/year (B). Spearman's coefficient (r) value and summarised p values are represented on both graphs.

3.4 Results: Part 2

3.4.1 Characterisation of RV-1B infection in BALB/c mice

3.4.1.1 RV-1B viral load

BALB/c mice were infected i.n. with RV-1B or UV-inactivated RV1B (UV-RV-1B) at a TCID₅₀ of 5×10^6 per mouse. Viral RNA was detected via qPCR in processed lung tissue that was harvested from mice at various time-points p.i.

In RV-1B-infected mice, viral load was significantly increased at 10h p.i. compared to levels in mice treated with UV-RV-1B ($P < 0.05$) (Figure 3.5). This increase persisted until day (d) 1 in infected mice (RV-1B vs UV-RV-1B $P < 0.05$), after which viral load decreased in both treated groups and was comparable at d7 p.i.

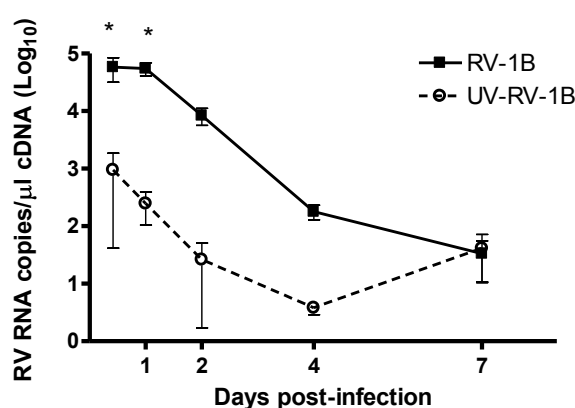


Figure 3.5 Quantification of RV-1B viral load in the mouse lung BALB/c mice were infected i.n with RV-1B or dosed with UV-inactivated virus (UV-RV-1B) as a negative control. Lungs were harvested at 10h and at days 1, 2, 4 and 7 p.i. and processed by extracting RNA and synthesizing cDNA to quantify viral RNA copies via qPCR. Data is expressed as the number of RNA copies per μl of cDNA added per qPCR reaction. Data is representative of at least 2 experiments. Each group of mice (n=4-6) is represented as the mean (+/-SEM) while statistical significance is denoted for comparison between RV-1B and UV-RV-1B treatment at the indicated time-points p.i. (* $P < 0.05$)

3.4.1.2 Airway leukocyte infiltration

To confirm successful RV-1B infection of mice (as previously characterised by early neutrophilia and delayed lymphocytosis²⁰²), differential cell counts were carried out on BAL cells from RV-1B-infected and UV-RV-1B-treated mice. The airways leukocyte profile was characterised by differential staining and cellular morphology in BAL cells fixed to cytopsin slides.

RV-1B induced rapid neutrophilia as early as 10h p.i. (RV-1B vs UV-RV-1B $P<0.01$) and peaked at d1 p.i. (RV-1B vs UV-RV-1B $P<0.001$) (Figure 3.6 A). This influx of neutrophils accounted for approximately 80% of total BAL cells at 10h and d1 p.i (Figure 3.6 B). Neutrophilic inflammation in infected mice had decreased by d2 and was resolved by d4 and d7 p.i. RV-1B also induced a significant infiltration of BAL lymphocytes at d2 (RV-1B vs UV-RV-1B $P<0.05$), d4 (RV-1B vs UV-RV-1B $P<0.05$) and d7 p.i. (RV-1B vs UV-RV-1B $P<0.01$) (Figure 3.6 C). The peak of lymphocytosis was observed at d7 p.i. and accounted for approximately 10% of total BAL cells (Figure 3.6 D). Both neutrophilic and lymphocytic responses observed in RV-1B-infected mice were consistent with previously published results of this model²⁰². UV-inactivated virus failed to induce an inflammatory cellular response, suggesting that the observed increase in neutrophils and lymphocytes in infected mice was dependant on RV-1B replication.

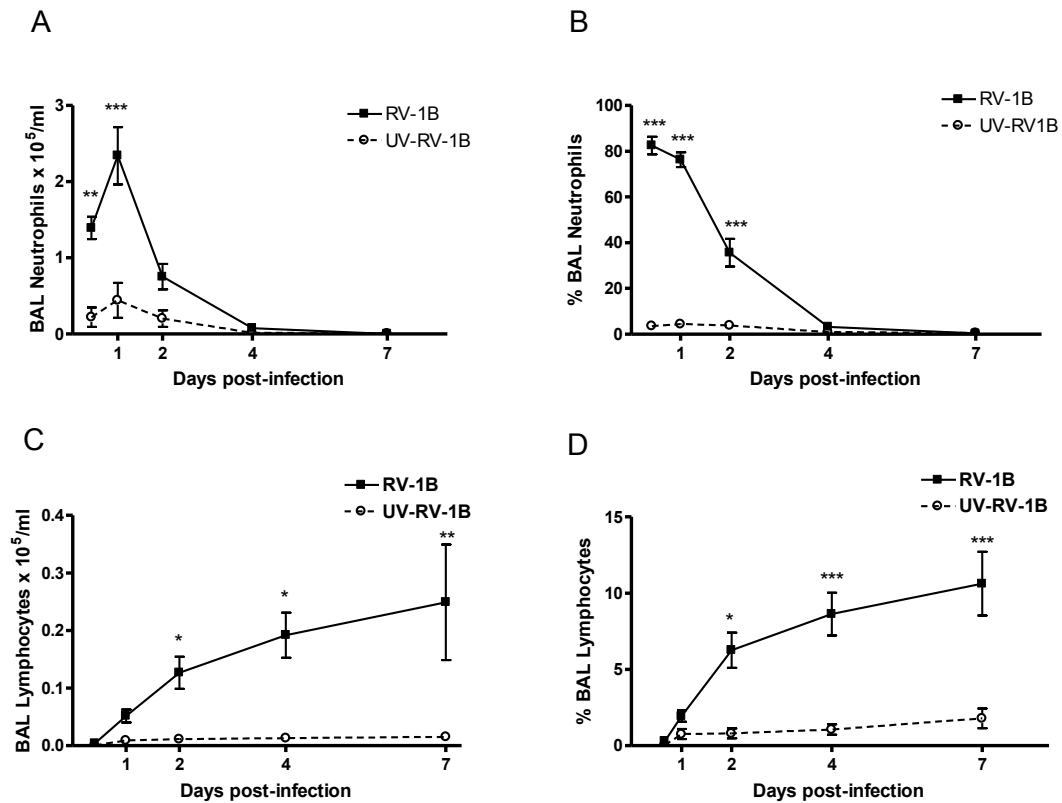


Figure 3.6 Kinetic airway leukocyte infiltration in response to RV-1B infection BALB/c mice were infected i.n with RV-1B or dosed with UV-inactivated virus (UV-RV-1B) as a negative control. BAL cells from mice were fixed to cytospin slides and stained to quantify total numbers and percentages of neutrophils (A and B respectively) and lymphocytes (C and D respectively). All slides were blinded to experimental treatments. Data is represented as the mean (+/-SEM) of 4-6 mice per group and is representative of at least 2 experiments. Statistical significance is denoted for RV-1B vs UV-RV-1B-treated mice at the indicated time-points p.i. (*P < 0.05) (**P < 0.01) (**P < 0.001).

3.4.1.3 Proinflammatory mediators in the BAL

The induction of proinflammatory chemokines and cytokines in response to RV-1B infection in mice and humans has previously been demonstrated^{202 39}. To confirm an effective RV-1B infection, proinflammatory mediators in the BAL fluid of mice were quantified by ELISA. A significant early increase in the proinflammatory cytokine, IL-6, was observed at 10h p.i. in RV-1B-infected mice (RV-1B vs UV-RV-1B P < 0.001) (Figure 3.7 A). This induction was still significant at d1 p.i. (RV-1B vs UV-RV-1B P < 0.001), after which levels decreased to baseline after d2 p.i. A similar trend in induction in response to RV-1B was observed with the antiviral

type III interferon, IFN- λ (Figure 3.7 B). Levels in infected mice were significantly higher at 10h (RV-1B vs UV-RV-1B $P<0.05$) and d1 p.i. (RV-1B vs UV-RV-1B $P<0.05$) and progressively decreased after d2 p.i. The neutrophil chemoattractant, CXCL1/KC, was induced concurrently with the onset of neutrophilia at 10h p.i. (RV-1B vs UV-RV-1B $P<0.001$) and remained significantly higher at d2 p.i. (RV-1B vs UV-RV-1B $P<0.001$) in RV-1B-infected mice compared with treated controls (Figure 3.7 C). A comparatively later peak in the T cell chemokine, CCL5/RANTES, was observed at d1 (RV-1B vs UV-RV-1B $P<0.001$) and d2 p.i (RV-1B vs UV-RV-1B $P<0.001$) in RV-1B-infected mice (Figure 3.7 D) which preceded the significant infiltration of lymphocytes observed at d4 and d7 p.i.

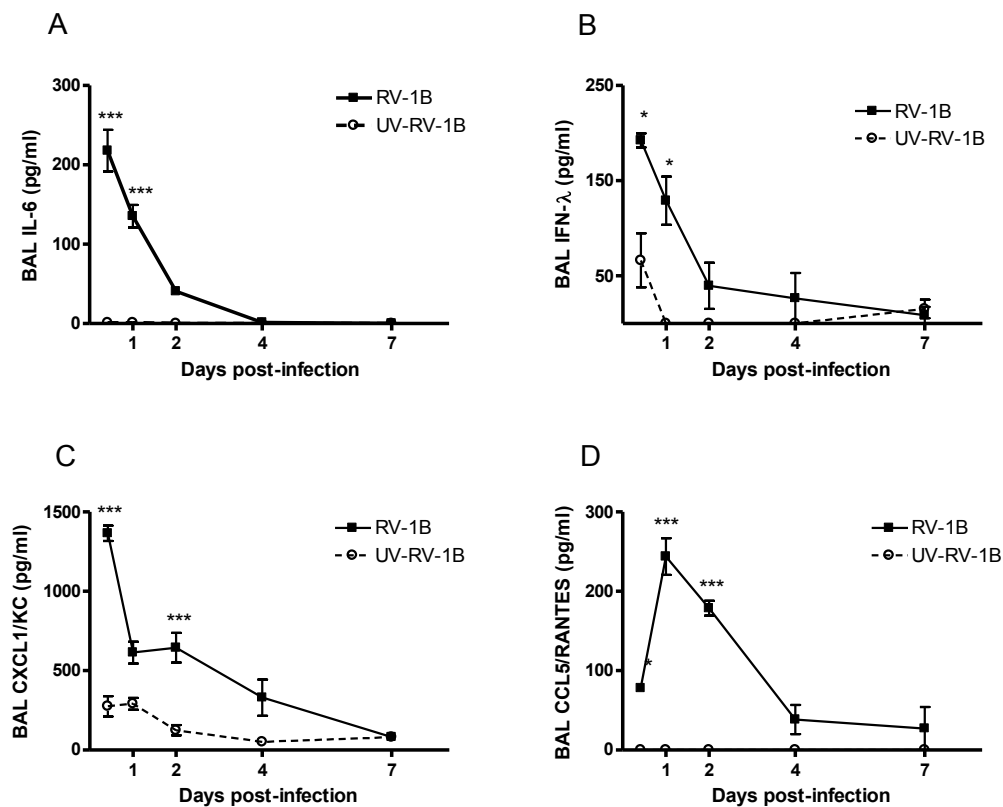


Figure 3.7 Quantification of proinflammatory mediators in response to RV-1B infection BAL from RV-1B-infected and UV-RV-1B-treated controls was collected at the indicated time-points and BAL supernatant was used to measure IL-6 (A), IFN- λ (B), CXCL1/KC (C) and CCL5/RANTES (D) by ELISA. Data is represented as the mean (+/-SEM) of 4-6 mice per group and is representative of at least 2 experiments. Statistical significance is denoted for RV-1B vs UV-RV-1B-treated mice at the indicated time-points p.i. (* $P < 0.05$) (** $P<0.01$) (***) $P<0.001$).

3.4.2 IL-25 expression in response to RV-1B infection

3.4.2.1 IL-25 gene expression in the BAL cells and lung tissue

Studies in mouse models have demonstrated induction of IL-25 during Th2-mediated immune responses; however the induction of IL-25 by RV infection *in vivo* has not previously been reported. Based on results from part 1 of this chapter which indicated that RV alone upregulated IL-25 *in vitro*, we sought to investigate IL-25 expression in mice infected with RV-1B.

IL-25 gene expression was quantified by qPCR in lung tissue and BAL cells from RV-1B-infected mice and UV-RV-1B-treated controls. RV-1B induced a significant early increase of IL-25 mRNA in lung tissue (RV-1B vs UV-RV-1B $P<0.01$) (Figure 3.8 A) and BAL cells (RV-1B vs UV-RV-1B $P<0.05$) (Figure 3.8 B) which was significantly greater than treated controls at 10h p.i. After d1 p.i., IL-25 gene expression levels decreased steadily in infected mice and by d7 p.i. quantities were comparable to mRNA levels in treated control.

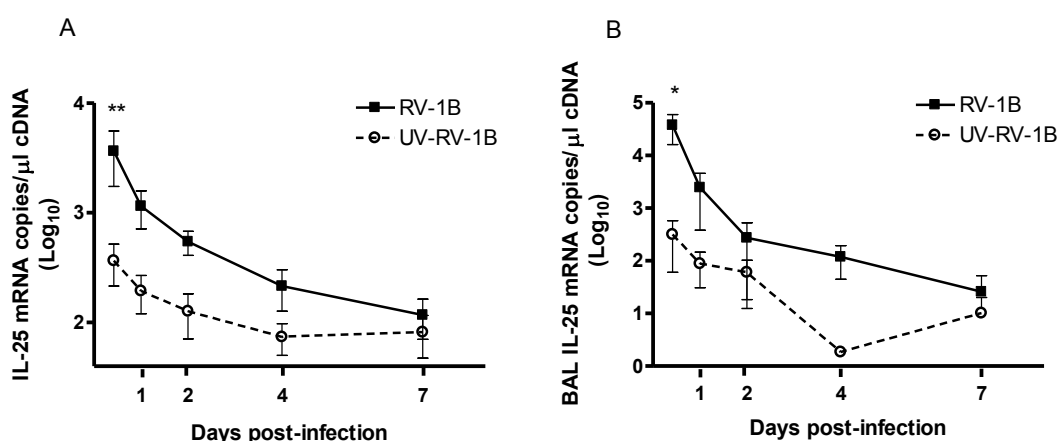


Figure 3.8 Measurement of IL-25 mRNA in the BAL cells and lung tissue from RV-1B-infected mice BALB/c mice were infected i.n with RV-1B or dosed with UV-inactivated virus (UV-RV-1B) as a negative control. BAL cells were harvested by flushing lungs before excising the lung tissue at the time-points indicated p.i. Lung tissue (A) and BAL cells (B) were processed by extracting RNA and synthesizing cDNA to measure IL-25 gene expression by qPCR. Data is expressed as the number of mRNA copies per μl of cDNA added per qPCR reaction. Data is representative of at least 3 experiments. Each group of mice (n=4-6) is represented as the mean (+/-SEM) while statistical significance is denoted for comparison between RV-1B and UV-RV-1B treatment at the indicated time-points p.i. (* $P<0.05$) (** $P<0.01$).

3.4.2.2 IL-25 protein expression

As IL-25 protein was undetectable in the BAL, processed lung homogenate from RV-1B-infected mice and UV-RV-1B-treated controls was used to quantify protein levels by ELISA while a PBS-treated control group was also included (baseline). RV-1B induced an initial sharp increase in IL-25 protein expression at d1 p.i. and from d2-d7 p.i. levels remained significantly elevated compared with UV-RV-1B-treated controls (d2 $P<0.05$; d4 $P<0.01$; d7 $P<0.01$) (Figure 3.9). Inactivated virus triggered IL-25 expression that was comparable to levels measured in mice at 10h and emulated expression patterns observed in infected mice. However, these amounts were on average ~2-fold lower in UV-RV-1B-treated groups compared with infected groups from d2-d7 p.i.

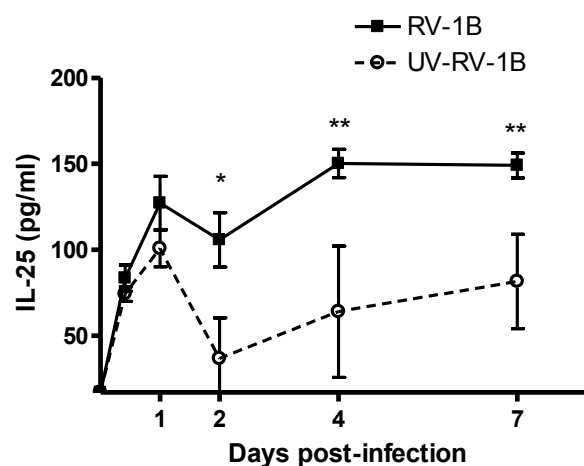


Figure 3.9 Measurement of IL-25 protein in lung tissue from RV-1B-infected mice BALB/c mice were infected i.n. with RV-1B or UV-activated virus (UV-RV-1B) or PBS (baseline) as a control. Lung tissue from mice was collected at the indicated time-points and used to measure IL-25 protein expression in processed lung homogenate by ELISA. Each group of mice (n=4-6) is represented as the mean (+/-SEM) while statistical significance is denoted for comparison between RV-1B and UV-RV-1B at the indicated time-points p.i. (* $P < 0.05$) (** $P<0.01$). Data is representative of at least 3 experiments.

3.4.2.3 Immunostaining for IL-25 protein expression

To further analyse IL-25 protein expression and identify cellular sources of IL-25 *in vivo*, immunostaining was carried out on paraffin-embedded lung tissue from RV-1B-infected mice and UV-RV-1B-treated controls at 10h, d1, d2 and d4 p.i. while a PBS-treated control

group was also included (baseline). IL-25 immunoreactivity was observed in the bronchial epithelium and in infiltrating inflammatory cells in the airway lamina propria of RV-1B-infected mice.

i.) Airway epithelial cells

Airway epithelium was semi-quantitatively scored by Dr Jie Zhu (Royal Brompton Hospital, Imperial College London) and IL-25 immunoreactivity was detected by brown staining (Figure 3.12 A-K). IL-25 protein expression in the bronchial epithelium of RV-1B-infected mice was significantly greater at 10h (RV-1B vs UV-RV-1B $P<0.05$) and d1 p.i. (RV-1B vs UV-RV-1B $P<0.01$) compared with treated controls (Figure 3.10). At d2 p.i., IL-25 immunoreactivity was reduced in RV-1B-infected mice and by d4 p.i., levels were similar to UV-RV-1B-treated controls. Epithelial IL-25 immunostaining was also observed in UV-RV-1B-treated controls; however significantly greater levels observed in RV-1B-infected mice early after infection indicated that IL-25 protein expression was predominantly virus replication dependant.

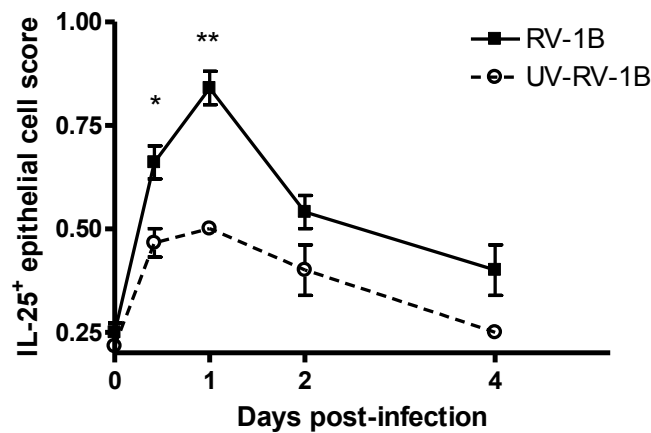


Figure 3.10 Measurement of IL-25+ epithelial cells in lung tissue from RV-1B-infected mice BALB/c mice were infected i.n. with RV-1B or UV-activated virus (UV-RV-1B) or PBS (baseline) as a control. Lungs were harvested at the indicated time-points and immunostained to quantify IL-25+ epithelial cells. Immunostaining intensity for IL-25 protein was scored semi-quantitatively between 0 to 1.5 (0 = negative; 0.5 =weak staining, 1 = moderate staining, and 1.5 =strong staining). All counts were performed by one investigator and slides were blinded to experimental treatments. Each group of mice (n=5) is represented as the mean (+/-SEM) while statistical significance is denoted for comparison between RV-1B and UV-RV-1B at the indicated time-points p.i. (* $P<0.05$) (** $P<0.01$)

ii.) Inflammatory cells

Infiltrating IL-25+ inflammatory cells were also quantified and expressed as the number of positive inflammatory cells per millimeter length of the reticular basement membrane (Figure 3.13 A-E). Significantly increased numbers of IL-25 immunoreactive cells were quantified in the airway lamina propria at d1 (RV-1B vs UV-RV-1B $P<0.01$; RV-1B) and d2 p.i. (RV-1B vs UV-RV-1B $P<0.001$) compared with treated controls (Figure 3.11). By d4 p.i., these levels were greatly reduced but still remained higher than levels in UV-RV-1B-treated mice. Although double staining IHC would be required to verify results, the majority of IL-25+ cells at d1 p.i. appeared to be macrophages and neutrophils while some IL-25+ lymphocytes were observed at d2 p.i. based on morphological identification.

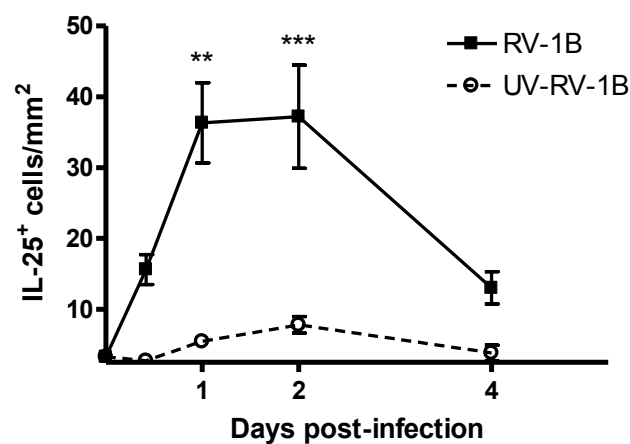
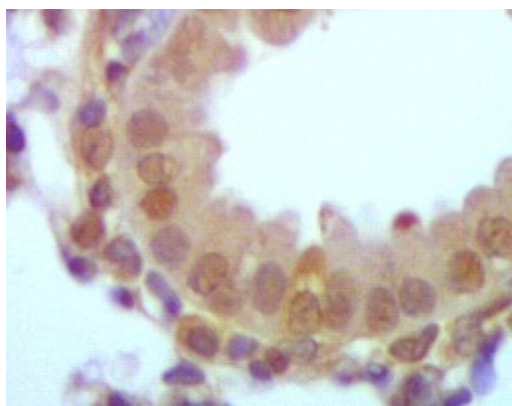
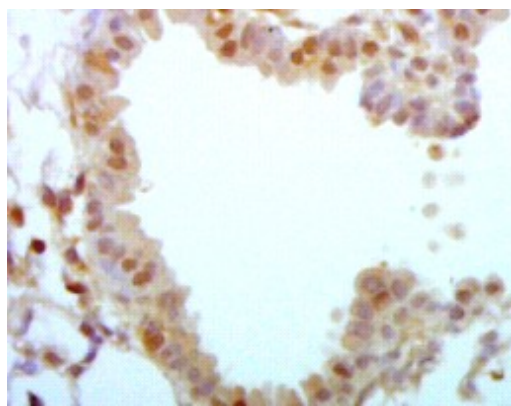


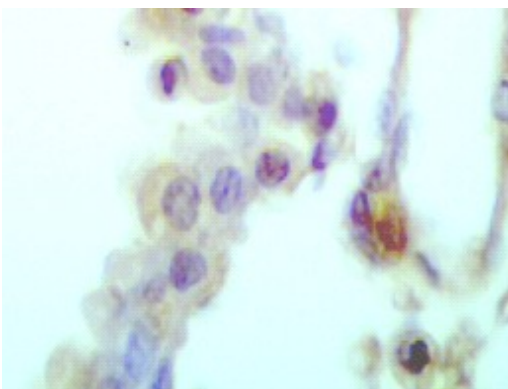
Figure 3.11 Measurement of IL-25+ inflammatory cells in lung tissue from RV-1B-infected mice BALB/c mice were infected i.n. with RV-1B or UV-activated virus (UV-RV-1B) or PBS (baseline) as a control. Lungs were harvested from mice at the indicated time-points and immunostained to quantify infiltrating IL-25+ inflammatory cells in the airway lamina propria. Data is expressed as the number of positive inflammatory cells per millimetre length of the reticular basement membrane. All counts on histology slides were performed by one investigator and slides were blinded to experimental treatments. Each group of mice (n=5) is represented as the mean (+/-SEM) while statistical significance is denoted for comparison between RV-1B and UV-RV-1B treatment at the indicated time-points p.i. (** $P<0.01$) (***) $P<0.001$)



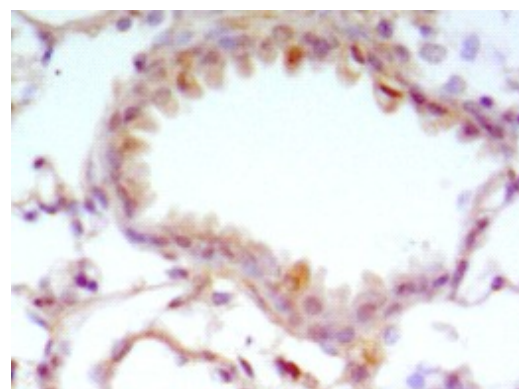
(A) 10hRV-1B p.i. (X40)



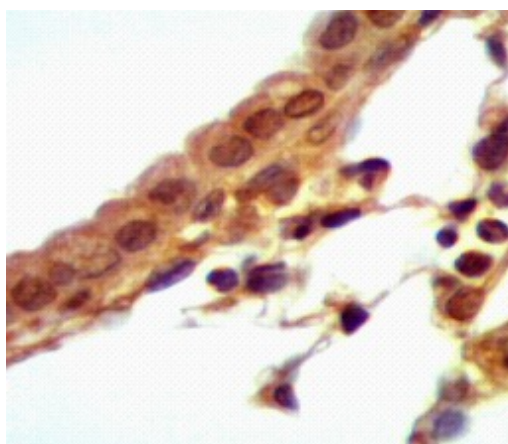
(B) 10h RV-1B p.i. (X20)



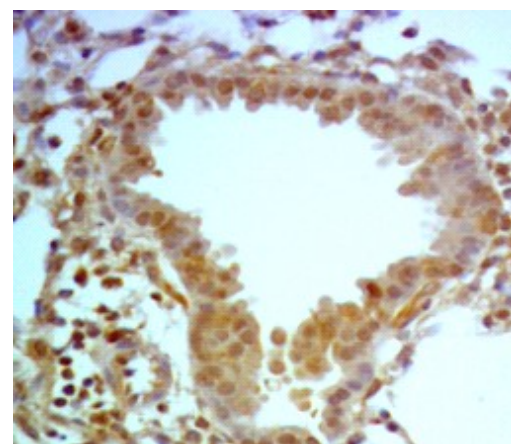
(C) 10h UV-RV-1B p.i. (X40)



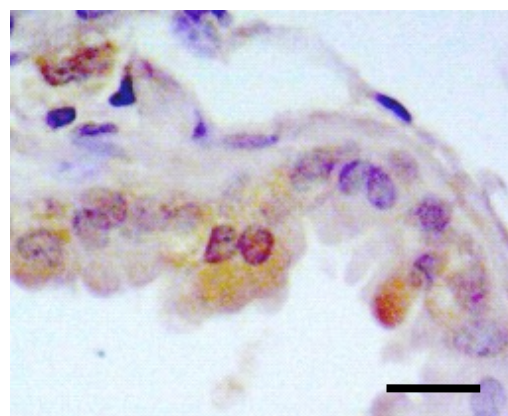
(D) 10h UV-RV-1B p.i. (X20)



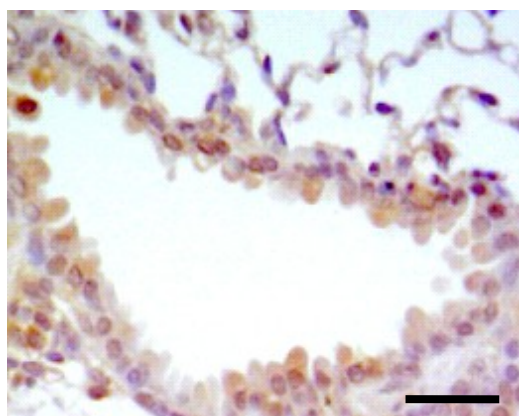
(E) d1 RV-1B p.i. (X40)



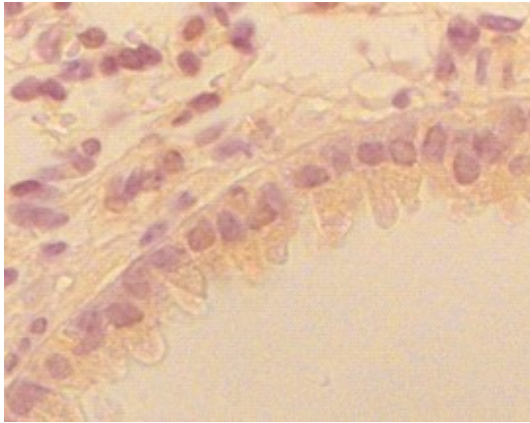
(F) d1 RV-1B p.i. (X20)



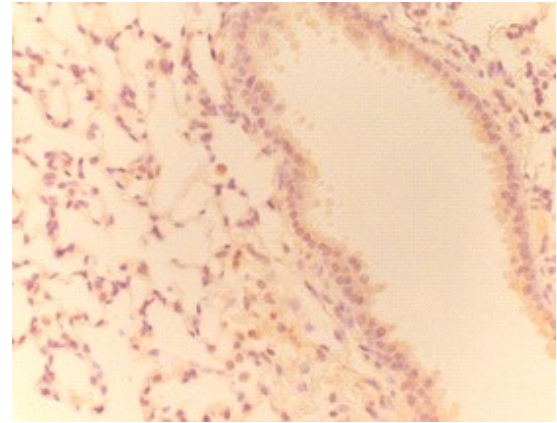
(G) d1 UV-RV-1B p.i. (X40)



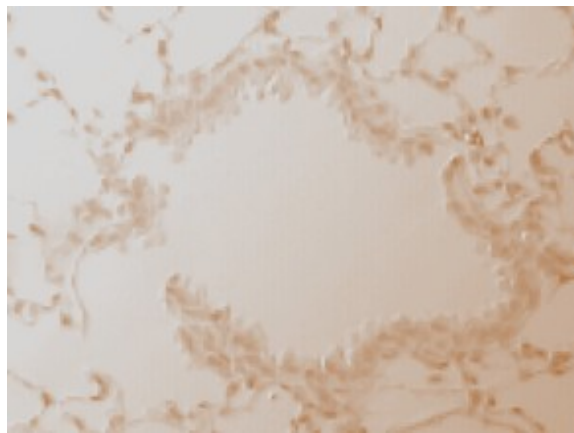
(H) d1 UV-RV-1B p.i. (X20)



(I) d1 PBS-mock (X40)



(J) d1 PBS-mock (X20)



(K) Isotype control (X20)

Figure 3.12 Representative immunohistochemistry staining for IL-25 in the lung epithelium in tissue from RV-1B-infected, UV-RV-1B and PBS-treated mice BALB/c mice were infected i.n. with RV-1B or UV-activated virus (UV-RV-1B) and PBS (baseline) as negative controls. IL-25 immunoreactivity is represented by brown staining in RV-1B-infected mice at 10h (A-B) and d1 p.i. (E-F) and in UV-RV-1B-treated (C-D; G-H) and PBS-treated controls (I-J) at a magnification of X20 (scale bar=150 μ m) and X40 (scale bar=75 μ m). An isotype control (K) at a magnification of X20 is also represented.

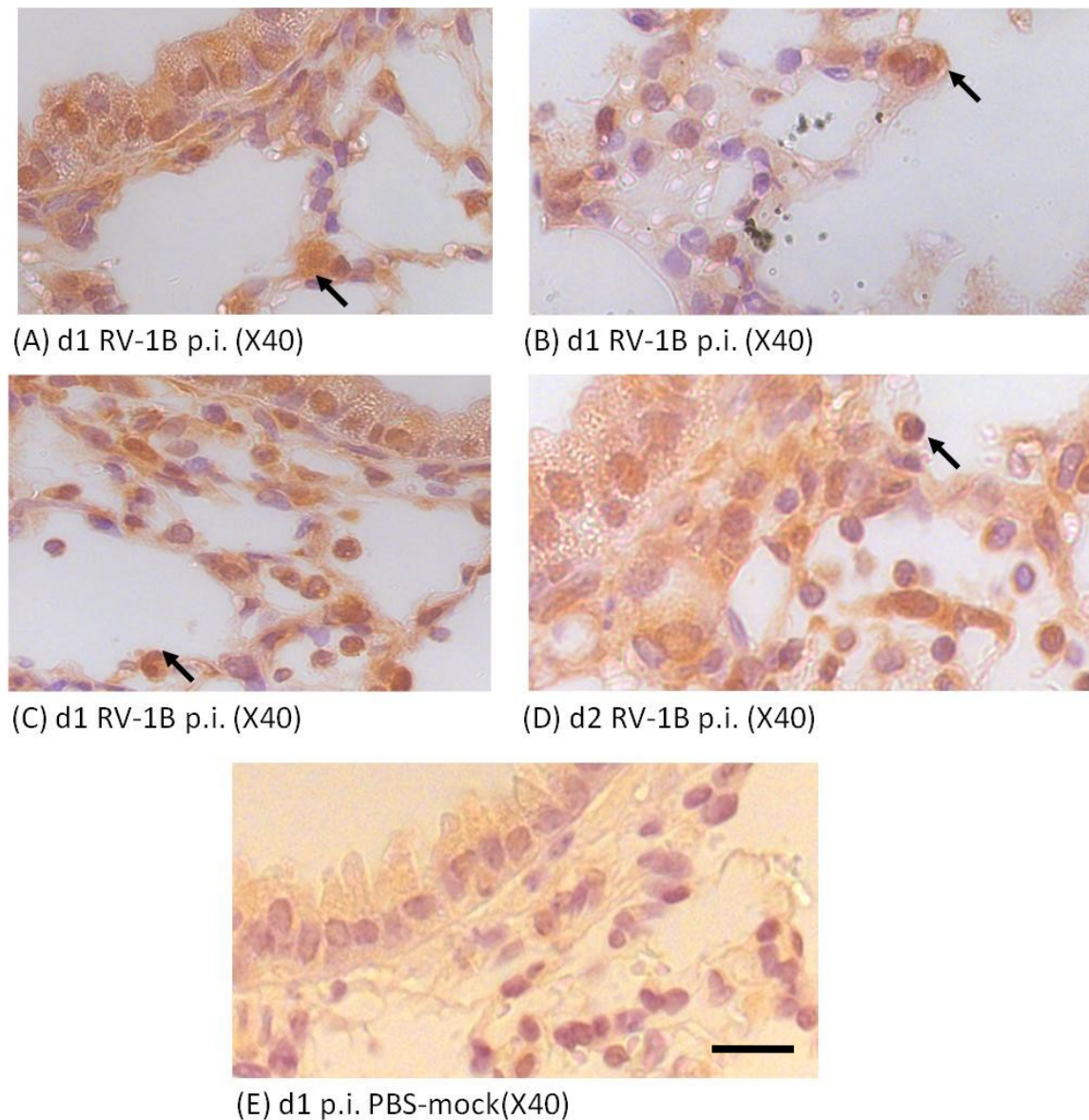


Figure 3.13 Representative immunohistochemistry staining for IL-25+ inflammatory cells Black arrows point to IL-25+ macrophages (A and B) and neutrophils (C) in RV-1B-infected mice at d1 p.i. as well as IL-25+ lymphocytes (D) at d2 p.i. which were identified based on morphology. PBS-mock controls (E) are also represented. All slides are represented at a magnification of X40 (scale bar=75 μ m).

3.4.3 Quantification of Th2 cytokines

Due to fact that RV induced IL-25, the classical Th2-associated cytokines, IL-4, IL-5 and IL-13 were also measured in BAL cells and lung tissue from RV-1B-infected mice and UV-RV-1B-treated controls. All measured cytokines were undetectable in all treated groups (results not shown).

3.5 Summary

In part one of this chapter, we reported that RV-1B infection of the human bronchial epithelial cell line, BEAS-2B, induced IL-25 gene expression; however, gene induction was not detected in other investigated cell lines or commercially purchased primary HBECs. Furthermore, IL-25 protein in cell supernatants from RV-1B-infected BEAS-2B cells was undetectable as measured by ELISA. In contrast, IL-25 mRNA and protein was detected in the majority of RV-1B-infected and media-treated HBECs from AAs and NANAs. Moreover, RV-1B induced significantly higher IL-25 gene expression at 8h p.i. and protein at 24h p.i. in cells from AA patients compared with media-treated HBECs from healthy controls. These findings are novel and indicate intrinsic differences in the bronchial epithelium of asthmatics compared with healthy subjects. Furthermore, enhanced IL-25 protein expression was associated with asthma-associated outcomes; enhanced protein levels at 24h p.i. significantly correlated with sensitisation to a greater number of common environmental allergens as indicated by a positive wheal and flare responses in AA patients. Furthermore, an association between cells from AA patients producing more RV-induced IL-25 protein and increased numbers of recorded asthma exacerbations per year in subjects was also evident.

In part two of this chapter, we demonstrated IL-25 induction in response to RV infection *in vivo* and confirmed that the release of mediators in BALB/c mice were characteristic of experimental RV infections in patients and *in vitro* studies of HBECs^{40,205}. These read-outs included an early robust neutrophilic response followed by lymphocytic inflammation as well as strong induction of the mouse CXCR2-specific chemokine, CXCL1/KC, the proinflammatory cytokine, IL-6 and CCL5/RANTES in the airways of RV-infected mice. IL-25 induction was initially confirmed by a significant early increase in IL-25 gene expression in the lung and airways of RV-infected mice. Although IL-25 protein was undetectable in the BAL, increased protein expression in the lung tissue of infected mice was confirmed by ELISA and IHC results. Immunostaining confirmed findings from part 1 demonstrating that the bronchial epithelium is a major source of early RV-induced IL-25 expression. Macrophages, neutrophils and lymphocytes were also identified as potential IL-25 producers in the airway lamina propria.

In summary, *in vitro* results indicated that HBECs from asthmatics possess a greater propensity to produce IL-25 than cells from non-atopic non-asthmatic individuals. Based on

the ability of IL-25 to augment an allergic response, this induction may represent a mechanism of exacerbated RV-induced allergic inflammation in the asthmatic lung. *In vivo* results demonstrated that IL-25 was upregulated in RV-1B-infected mice; however it was apparent from the absence of Th2 inflammation (as demonstrated by undetectable IL-4, IL-5 and IL-13 and the absence of airway eosinophilia) that enhanced IL-25 alone was not sufficient to initiate an allergic phenotype. In the next chapter we sought to investigate the effects of RV infection and allergen on IL-25 production in a mouse model of RV-induced allergic airways disease with the ultimate aim of assessing if IL-25 plays a role in exacerbation of Th2 allergic disease.

Chapter 4: Results

IL-25 induction in response to allergic and viral interactions in a model of RV-induced allergic airways disease

4.1 Introduction

Epidemiological and experimental studies have demonstrated an interaction between allergen exposure, RV infection and the risk of exacerbations of asthma^{92,95}. Notably, two seminal clinical studies showed that the combination of allergic sensitisation, exposure to high levels of allergen and viral infection, including RV, was strongly associated with the risk of hospital admittance with acute asthma in both children and adults^{92,95}. While Th2-mediated inflammation is clearly implicated in the asthmatic response, it is unknown how the immune response to RV infection interacts with Th2 immunity to cause an asthma exacerbation.

To elucidate potential mechanisms of interplay between allergen exposure and RV infection in a sensitised system, a mouse model of RV-induced allergic airways disease was developed in our laboratory²⁰². Using this model, we aimed to test the hypothesis that a Th2 pulmonary environment in conjunction with virus synergistically enhances IL-25.

The upregulation of IL-25 in response to allergen alone has been reported in several *in vivo* and *in vitro* studies while clinical findings have shown an increase in IL-25 mRNA and its receptor in tissue from chronic asthmatics^{70,127,169,170}. However, the induction of IL-25 by RV, with or without concomitant exposure to allergen, has not been investigated.

Studies in mouse models of allergic airways inflammation have demonstrated that IL-25 has the potential to act directly on both innate and adaptive Th2 cytokine-producing cells in the lung^{76,169,187}. Therefore, based on this evidence we postulated that RV-induced IL-25 augments allergic inflammation via activation of Th2-associated cells which are recruited to the mouse lung by allergen sensitisation and challenge, thus representing a link between antiviral responses and Th2 driven inflammation. For these reasons, IL-25 and Th2-related innate and adaptive responses were investigated in our model of RV-induced allergic airways disease by assessing various time-points after infection including early (10h p.i.) and later (d7 p.i.) events.

4.2 Hypotheses and Aims

4.2.1 Hypotheses

- i.) RV infection in an allergen sensitised and challenged mouse enhances IL-25 expression compared with either allergen exposure or viral infection alone
- ii.) RV infection increases allergen driven Th2-associated responses potentially regulated by IL-25

4.2.2 Aims

To investigate the effects of allergen and RV on IL-25 expression as well as characterise RV-induced enhancement of Th2 inflammation potentially regulated by IL-25 using a mouse model of RV-induced allergic airways disease

- i.) To determine the magnitude and kinetics of IL-25 in the mouse lung by qPCR to measure gene expression and ELISA to detect protein
- ii.) To measure potential IL-25-regulated Th2 outcomes in mice including:
 - AHR using whole-body and double-chamber plethysmography
 - Viral load in the lung tissue using qPCR
 - Inflammatory airway leukocyte infiltration in the BAL via differential cell counts
 - Th2-associated cytokines and chemokines in the BAL and lung tissue by ELISA
 - Respiratory mucins including MUC5ac and MUC5b in the BAL by ELISA
 - Total and allergen-specific IgE in mouse serum by ELISA

4.3 Results

In this chapter, a mouse model of RV-induced allergic airways disease was employed and treatment groups, as previously described in section 2.2.4 are summarised in the table below for clarification.

Experimental groups	Sensitisation (OVA/Alum i.p.)	Challenge (OVA i.n)	RV-1B infection (RV-1B i.n./UV-RV-1B control)
RV OVA	Yes	Yes	Yes
UV OVA	Yes	Yes	No
RV PBS	Yes	No	Yes
UV PBS	Yes	No	No

Table 4.1 Treatment regime and nomenclature of groups in a model of RV-induced allergic airways disease

4.3.1 Airway responses

We employed whole-body (as measured by enhanced pause [Penh]) and double-chamber (as measured by specific airway resistance [SRaw]) plethysmography to assess the exacerbating effects of RV infection in OVA-sensitised and challenged mice on AHR. At d1 p.i./allergen challenge, RV OVA mice showed enhancement in airway obstruction as assessed by Penh (Figure 4.2 A). Specifically, at a dose of 30mg/ml of methacholine, Penh was significantly greater in RV OVA-treated mice compared with UV PBS controls ($P < 0.05$) while at 100mg/ml RV OVA mice displayed significantly greater Penh compared with PBS-challenged groups (RV OVA vs RV PBS $P < 0.05$; RV OVA vs UV PBS $P < 0.001$). There was a trend towards increased Penh in RV OVA groups vs UV OVA groups however this was not statistically significant. Whole-body plethysmography data was confirmed by measurement of specific airway resistance (SRaw) using double-chamber plethysmography, which demonstrated increased AHR in RV OVA mice compared with UV PBS controls ($P < 0.05$) at a dose of 20mg/ml of methacholine (Figure 4.2 B). Furthermore, RV OVA mice displayed significantly greater AHR compared with UV OVA mice, indicating that RV infection exacerbated allergen-induced allergic airways disease at a dose of 20mg/ml of methacholine.

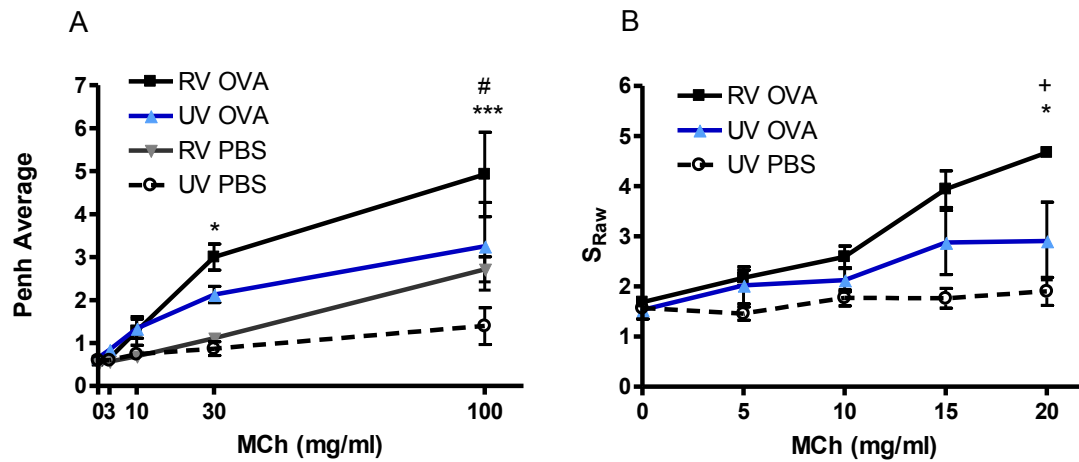


Figure 4.2 Airway responses in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. At d1 p.i./final allergen challenge, Penh (A) and SRaw (B) were measured in response to increasing doses (in mg/ml) of aerosolised methacholine (MCh). Penh and SRaw were measured over 5 minute periods after 1 minute aerosolisation challenge to methacholine. For both plethysmograph experiments, aerosolised water was used to determine baseline values. Data is represented as the mean (+/-SEM) of 5 mice per treatment and is representative of at least 2 independent experiments (Penh data) or one experiment (sRaw data). + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (*/#/+P < 0.05; *** P<0.001)

4.3.2 RV-1B viral load

To assess if enhanced AHR observed in RV OVA mice was associated with increase viral load, RV RNA copies were quantified by qPCR in the lung tissue of all treated groups of mice to compare viral load in the presence or absence of allergen challenge.

RV-1B viral RNA was significantly increased at 10h p.i. in RV OVA mice compared with all other treated groups (RV OVA vs UV OVA P<0.001; RV OVA vs RV PBS P<0.001; RV OVA vs UV PBS P<0.001) (Figure 4.3). By d1 p.i., levels of viral RNA copies had decreased sharply in RV OVA mice and by d2 p.i. levels were comparable to viral RNA in RV PBS-treated mice which displayed a peak in viral load at this time-point. Thus, OVA challenge significantly enhanced early viral load in RV OVA mice compared with RV infection alone.

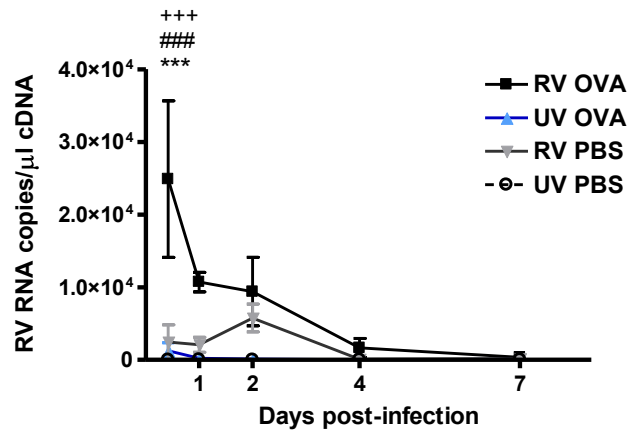


Figure 4.3 Viral load in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. Lungs were harvested at 10h and days 1, 2, 4 and 7 p.i. and processed by extracting RNA and synthesizing cDNA to quantify viral RNA copies via qPCR. Data is expressed as the number of RNA copies per μl of cDNA added per qPCR reaction. Each group of mice ($n=5$) is represented as the mean ($\pm$ SEM) and data is representative of at least 2 experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (+++/###/*** $P < 0.001$)

4.3.3 Airway leukocyte infiltration

In human experimental infection models, RV infection has been reported to increase recruitment of neutrophils, lymphocytes and eosinophils in the asthmatic lung⁹³. In accordance with this data, RV OVA mice displayed an early increase in neutrophilia and later enhanced eosinophilia as characterised by differential staining of BAL cell cytopins. Neutrophilia at d1 p.i. in RV OVA mice was approximately 5 fold higher compared with RV PBS mice (RV OVA vs RV PBS $P < 0.001$) and remained significantly elevated until d2 p.i. compared with all other groups (RV OVA vs UV OVA $P < 0.001$; RV OVA vs RV PBS $P < 0.001$; RV OVA vs UV PBS $P < 0.001$) (Figure 4.4 A). Eosinophilia was observed in OVA-challenged mice exclusively with RV OVA mice displaying the greatest levels at d4 and d7 p.i (Figure 4.4 B). A prolonged increase in lymphocytes from d2 to d7 p.i. was observed in RV OVA mice which were significantly increased compared with RV PBS and UV PBS mice but not compared with UV OVA groups (Figure 4.4 C). This kinetic analysis of BAL cells is consistent with previously

published data of this model indicating enhancement of allergic airways inflammation by RV infection²⁰².

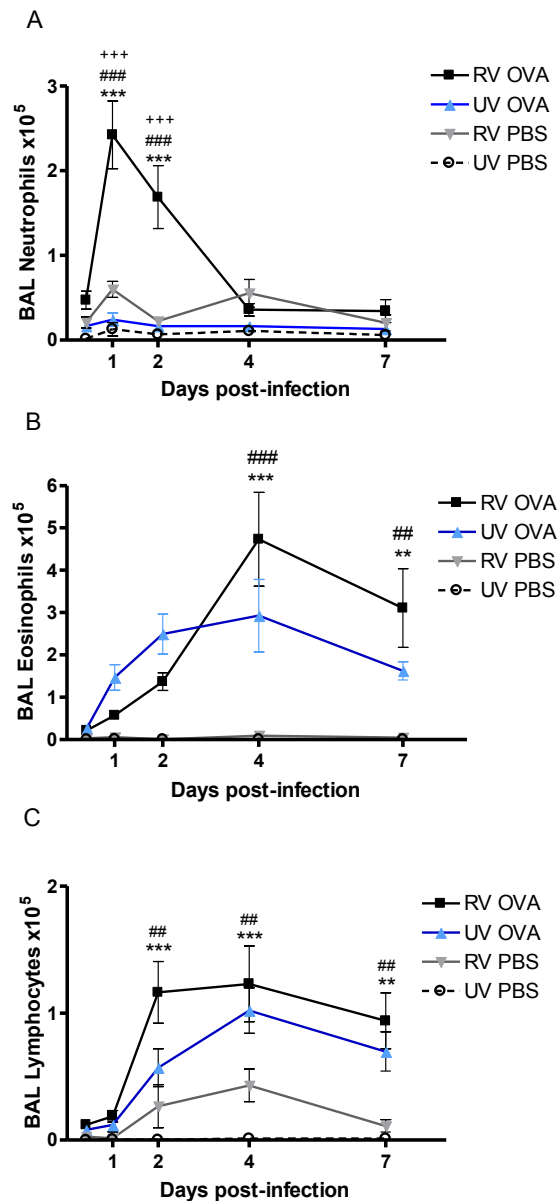


Figure 4.4 Airway leukocyte infiltration in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. BAL cells from mice were fixed to cytopsin slides and stained to quantify total numbers of neutrophils (A), eosinophils (B) and lymphocytes (C) at the indicated time-points p.i. All slides were blinded to experimental treatments. Each group of mice (n=5) is represented as the mean (+/-SEM) and data is representative of at least 2 experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (##/** P<0.01; +++/###/** P < 0.001)

4.3.4 BAL chemokines

Given that eosinophil and Th2 lymphocyte recruitment and activation are associated with asthma pathogenesis, the chemokines CCL5/RANTES, CCL11/Eotaxin 1, CCL24/Eotaxin 2 and CCL17/TARC were measured in the BAL of all treated mice by ELISA.

CCL5/RANTES, which has been demonstrated to be upregulated in virus-induced asthma exacerbation in humans, is a potent chemoattractant for eosinophils as well as memory T cells²⁰⁶. Here, we showed that RV infection significantly induced early CCL5/RANTES expression in OVA-challenged mice (RV OVA vs UV OVA $P<0.01$, RV OVA vs RV PBS $P<0.05$, RV OVA vs UV PBS $P<0.01$) which persisted to d1 p.i. (RV OVA vs UV OVA $P<0.05$, RV OVA vs UV PBS $P<0.01$) (Figure 4.5 A). This was consistent with enhanced lymphocytosis and eosinophilia observed in RV OVA mice as illustrated in Figure 4.4. Similarly, the eosinophil chemoattractants, CCL11/Eotaxin 1 and CCL24/Eotaxin 2, were significantly upregulated in RV OVA mice with an early increase in CCL11/Eotaxin 1 at 10h p.i. (RV OVA vs UV OVA $P<0.05$, RV OVA vs UV PBS $P<0.05$) (Figure 4.5 C) and a comparatively delayed peak of CCL24/Eotaxin 2 protein at d1 p.i. (RV OVA vs UV OVA $P<0.05$, RV OVA vs RV PBS $P<0.001$, RV OVA vs UV PBS $P<0.001$) (Figure 4.5 D). Both groups of OVA-challenged mice displayed late enhancement of the Th2 cell chemoattractant, CCL17/TARC, at d7 p.i. and while levels were higher in RV OVA mice, these quantities were not significantly different (Figure 4.5 B).

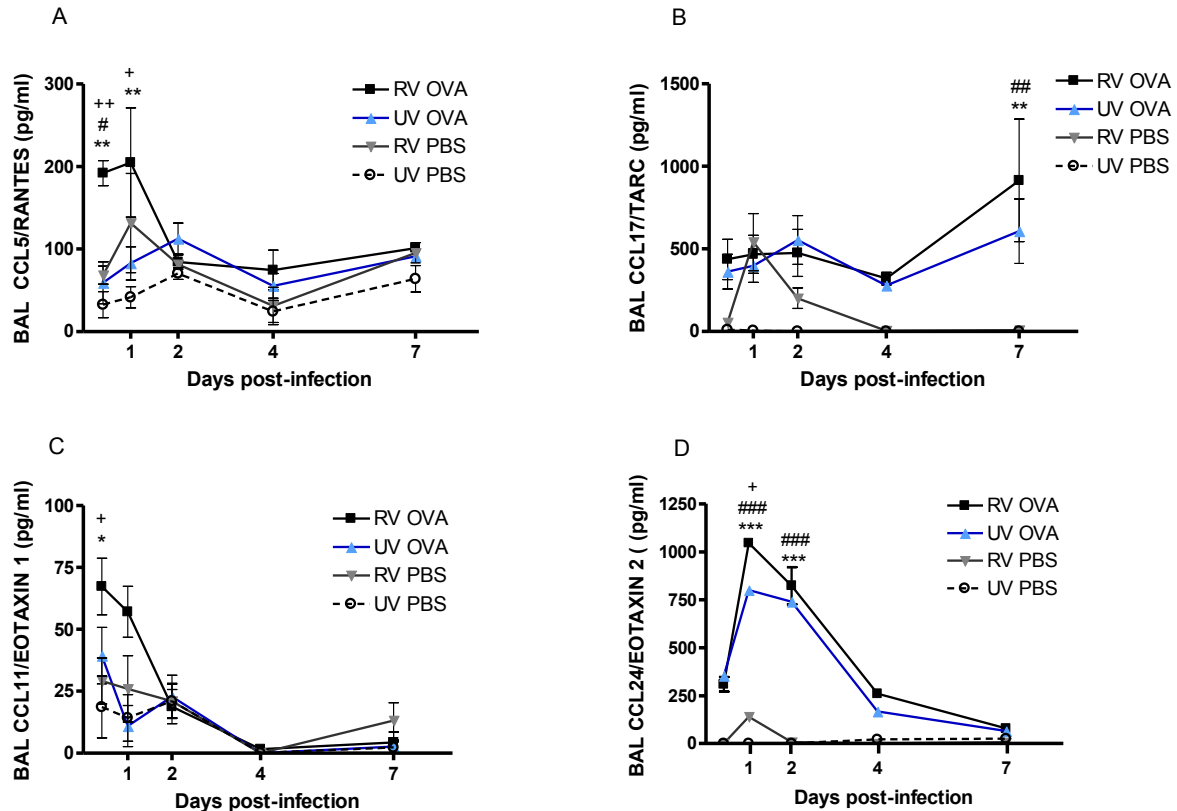


Figure 4.5 BAL chemokines in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. BAL from mice was collected at the indicated time-points and BAL supernatant was used to quantify CCL5/RANTES (A), TARC/CCL17 (B), CCL11/Eotaxin1 (C) and CCL24/Eotaxin2 (D) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of at least 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (+/#/* $P < 0.05$; ++/##/** $P < 0.01$; +++/###/*** $P < 0.001$)

4.3.5 BAL IL-6

Increases in the proinflammatory cytokine IL-6 have been reported in atopic asthmatics experimentally infected with RV which remained elevated during convalescence²⁰⁵. We measured BAL IL-6 in our mouse model of RV-induced allergic airways disease by ELISA and observed an approximate 6-fold increase in RV OVA mice compared with UV OVA mice at 10h p.i. (RV OVA vs UV OVA $P < 0.001$, RV OVA vs RV PBS $P < 0.001$, RV OVA vs UV PBS $P < 0.001$) (Figure 4.6). By d1 p.i., these levels had decreased but still remained significantly

elevated compared with all treated groups (RV OVA vs UV OVA $P < 0.001$, RV OVA vs RV PBS $P < 0.001$, RV OVA vs UV PBS $P < 0.001$).

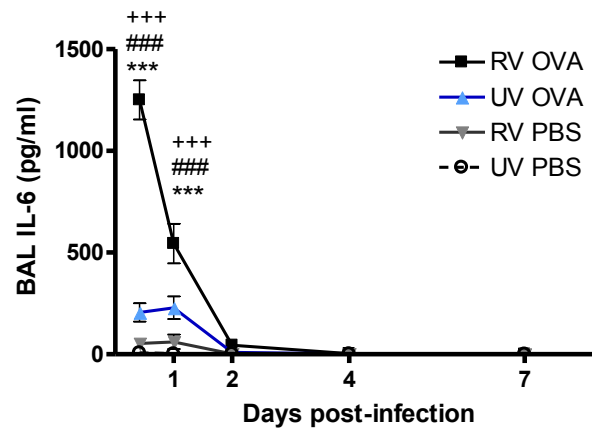


Figure 4.6 BAL IL-6 in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. BAL from mice was collected at the indicated time-points and BAL supernatant was used to quantify IL-6 by ELISA. Data is represented as the mean ($\pm$ SEM) of 5 mice per group and is representative of at least 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; * RV OVA vs UV PBS (+++/###/*** $P < 0.001$)

4.3.6 Th2 cytokine expression

It is well established that asthma pathogenesis is largely mediated by the Th2 cytokines, IL-4, IL-5 and IL-13²⁰⁷⁻²⁰⁹. Previously published data from our mouse model demonstrated significantly greater levels of IL-4 and IL-13 at d1 p.i. in the BAL of RV OVA mice compared with OVA-challenged mice alone²⁰². Given the potential importance of IL-25-regulated Th2 cytokines in the pathogenesis of RV-induced asthma exacerbations, we undertook a thorough investigation of Th2 cytokine production in the BAL and lung homogenate by ELISA and included an earlier time-point at 10h p.i. which has not previously been studied.

4.3.6.1 BAL Th2 cytokines

BAL Th2 cytokines were detectable only in allergen-sensitised and challenged mice. The combination of RV infection and OVA challenge induced significantly higher levels of BAL IL-4 (RV OVA vs UV OVA $P < 0.001$; RV OVA vs RV PBS $P < 0.001$; RV OVA vs UV PBS $P < 0.001$) (Figure 4.7 A) and IL-13 (RV OVA vs UV OVA $P < 0.05$; RV OVA vs RV PBS $P < 0.001$; RV OVA vs UV PBS $P < 0.001$) (Figure 4.7 B) early on (10h p.i.). Furthermore, RV OVA mice produced approximately two-fold more IL-4 protein in the airways than OVA-challenged mice alone. By d1 p.i. quantities of IL-4 and IL-13 in RV OVA mice were comparable to OVA-challenged mice alone and by d4 and d7 p.i. levels in both groups were either low or undetectable. Concentrations of BAL IL-5 were comparable in both OVA-challenged groups at 10h p.i. while a later peak in RV OVA mice was observed at d1 p.i. (Figure 4.7 C). These levels were significantly increased compared with RV-infected (RV OVA vs RV PBS $P < 0.001$) and allergen-challenged groups (RV OVA vs UV OVA $P < 0.01$). This data confirmed that RV infection enhances early Th2 production in the airways of allergen-challenged mice and identified 10h p.i. as a key time-point for analysis of these increases.

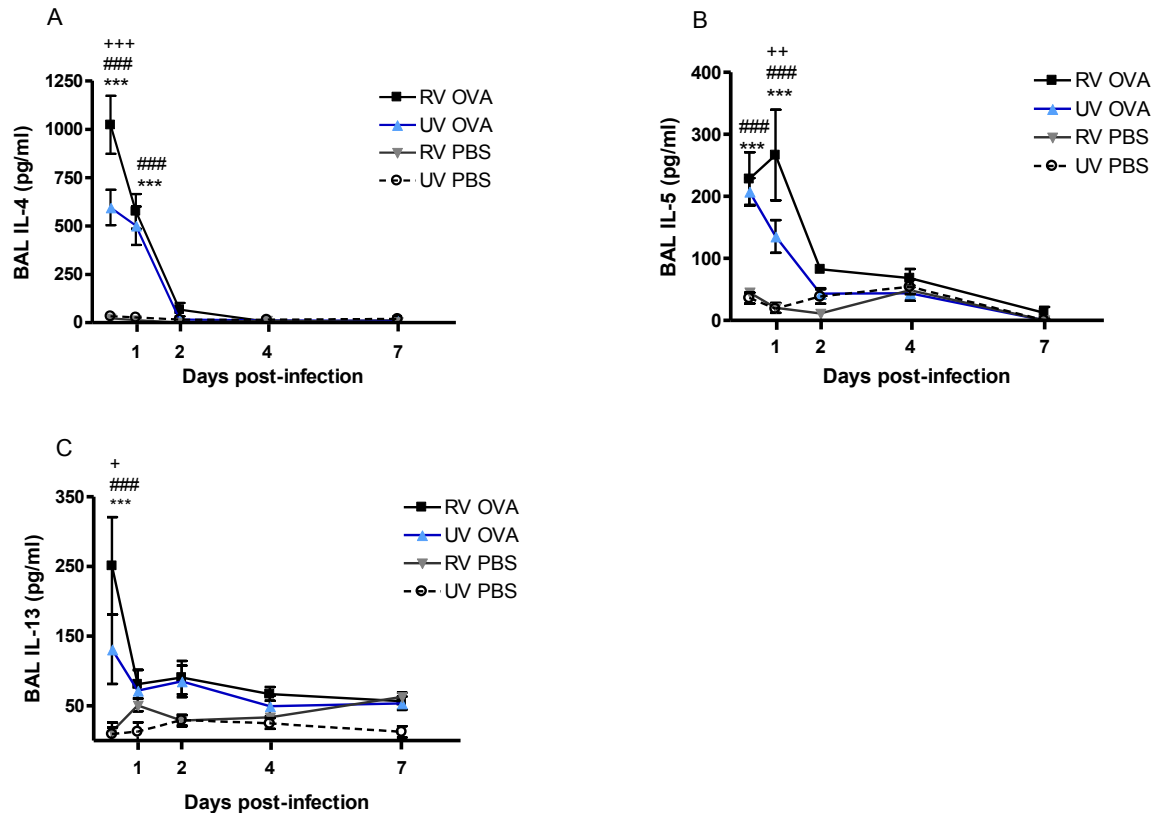


Figure 4.7 BAL Th2 cytokines in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. BAL from mice was collected at the indicated time-points and BAL supernatant was used to measure IL-4 (A), IL-5 (B) and IL-13 (C) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of at least 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (+ P<0.05; ++ P<0.01; +++/###/*** P < 0.001)

4.3.6.2 Th2 cytokines in lung homogenate

To assess if Th2 cytokine profiles in the BAL reflected those in the lung parenchyma as well as gain a thorough profile of the magnitude and kinetics of expression, Th2 cytokines were also measured in lung homogenate by ELISA. Results indicated varied temporal peaks in cytokine expression in lung tissue compared with those observed in the BAL. Specifically, delayed peaks at d4 p.i. in both OVA-challenged groups were detected for all cytokines (Figure 4.8). Although levels were generally higher in RV OVA mice at d4 p.i., only IL-5 amounts reached significance in RV OVA groups compared to UV OVA groups (RV OVA vs UV

OVA $P < 0.05$) (Figure 4.8 B) . In addition, an earlier lower peak was observed at d1 p.i. in RV-infected and OVA-challenged mice that was absent in OVA-challenged mice alone. At this time-point, RV infection significantly enhanced IL-4 (RV OVA vs UV OVA $P < 0.01$) (Figure 4.8 A), IL-5 (RV OVA vs UV OVA $P < 0.01$) and IL-13 (RV OVA vs UV OVA $P < 0.05$) (Figure 4.8 C) expression in RV-infected OVA-challenged mice compared with OVA-challenged mice alone.

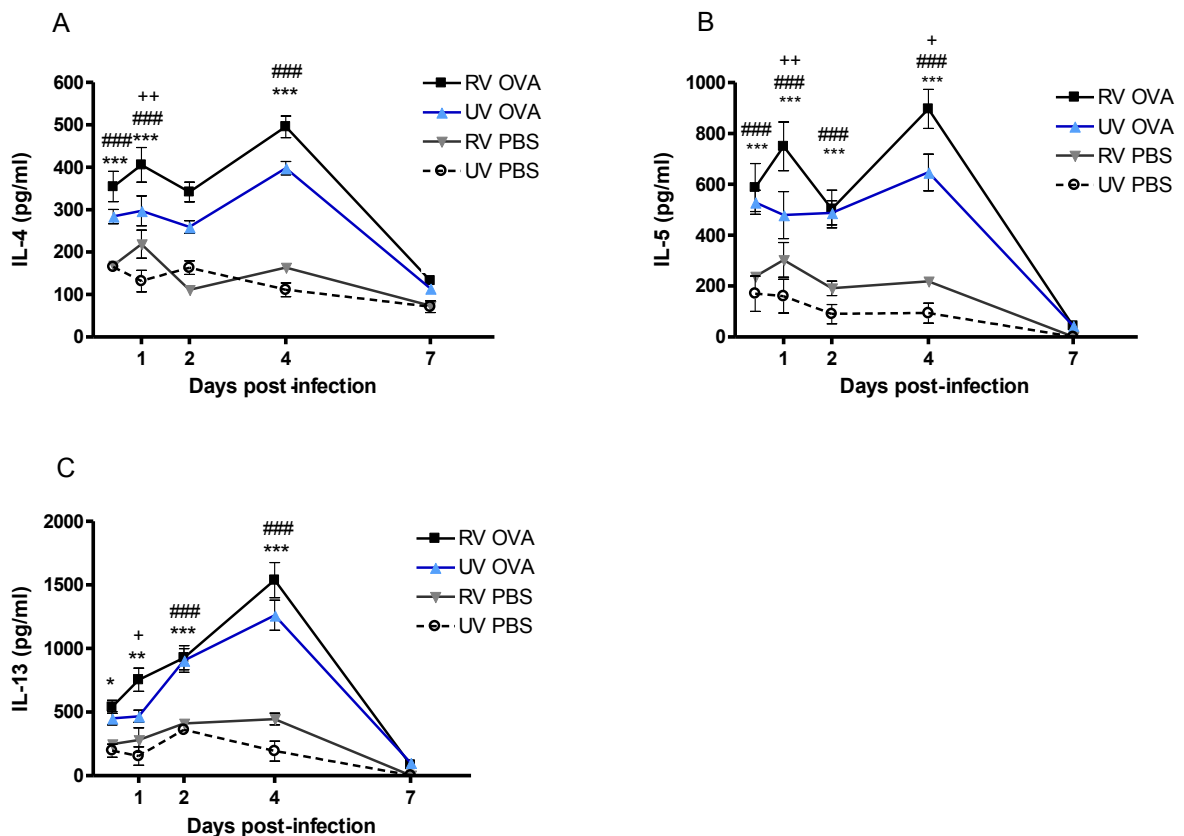


Figure 4.8 Th2 cytokines in lung tissue in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. Lung tissue from mice was collected at the indicated time-points and processed lung homogenate was used to measure IL-4 (A), IL-5 (B) and IL-13 (C) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of at least 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (+/* P<0.05; +/* P<0.01; +++/###/*** P < 0.001)

4.3.7 IL-25 expression

Enhanced IL-25 expression in response to allergen exposure *in vivo* and *in vitro* has been reported in several studies and clinical findings have shown an increase in both IL-25 and its receptor mRNA in asthmatic tissue^{70,127,170}. Findings from chapter 3 demonstrate increased IL-25 expression in RV-infected asthmatic cells compared with cells from healthy individuals suggesting an interaction between allergic inflammation and infection leading to increased IL-25 in asthma. To investigate the interaction between allergen-driven Th2 immunity and RV infection *in vivo*, IL-25 expression was measured in a mouse model of RV-induced allergic airways disease.

IL-25 gene expression was quantified by qPCR in lung tissue while protein was detected in lung homogenate by ELISA as IL-25 was undetectable in the BAL. RV infection induced significantly higher levels of IL-25 mRNA in OVA-challenged mice early on (10h p.i.) compared with all other treated groups (RV OVA vs UV OVA $P<0.05$; RV OVA vs RV PBS $P<0.05$; RV OVA vs UV PBS $P<0.05$) (Figure 4.9 A). By d1 and d2 p.i., mRNA levels in RV OVA mice had decreased and were comparable to UV OVA mice. IL-25 levels peaked in both UV OVA and RV PBS mice at d2 p.i. with ~10-fold more IL-25 expressed in the lungs of UV OVA mice compared with infection alone.

IL-25 protein displayed a similar expression pattern as Th2 cytokines measured in mouse lung homogenate (Figure 4.8). Specifically, an early peak in IL-25 expression was observed exclusively in RV OVA mice at d1 p.i. (RV OVA vs UV OVA $P<0.05$; RV OVA vs UV PBS $P<0.01$) (Figure 4.9 B). This was followed by a second larger peak at d4 p.i. which was detected in both OVA-challenged groups, although levels were moderately higher in RV OVA mice.

Consistent with results from chapter 3 (Figure 3.9), RV infection alone induced IL-25 protein which were comparable to levels measured in UV OVA mice. However, unlike sustained levels observed in our model of RV infection which employed a higher viral titre, levels had decreased to baseline by d7 p.i. in RV PBS mice suggesting that the lower titre used in this model may result in decreased, varied kinetics expression.

In summary, we observed upregulation of IL-25 mRNA and protein in OVA-challenged mice further demonstrating that RV infection in OVA-challenged mice enhanced allergen-driven IL-25 production.

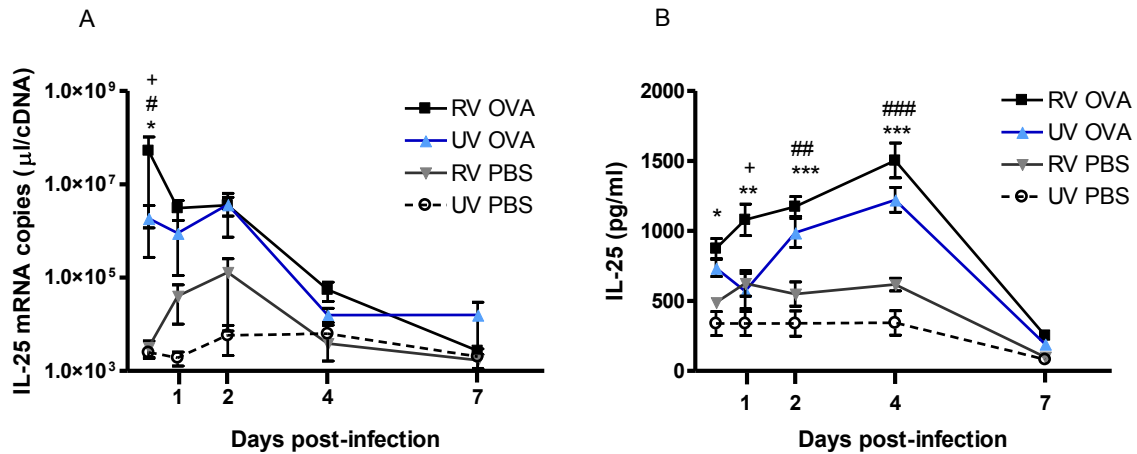


Figure 4.9 IL-25 gene and protein expression in a model of RV-induced allergic airways disease

OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected UV-inactivated virus. Lung tissue from mice was collected at the indicated time-points and used to measure IL-25 mRNA (A) and protein expression (B). For measurement of IL-25 gene, RNA was extracted and cDNA synthesized by qPCR. Data is expressed as the number of mRNA copies per μ l of cDNA added per qPCR reaction. IL-25 protein was detected in processed lung homogenate by ELISA. Data is represented as the mean ($\pm$ -SEM) of 5 mice per group and is representative of at least 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (+/#/* $P < 0.05$; ##/** $P < 0.01$; ###/*** $P < 0.001$)

4.3.8 IL-33 and TSLP expression

Unpublished data from our laboratory indicates that IL-33 is significantly upregulated in atopic asthmatic patients experimentally infected with RV compared with healthy controls (Jackson, D. *et al*). In addition, asthmatic epithelial cells have been reported to generate increased amounts of the pro-Th2 cytokine, TSLP, in response to RV infection which was enhanced by supplementing IL-4 in the media *in vitro*²¹⁰. Based on the fact that IL-25, IL-33 and TSLP are all epithelial-derived and have been reported to interact predominantly in the innate Th2 response, we aimed to measure IL-33 and TSLP in our model of RV-induced allergic airways disease.

Like IL-25, IL-33 and TSLP protein was undetectable in the BAL, therefore levels were measured in lung homogenate by ELISA. Unlike Th2 cytokines measured in lung tissue above (Figures 4.8 and 4.9 B), IL-33 levels peaked in RV OVA mice at d1 p.i. with no subsequent larger peak at d4 p.i. (Figure 4.10 A). Though IL-33 levels were significantly higher in RV OVA

groups compared with both PBS-challenged groups at 10h (RV OVA vs RV PBS $P<0.01$; RV OVA vs UV PBS $P<0.001$) and d1 p.i. (RV OVA vs RV PBS $P<0.001$; RV OVA vs UV PBS $P<0.001$), no significant differences were observed between OVA-challenged groups. Interestingly, levels measured in OVA-challenged mice were in excess of $\sim 9000\text{pg/ml}$ at d1 p.i., an amount ~ 6 -fold higher than other Th2 cytokine quantities in the lung homogenate (Figures 4.8 and 4.9 B).

In contrast to results from Th2 cytokines measured thus far, TSLP protein expression appeared to be largely allergen-driven as indicated by increased levels in UV OVA mice at 10h-d4 p.i (Figure 4.10 B). At d7 p.i., RV infection induced a delayed peak in expression in OVA-challenged mice and though these levels were significant compared with UV PBS groups ($P<0.01$), levels between OVA-challenge mice were not significantly different.

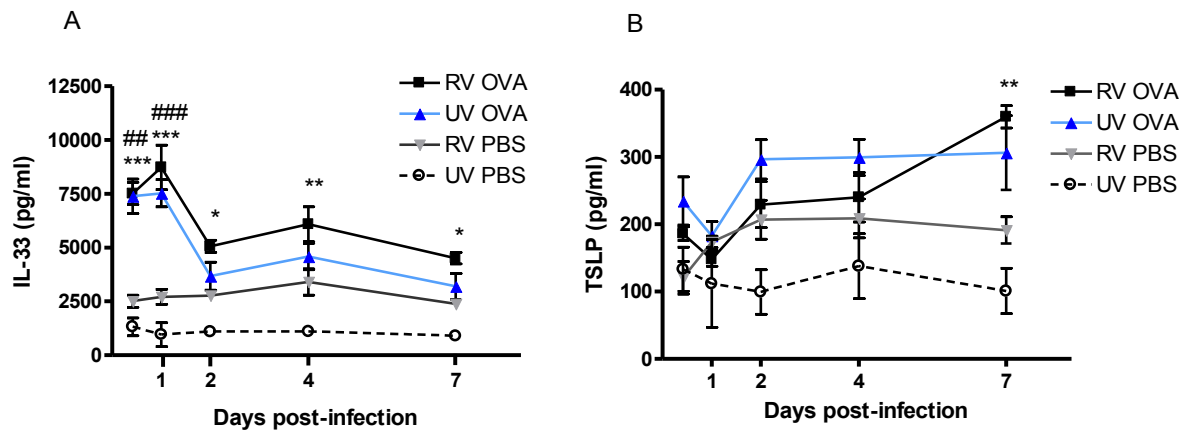


Figure 4.10 IL-33 and TSLP in lung tissue in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. Lung tissue from mice was collected at the indicated time-points and processed lung homogenate was used to measure IL-33 (A) and TSLP (B) by ELISA. Data is represented as the mean ($\pm$ -SEM) of 5 mice per group and is representative of at least 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (* $P<0.05$; ##/** $P<0.01$; ###/** $P<0.001$)

4.3.9 BAL mucins

Enhanced mucus secretion is associated with acute exacerbations and decreased lung function in asthmatic patients²¹¹. The major respiratory mucins, Mucin5, subtypes a and c (MUC5ac and MUC5b), have been shown to be upregulated in response to RV infection alone while the combination of allergen and RV infection resulted in further enhancement²⁰². Therefore, we measured MUC5ac and MUC5b in the BAL of all treated mice by ELISA. Both mucins were predominantly induced in the OVA-challenged groups as observed by comparable levels in RV OVA and UV OVA mice up until d4 p.i. for MUC5ac (Figure 4.11 A) and until d7 p.i. for MUC5b (Figure 4.11 B). Consistent with previously published results of this model, MUC5ac was significantly increased in RV OVA mice at d7 p.i. compared with all other groups (RV OVA vs UV OVA $P < 0.001$, RV OVA vs RV PBS $P < 0.001$, RV OVA vs UV PBS $P < 0.001$). There were no significant differences in MUC5b levels between OVA-challenged mice.

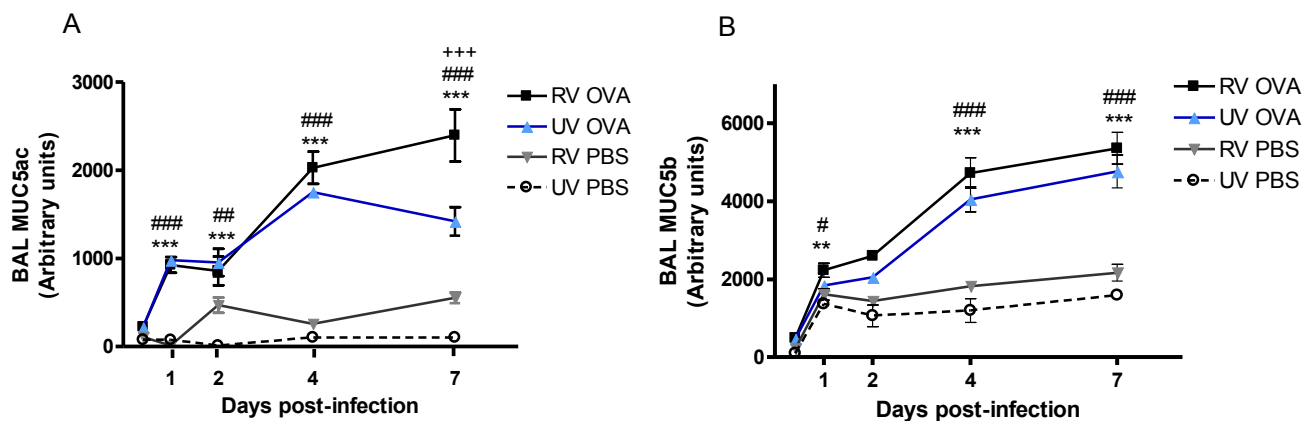


Figure 4.11 Mucins in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or UV-inactivated virus. BAL from mice was collected at the indicated time-points and BAL supernatant was used to quantify MUC5ac (A) and MUC5b (B) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of at least 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (# $P < 0.05$; ** $P < 0.01$; +++/###/*** $P < 0.001$)

4.3.10 Serum IgE

Elevated IgE levels to common environmental allergens is a general marker of atopic asthma and provides the trigger for the immediate allergic response upon allergen exposure⁵⁵. In addition to allergen-specific IgE, viruses including RSV and RV have been demonstrated to enhance total IgE^{108,212}. Therefore, we measured OVA-specific and total IgE in serum from mice harvested at d7 p.i. by ELISA. Although differences between treated groups were not significant, total (Figure 4.12 A) and OVA-specific IgE (Figure 4.12 B) was generally higher in RV OVA mice with comparable levels of total IgE in UV OVA and RV PBS mice.

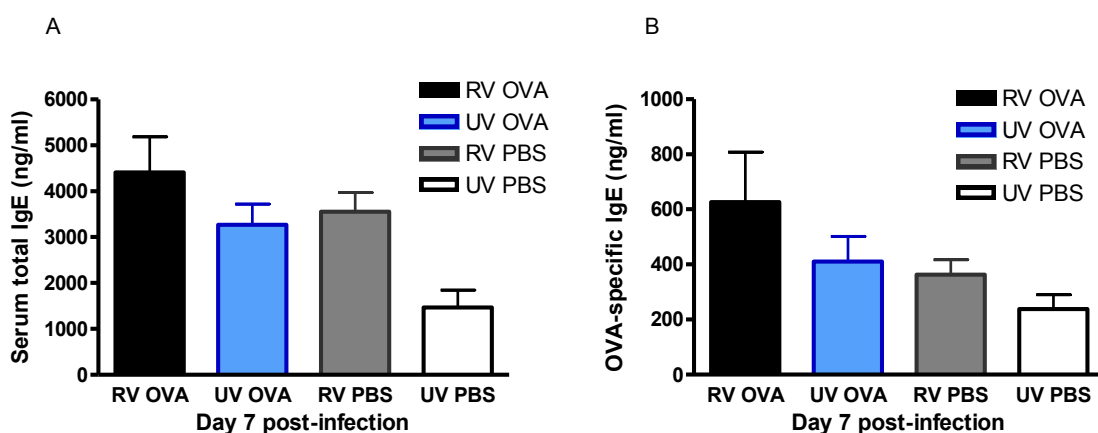


Figure 4.12 Total and OVA-specific IgE in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or UV-inactivated virus. Blood was collected from the carotid artery of mice at d7 p.i. and serum was separated to measure total (A) and OVA-specific (B) IgE by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of at least 2 independent experiments.

4.4 Summary

The studies described in this chapter were designed to utilise an established mouse model to identify RV-induced exacerbation-associated responses potentially regulated by IL-25 as well as the effects of infection and allergen on IL-25 expression.

The combination of RV infection and OVA sensitisation and challenge in mice enhanced numerous read-outs of Th2 allergic inflammation compared with OVA treatment alone. One of the clearest indications of this effect was the significantly enhanced BAL Th2 cytokines, including IL-4, IL-5 and IL-13, in the airways and lungs of RV OVA mice shortly after infection (10h p.i.). In addition to the classical Th2 cytokines, IL-25 mRNA and protein was also

significantly induced over allergen-treated or infected mice alone. Though IL-25 was undetectable in the BAL, protein expression in the lung tissue followed a similar expression pattern as IL-4, IL-5, and IL-13; namely, a peak at d1 p.i. that was unique to RV OVA mice and significantly upregulated above OVA-treated mice alone. In contrast, the innate, epithelial-derived Th2 cytokines, IL-33 and TSLP, were not significantly induced by RV infection and allergen treatment, with TSLP induction appearing to be predominantly allergen-driven.

The combination of RV infection and OVA treatment also significantly enhanced early airway neutrophilia while eosinophilia and lymphocytosis was generally higher in RV OVA mice. This exacerbated airway inflammatory response was also confirmed by significantly upregulated levels of the eosinophil chemoattractants, CCL11/Eotaxin 1 and CCL24/Eotaxin 2, and the T cell and eosinophil chemokine, CCL5/RANTES, in the airways. Increased airway inflammation also coincided with enhanced AHR (as measured by SRaw) in RV OVA mice which was significantly greater than UV OVA groups in double-chamber plethysmography experiments.

RV infection also enhanced viral RNA copy numbers as well as early expression of the pro-inflammatory cytokine, IL-6, in OVA-treated mice. A later augmentation in the respiratory mucin, MUC5ac, was also observed in RV OVA mice at d7 p.i. with no discernable differences in MUC5b expression between OVA-challenged groups. Total and OVA-specific IgE displayed a trend towards increased levels in RV OVA mice, however a time-point later than d7 p.i. may be required to distinguish greater differences in humoral immune responses between groups.

In summary, RV infection of an allergen-sensitised and challenged mouse exacerbated Th2 inflammation in addition to enhancing viral load and disease severity as demonstrated by augmented AHR. The interaction of virus and allergen resulted in the greatest induction of IL-25 gene and protein expression compared with either factor alone verifying our hypothesis. To further investigate the resultant effects of enhanced IL-25 in our model of RV-induced allergic airways disease, we aimed to characterise IL-25-responsive cells via flow cytometry as demonstrated in the next chapter.

Chapter 5: Results

IL-25-responsive cell populations in a model of RV-induced allergic airways disease

5.1 Introduction

The ability of IL-25 to amplify Th2 immunity is dependent on non-covalent binding to its principal receptor, IL-17RB, which forms a heterodimer with IL-17RA¹⁷⁹. Several cell types including airway smooth muscle cells, invariant natural killer T cells, Th9 cells and Th2 memory cells have been demonstrated to express IL-17RB in the lung^{127,185,187,189,213}. In addition, the majority of recent IL-25-based studies have focussed on characterising IL-17RB-expressing ILC2 populations which secrete Th2 cytokines upon IL-25 binding^{77,78,193}. Grouped under this heading, and of particular interest, are the IL-25-responsive novel population termed nuocytes which have been reported to infiltrate the mouse lung in response to OVA sensitisation and challenge and induce AHR via the secretion of IL-13⁷⁶. Furthermore, OVA sensitisation and challenge in mice has been demonstrated to enhance adaptive Th2 immunity via IL-25-dependant recruitment of CD4+ cells to the lung¹⁶⁹. Wang *et al.* further established that Th2 memory cells secrete IL-4, IL-5 and IL-13 upon IL-25 binding¹²⁷. In this chapter, we aimed to characterise potential IL-25-responsive cells by measuring expression of IL-17RB in our mouse model of RV-induced allergic airways disease by flow cytometry analysis.

We hypothesised that RV-induced IL-25 acts on IL-17RB+ innate cell populations and IL-17RB+ adaptive cells which are recruited into the mouse lung by OVA sensitisation and challenge resulting in exacerbated Th2 inflammation. We also hypothesised that enhanced levels of IL-25 previously observed in RV-infected and OVA-challenged mice would cause increased recruitment of IL-17RB-expressing leukocytes to the lung providing a potential mechanism whereby RV infection may exacerbate allergen-driven Th2 immunity. To test this, IL-17RB-expressing cells were characterised by flow cytometry in our model of RV-induced allergic airways disease.

5.2 Hypotheses and Aims

5.2.1 Hypotheses

- i.) Significantly enhanced IL-25 levels measured in RV-infected and allergen-sensitised and challenged mice correspond with recruitment of IL-17RB expressing cells to the lung
- ii.) RV infection in an allergen-sensitised and challenged mouse results in recruitment of both innate and adaptive IL-25-responsive cell populations

5.2.2 Aims

To investigate the effects of RV infection and OVA sensitisation and challenge on IL-17RB-expressing cell populations using a mouse model of RV-induced allergic airways disease

- i.) To determine the magnitude and kinetics of total IL-17RB+ cell numbers in the mouse lung and airways by flow cytometry
- ii.) To characterise the magnitude and kinetics of innate and adaptive IL-25-responsive cell populations by analysing:
 - Expression of IL-17RB and co-expression of markers associated with innate cell populations and Th2-associated markers in the lungs and airway using flow cytometry analysis
 - Adaptive cells expressing IL-17RB and co-expressing Th2-associated markers in the lungs and airways using flow cytometry analysis

5.3 Results

5.3.1 Total IL-17RB+ lymphocyte populations

Using a mouse model of RV-induced allergic airways disease, we sought to determine the effects of infection and OVA-sensitisation and challenge on IL-17RB-expressing cells by staining for IL-17RB in whole lung cell preparations and BAL cells and analysing by flow cytometry.

Approximately 99% of IL-17RB+ lung cells were encompassed within the 'lymphocyte' gate based on forward scatter (FSC) vs side scatter (SSC) analysis. The data represented is annotated as percentages and total cell numbers of IL-17RB+ lymphocytes, with the understanding that this gating strategy also incorporated non-lymphocyte populations including basophils and other innate cell populations.

The greatest number of IL-17RB-expressing cells in whole lung cell preparations were observed in RV OVA mice at d7 p.i. (RV OVA vs UV OVA $P < 0.001$; RV OVA vs RV PBS $P < 0.001$; RV OVA vs UV PBS $P < 0.001$) (Figure 5.1 A and 5.2.1). These cells comprised approximately 14% of the total lung lymphocyte population in RV OVA mice which was significantly higher compared with all other treated groups (RV OVA vs UV OVA $P < 0.001$; RV OVA vs RV PBS $P < 0.001$; RV OVA vs UV PBS $P < 0.001$) (Figure 5.1 B). At d1 p.i., RV OVA mice displayed a small elevation in total IL-17RB+ cell numbers compared with other treated groups which represented ~1% of total lung lymphocytes. In BAL IL-17RB+ cells, this peak was more pronounced and was significant compared with UV PBS control groups (RV OVA vs UV PBS $P < 0.05$) (Figure 5.1 C and 5.2.2). Consistent with findings in lung cells, the highest peak in BAL IL-17RB+ cells was observed at d7 p.i. in both OVA-challenged groups as well as in RV-infected mice alone.

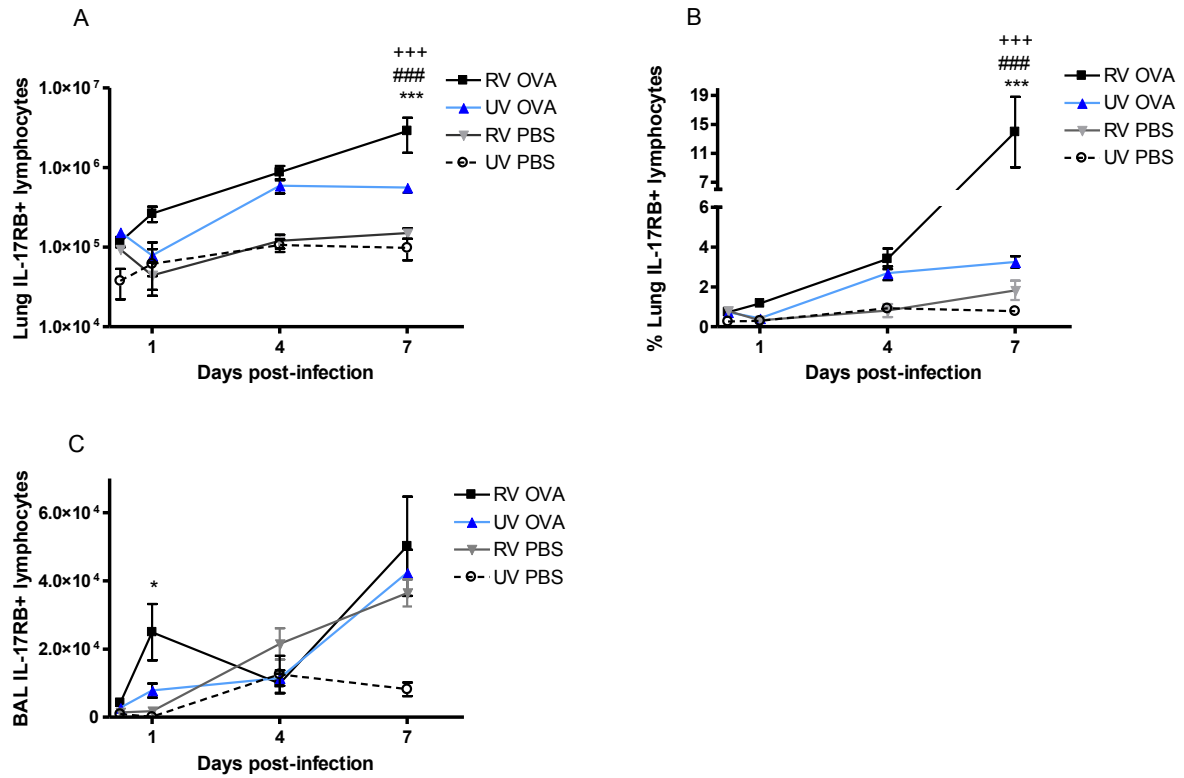


Figure 5.1 Total IL-17RB+ cells in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. Cells were stained for IL-17RB and total live, single cells per whole mouse lung (A) and the percentage of IL-17RB+ lung cells within the 'lymphocyte' gate (B) are represented, as well as total BAL IL-17RB+ cells (C). Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (* $P < 0.05$; +/+###/*** $P < 0.001$)

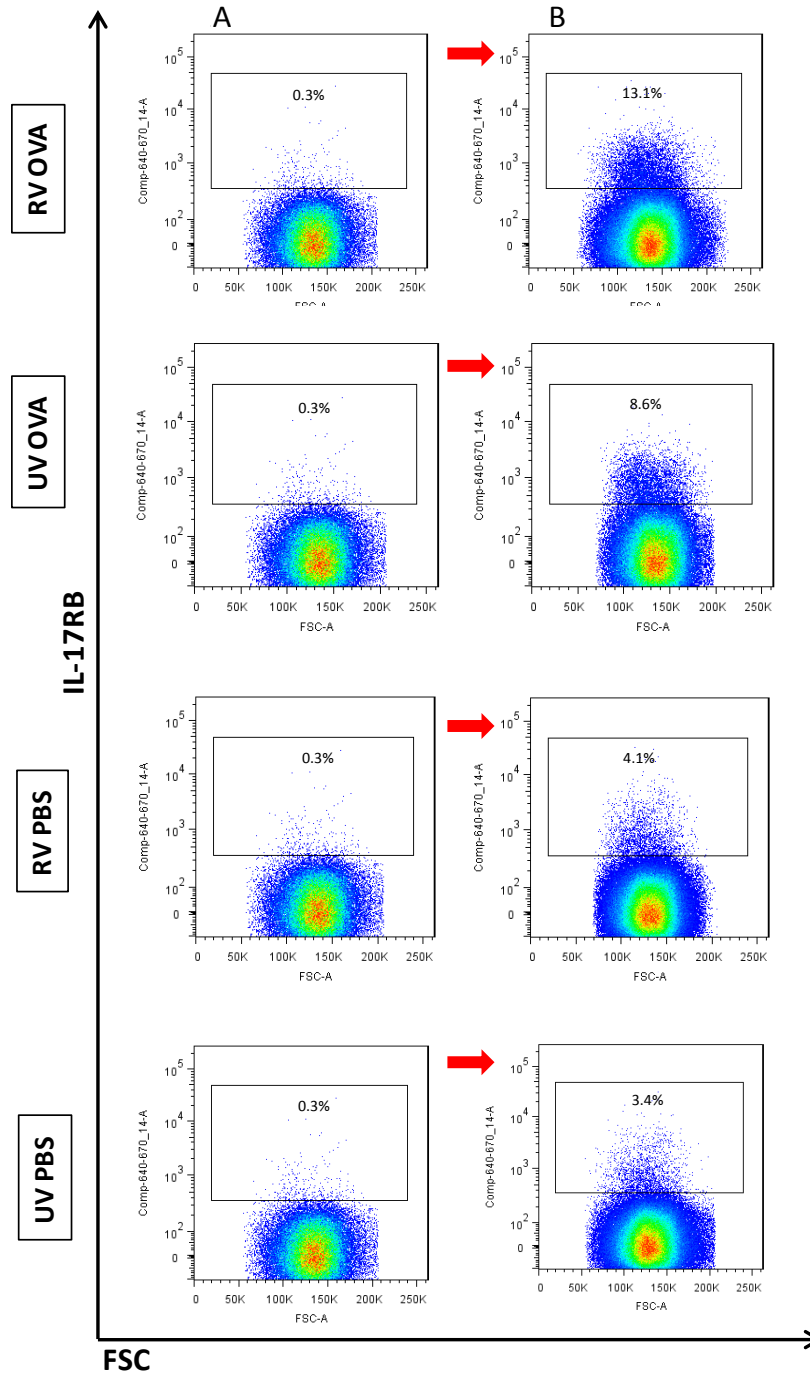


Figure 5.2.1 Representative flow plots of total IL-17RB+ cells in whole lung tissue in all treated groups at d7 p.i. After gating live, single lymphocytes as demonstrated in figure 2.4.1, lung cells stained with all markers with the exclusion of IL-17RB were used as a control to set gates (A) and define IL-17RB+ cells (B) in the lungs of RV OVA , UV OVA , RV PBS and UV PBS mice. Results are representative of 3 independent studies.

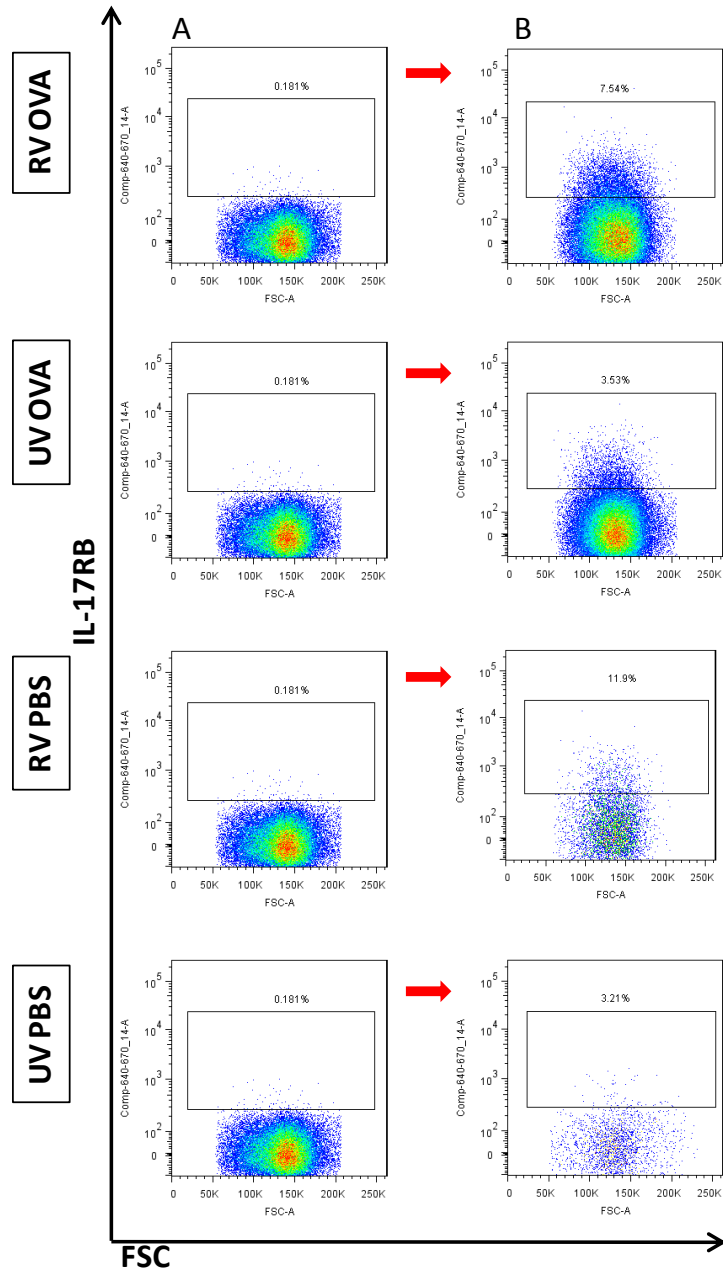


Figure 5.2.2 Representative flow plots of total BAL IL-17RB⁺ cells in all treated groups at d7 p.i. After gating live, single lymphocytes as demonstrated in figure 2.4.1, BAL cells stained with all markers with the exclusion of IL-17RB were used as a control to set gates (A) and define IL-17RB⁺ cells (B) in the lungs of RV OVA , UV OVA , RV PBS and UV PBS mice. Results are representative of 3 independent studies.

5.3.2 Innate cell populations

To gain a greater understanding of IL-17RB-expressing innate cell populations, a panel of various Th2-associated markers were used in conjunction with anti-IL-17RB to define cell phenotypes. These markers included the pan-NK cell and basophil marker, CD49b, the high affinity IgE receptor, FcεRI and the mast cell, basophil and Th2 cell marker, T1/ST2. We were also interested in characterising the newly termed ILC2 populations therefore a marker for ICOS in addition to the T cell markers, CD3 and CD4, were included to define T and non-T cell populations. Intracellular staining for IL-4 was also carried out. Using this panel, the cell populations listed below were identified.

5.3.2.1 Innate lymphoid cells (ILC) 2

Of the total IL-17RB⁺ lymphocytes in the lung and BAL, a population of T1/ST2⁺ ICOS⁺ IL-4⁻ FcεRI-CD49b-CD3-CD4⁻ were identified. Based on this expression, we hypothesised these cells represented one of the innate lymphoid cell populations recently grouped under the heading ILC2. Though additional markers to confirm a lineage negative phenotype would be required for definitive characterisation, this classification fitted most accurately based on recently published findings and will be defined as ILC2s throughout^{76,78}.

In lung tissue, this cell population was significantly elevated early on (6h p.i.) (RV OVA vs RV PBS $P < 0.001$; RV OVA vs UV PBS $P < 0.001$) and at d7 p.i. (RV OVA vs RV PBS $P < 0.001$; RV OVA vs UV PBS $P < 0.001$) in OVA-treated mice (Figure 5.3 A and 5.4.1). In between these 2 time-points, cell numbers were reduced to levels close to baseline at d1 and d4 p.i. in all treated groups. Where the highest levels were observed in OVA-challenged mice (d7 p.i.), cells represented a mere 0.45% of the total lymphocyte population (Figure 5.3 B).

In contrast to decreased levels observed in whole lung tissue at d1 p.i., ILC2s in the airways of RV OVA mice were significantly upregulated compared with all other treated groups (RV OVA vs UV OVA $P < 0.05$; RV OVA vs RV PBS $P < 0.01$; RV OVA vs UV PBS $P < 0.01$) (Figure 5.3 C and 5.4.2). Measurements at later time-points p.i. emulated trends observed in whole lung cell preparations; namely, decreased ILC2s in all treated mice at d4 p.i. and a delayed increased at d7 p.i. Though levels were elevated in both OVA-challenged groups at d7 p.i., significant differences were only recorded between RV OVA and UV PBS groups.

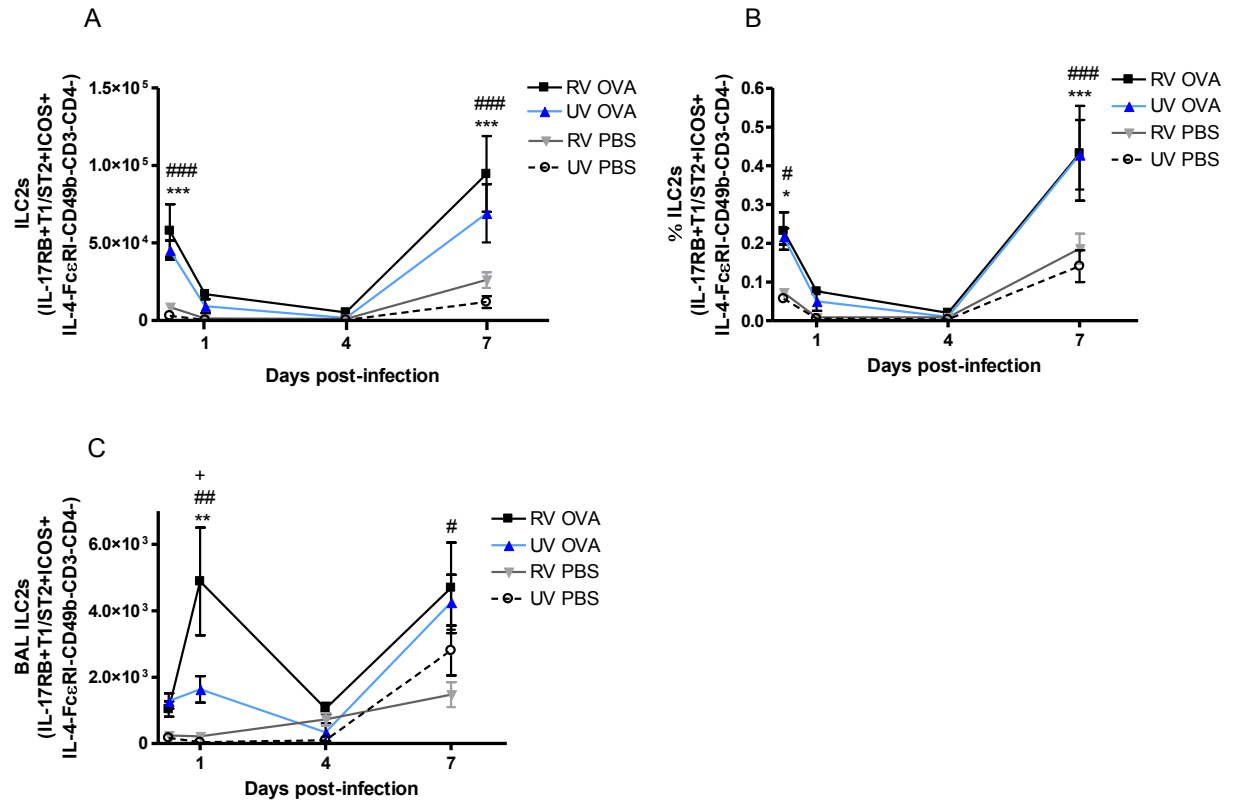


Figure 5.3 ILC2s in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected with UV-inactivated virus. Cells were stained for CD3, CD4, IL-17RB, T1/ST2, ICOS+, FcεRI, CD49b, and intracellular IL-4. The total cell numbers per whole mouse lung (A) and the percentage of ILC2s (CD3-CD4-IL-17RB+T1/ST2+ICOS+) lung cells within the ‘lymphocyte’ gate (B) are represented, as well as total BAL ILC2s (C). Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (+/# P<0.05; ##/** P<0.01; ###/*** P < 0.001)

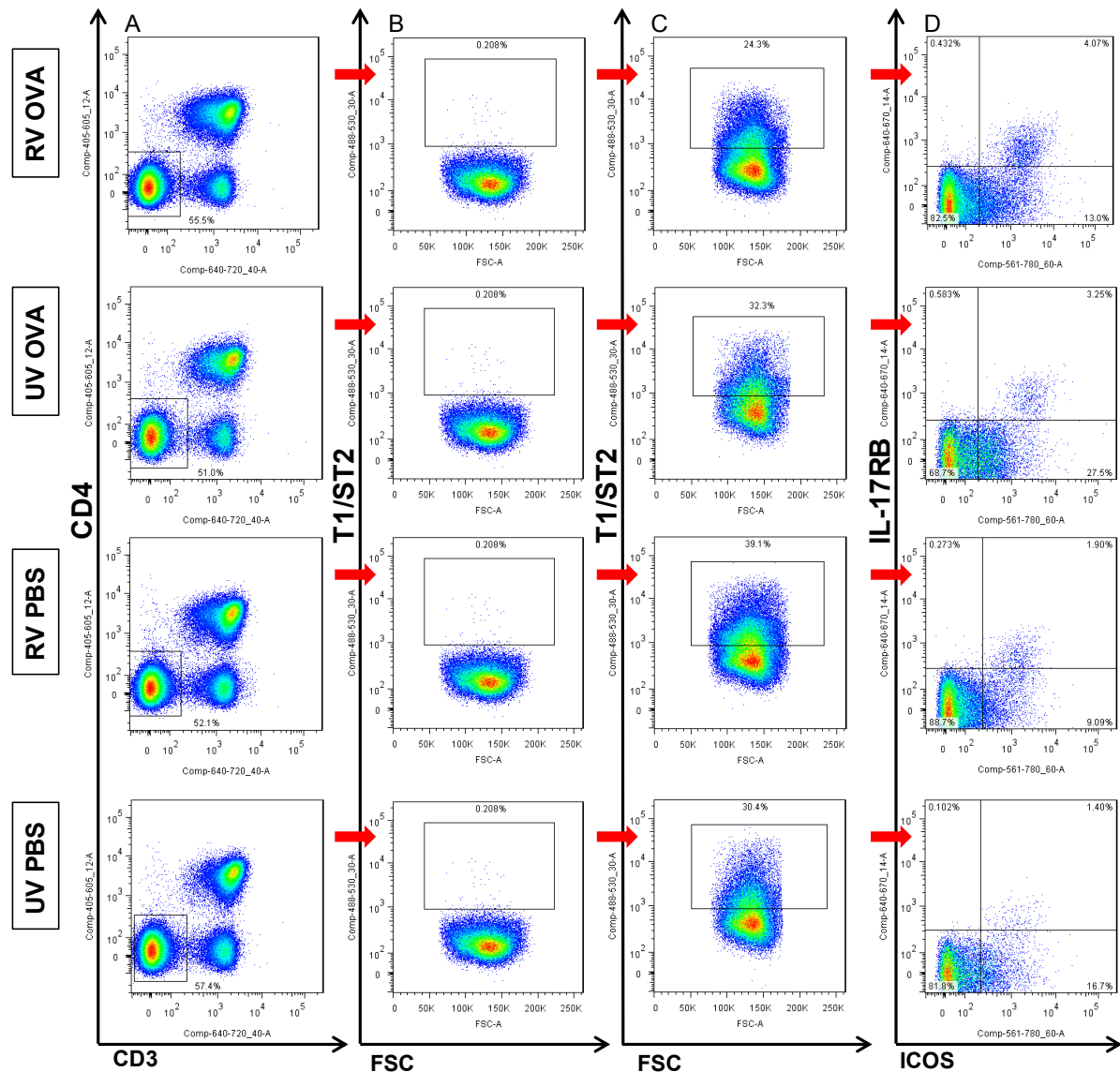


Figure 5.4.1 Representative flow plots of ILC2s in whole lung tissue in all treated groups at d7 p.i. Flow plots represent gated CD3-/CD4- (A) cells. In addition, lung cells stained with all markers with the exclusion of T1/ST2 were used as a control to set gates (B) and define T1/ST2+ cells (C), which were then gated on positive IL-17RB/ICOS expression (D) in the lungs of RV OVA , UV OVA , RV PBS and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 3 independent studies.

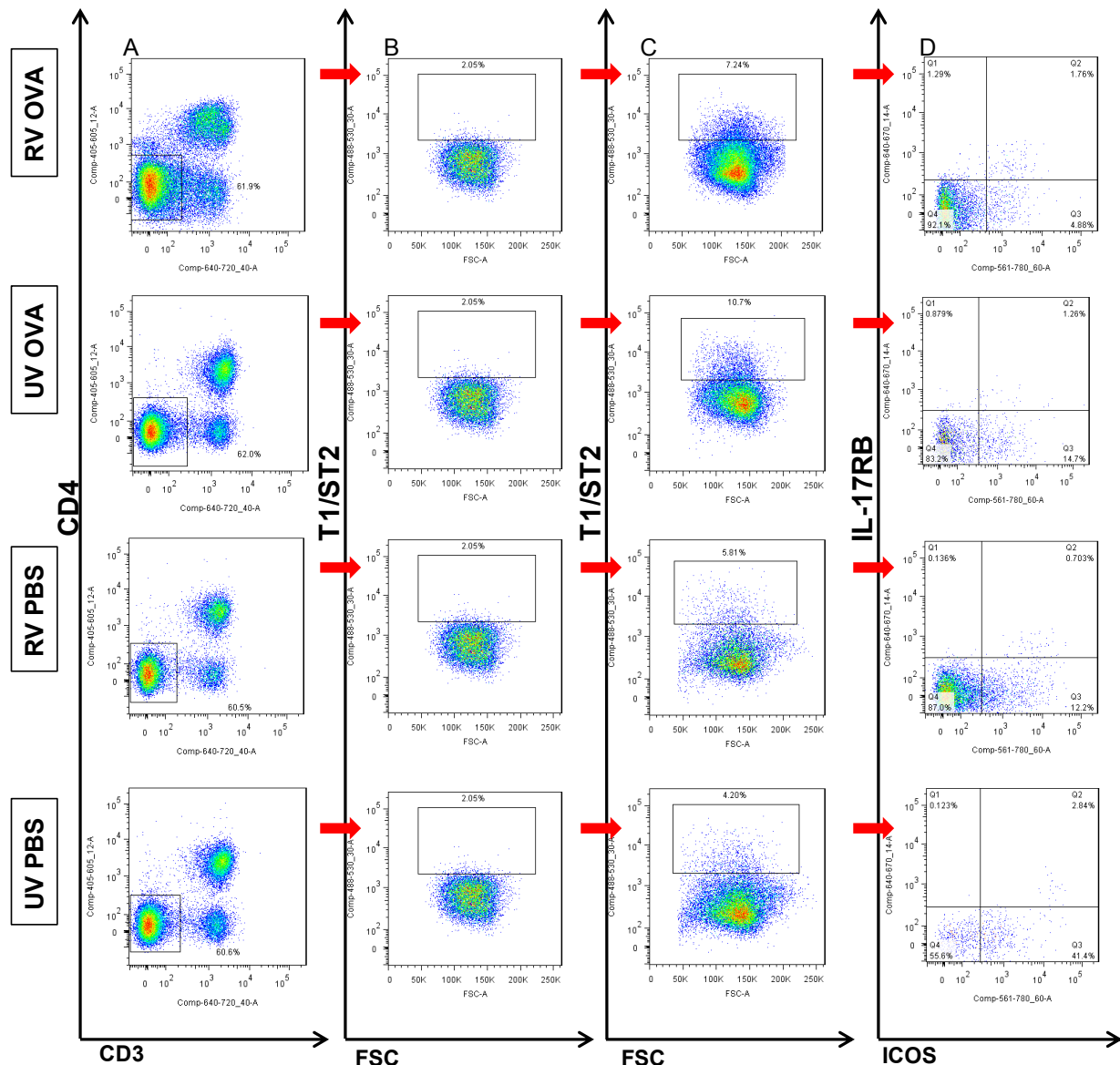


Figure 5.4.2 Representative flow plots of BAL ILC2s in all treated groups at d7 p.i. Flow plots represent gated CD3-/CD4- (A) cells. In addition, BAL cells stained with all markers with the exclusion of T1/ST2 were used as a control to set gates (B) and define T1/ST2+ cells (C), which were then gated on positive IL-17RB/ICOS expression (D) in the lungs of RV OVA , UV OVA , RV PBS and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 3 independent studies.

5.3.2.2 IL-4+ basophils

Based on the empirical observation that RV infection induced an early increase in IL-4 in the airways of OVA-challenged mice which was two-fold higher than levels in OVA-challenged mice alone (Figure 4.7 A), we sought to identify any IL-17RB+ cells that co-expressed IL-4. Though no non-T cell IL-4+IL-17RB+ cells were observed, we did identify an FSC^{lo}/SSC^{lo}CD3-CD4-FcεRI+T1/ST2+CD49b+ cell population in whole lung cell preparations and BAL leukocytes which produced IL-4, a surface marker and cytokine expression profile consistent with basophils.^{214,215 216}

Both OVA-challenged groups had increased levels of IL-4+basophils in whole lung tissue at 6h p.i. (RV OVA vs RV PBS P<0.01) and by d1 p.i. levels in RV OVA mice were significantly higher compared with other treatment groups (RV OVA vs UV OVA P<0.05; RV OVA vs RV PBS P<0.001; RV OVA vs UV PBS P<0.001) (Figure 5.5 A and 5.6.1). This peak in expression in RV OVA mice represented ~0.6% of total cells within the 'lymphocyte' gate (Figure 5.5 B). A similar trend was observed in the BAL; RV OVA mice displayed a peak in IL-4+basophils at d1 p.i. which was significantly elevated compared with PBS-challenged groups (RV OVA vs RV PBS P<0.001; RV OVA vs UV PBS P<0.001) but not compared with UV OVA mice (Figure 5.5 C and 5.6.2). Consistent with results from whole lung tissue, IL-4+basophils in the airways had declined by d4 and d7 p.i. in all groups.

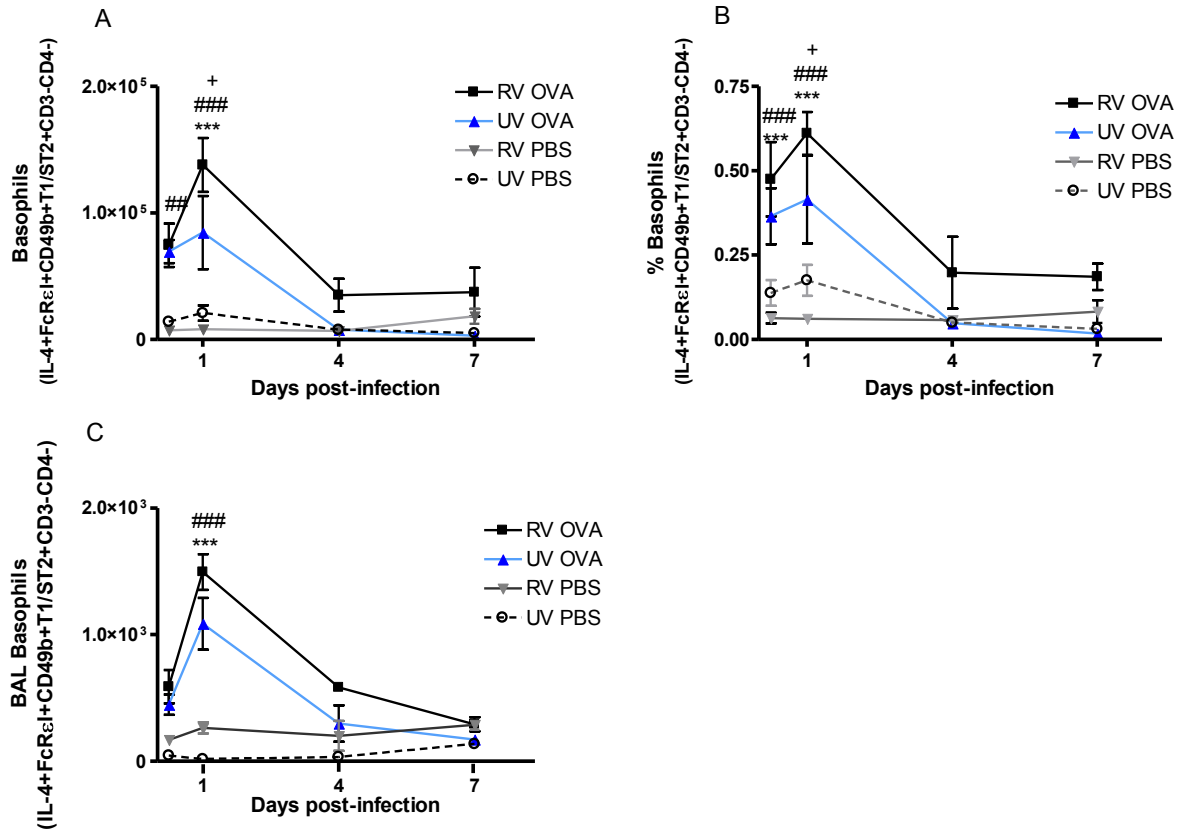


Figure 5.5 IL-4⁺ basophils in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or UV-inactivated virus. Cells were stained for IL-17RB, CD3, CD4, FcεRI, CD49b, T1/ST2, ICOS and intracellular IL-4. Total cell numbers per whole mouse lung (A) and the percentage of IL-4⁺ basophil (CD3⁺CD4⁻FcεRI⁺CD49b⁺T1/ST2⁺) lung cells within the 'lymphocyte' gate (B) are represented, as well as total IL-4⁺ basophils in BAL (C). Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (+P<0.05; ## P<0.01; ###/*** P < 0.001)

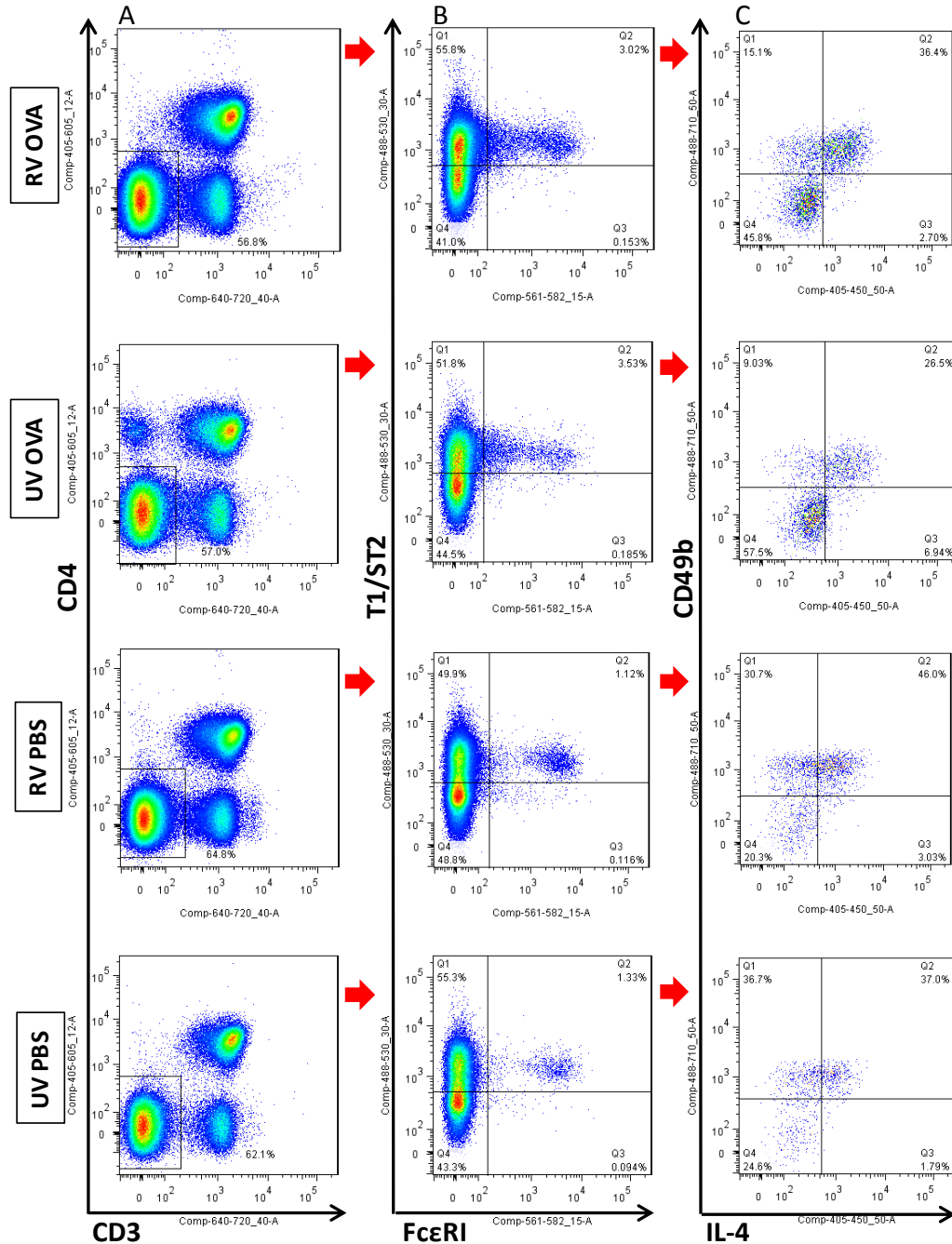


Figure 5.6.1 Representative flow plots of IL-4⁺ basophils in whole lung tissue in all treated groups at d1 p.i. Flow plots represent gated CD3⁺/CD4⁺ cells (A), T1/ST2⁺/FcεRI⁺ (B) and CD49b⁺/IL-4⁺ (C) cells in the lungs of RV OVA , UV OVA , RV PBS and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 2 independent studies.

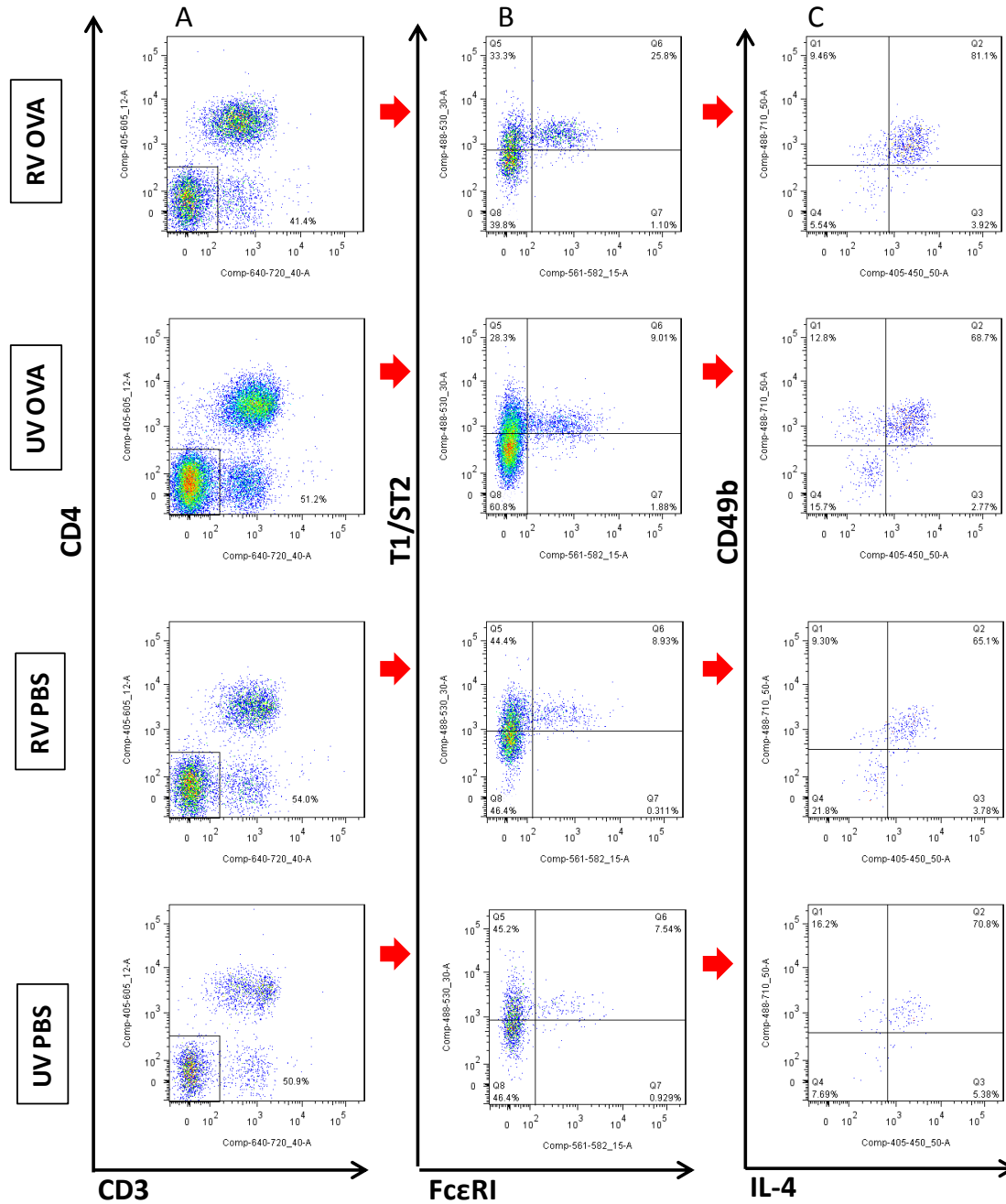


Figure 5.6.2 Representative flow plots of BAL IL-4⁺ basophil lung cells in all treated groups at d1 p.i. Flow plots represent gated CD3⁻/CD4⁻ cells (A), T1/ST2⁺/FcεRI⁺ (B) and CD49b⁺/IL-4⁺ (C) BAL cells in the lungs of RV OVA , UV OVA , RV PBS and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 2 independent studies.

5.3.3 IL-17RB+ T cell populations

5.3.3.1 IL-4+IL-17RB+CD4+ T cells

Next we measured IL-4+IL-17RB+ CD4+ T cells by staining lung and BAL leukocytes for IL-17RB, CD3, CD4 and intracellular IL-4.

In whole lung tissue, a robust increase in IL-4+IL-17RB+ CD4+ T cells was observed at d7 p.i. in RV OVA mice which was significantly enhanced compared with UV OVA and PBS-challenged mice (RV OVA vs UV OVA $P<0.001$; RV OVA vs RV PBS $P<0.001$; RV OVA vs UV PBS $P<0.001$) (Figure 5.7 A and 5.8.1). This increase accounted for the largest proportion of total IL-17RB-expressing cells at d7 p.i., making up approximately 5% of total cells within the 'lymphocyte' gate (Figure 5.7 B).

In the airways, an early increase in IL-4+IL-17RB+ CD4+ T cells was observed in RV OVA mice which increased up to d7 p.i (Figure 5.7 C and 5.8.2). Although levels in RV OVA mice were significantly elevated above PBS-challenged mice at d7 p.i., levels in OVA-challenged groups were not significantly different in the BAL.

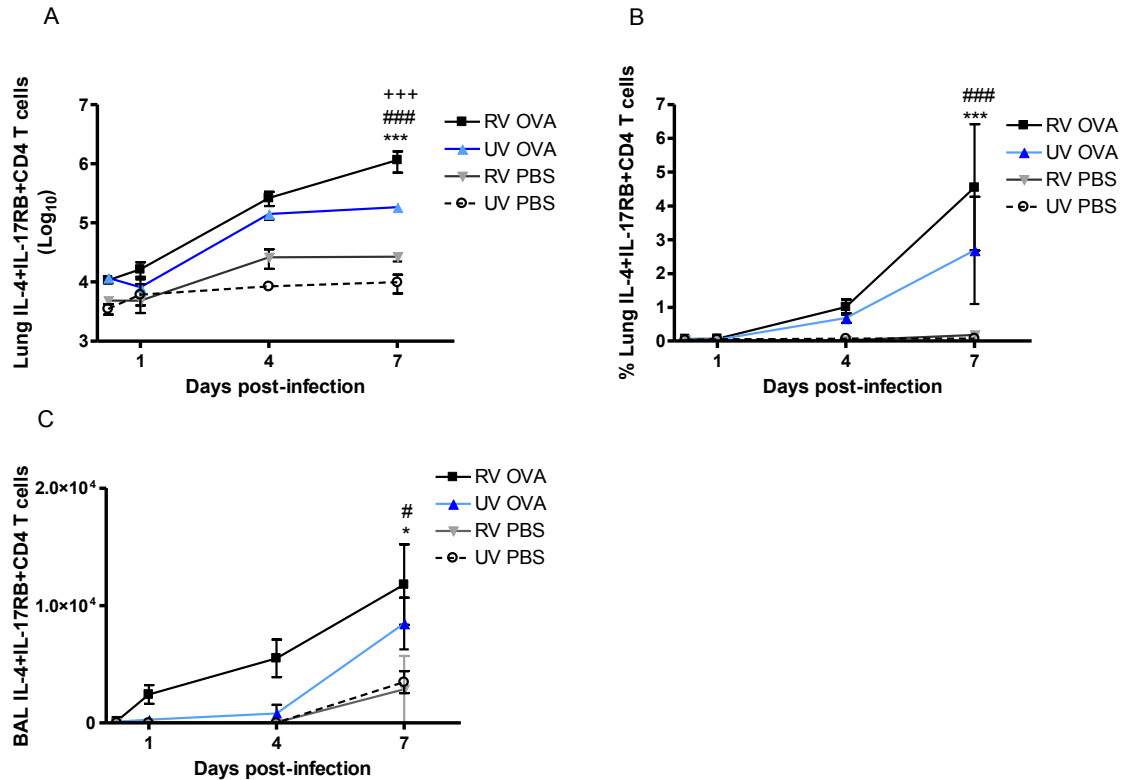


Figure 5.7 IL-4+IL-17RB+ CD4+ T cells in a model of RV-induced allergic airways disease OVA-sensitised BALB/c mice were challenged i.n. with OVA or PBS control and infected with RV-1B or mock-infected UV-inactivated virus. Cells were stained for IL-17RB, CD3, CD4, FcεRI, CD49b, T1/ST2, ICOS and intracellular IL-4. The total cells numbers per whole mouse lung (A) and percentage of IL-4+ CD4+ T cells in lung cells within the 'lymphocyte' gate (B) are represented, as well as total IL-4+ CD4+ T cells in BAL (C). Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. + RV OVA vs UV OVA; # RV OVA vs RV PBS; *RV OVA vs UV PBS (#/*P<0.05; +++/###/*** P < 0.001)

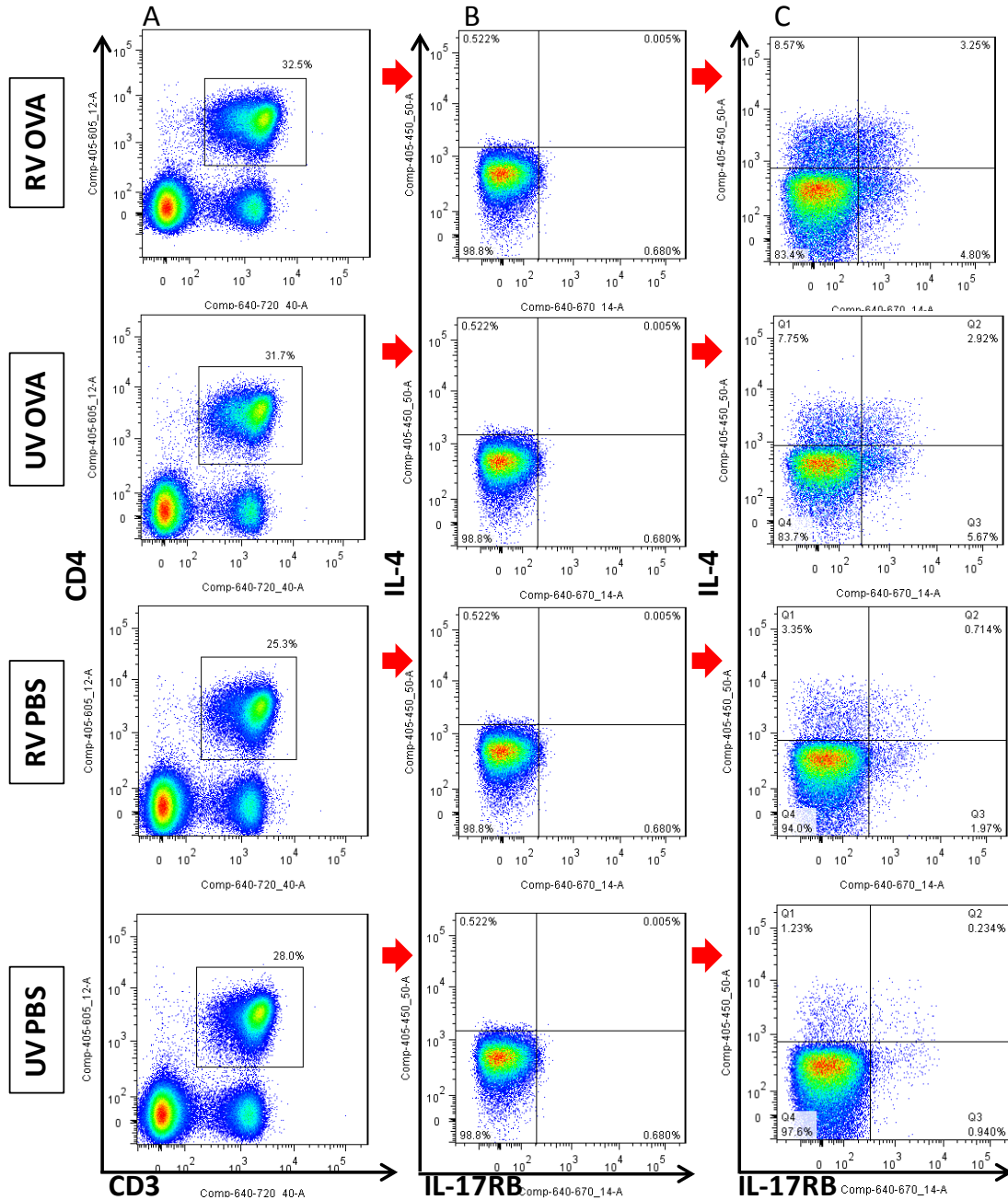


Figure 5.8.1 Representative flow plots of IL-4⁺IL-17RB⁺ CD4⁺ T cells in whole lung tissue in all treated groups at d7 p.i. Flow plots represent gated CD3⁺/CD4⁺ (A) cells while lung cells stained with all markers with the exclusion of IL-4 and IL-17RB were used as a control to set gates (B) and used to define IL-4⁺/IL-17RB⁺ (C) in the lungs of RV OVA , UV OVA , RV PBS and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 3 independent studies.

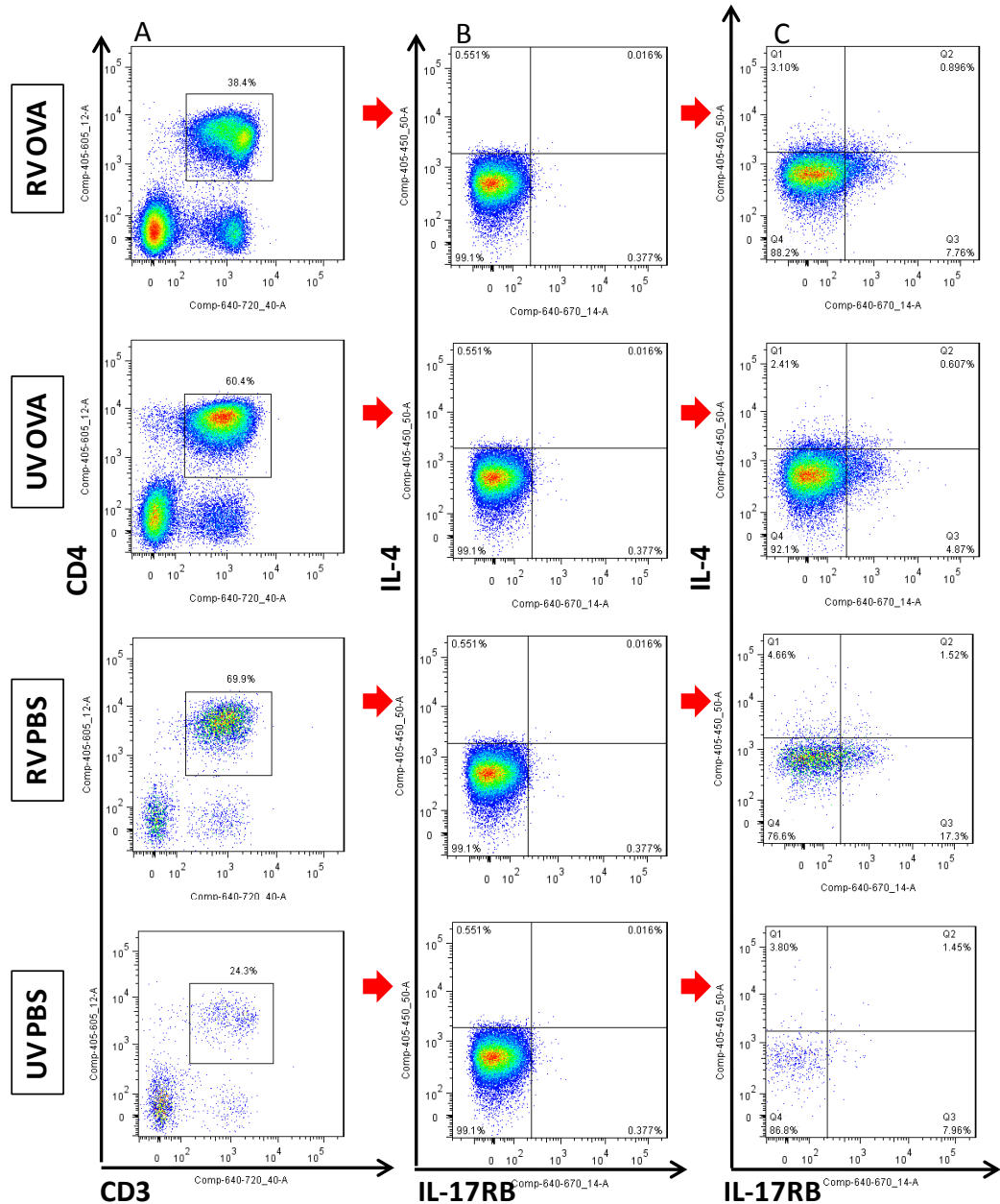


Figure 5.8.2 Representative flow plots of BAL IL-4+IL-17RB+ CD4+ T cells in all treated groups at d7 p.i. Flow plots represent gated CD3⁺/CD4⁺ (A) cells while BAL cells stained with all markers with the exclusion of IL-4 and IL-17RB were used as a control to set gates (B) and used to define IL-4+/IL-17RB+ (C) in the lungs of RV OVA , UV OVA , RV PBS and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 3 independent studies.

5.4 Summary

In this chapter, we identified non-T (ILC2s) and T cell (Th2) populations that expressed the IL-25 receptor, IL-17RB. In accordance with our hypothesis, RV infection in OVA-sensitised and challenged mice resulted in recruitment of IL-17RB-expressing cells in the total lung which coincided with increased IL-25 expression as demonstrated in chapter 4. Based on findings that activation of ILC2s and Th2 cells by IL-25 induces amplification of Th2 inflammation via the release of Th2 cytokines, this provides a potential mechanism whereby RV-induced IL-25 may enhance disease severity and exacerbate Th2 inflammation.

Of the total IL-17RB-expressing cells, an early infiltrating population that expressed IL-17RB, T1/ST2 and ICOS but was negative for CD3, CD4, CD49b, FcεRI and IL-4 was identified in the lung and airways of allergen-challenged mice. Based on their similarity to the newly grouped innate lymphoid populations, they were classified as ILC2s⁸¹. OVA-challenged mice also displayed a delayed peak at d7 p.i. which complemented published reports that nuocytes, which represent a cell subset grouped within ILC2s, persist through the adaptive response⁷⁶. Despite similar cell numbers in OVA-challenged mice in the lung, ILC2s were significantly enhanced in the BAL of RV OVA mice at d1 p.i. Due to their ability to amplify Th2 inflammation via the release of IL-5 and IL-13 in response to IL-25, upregulation of these innate cells in the BAL in response to RV and/or allergen together with increased expression of IL-25 may suggest a potential role in enhanced allergic inflammation.

An early source of IL-4+ cells that did not express IL-17RB was identified predominantly in the lungs and airways of RV OVA mice at d1 p.i. This cell subset was characterised as FSC^{lo}/SSC^{lo}CD3-CD4-FcεRI+T1/ST2+CD49b+ and accounted for approximately 85% of IL-4+ lymphocytes at 6h and d1 p.i. These cells closely resembled basophils and based on the fact that they made up the majority of IL-4+ cells, we speculated that they were a major source of IL-4 in the airways of OVA-challenged mice as demonstrated in chapter 4.

Characterisation of IL-17RB+ adaptive cells demonstrated that Th2 cells were enhanced in the lungs of OVA-challenged mice at d7 p.i. RV infection had a striking effect on Th2 cell populations in allergen-sensitised and challenged mice resulting in a significant increase over allergen-treated mice alone which accounted for approximately 5% of total IL-17RB+

lymphocytes. This is pertinent given that asthma pathogenesis is largely mediated by Th2 cells.

The studies described in this chapter facilitated identification of cell populations that IL-25 may mediate its biological effects through in RV-induced exacerbation. Using the same anti-IL-17RB antibody to block IL-25-mediated signalling *in vivo*, we sought to investigate any inhibitory effects on IL-17RB-expressing cell populations as well as on RV-mediated Th2 responses measured in chapter 4 with the aim of defining a functional role of RV-induced IL-25 in a model of allergic airways disease.

Chapter 6: Results

6.1 Blocking IL-25 signalling in a model of RV-induced allergic airways disease

RV infections are the principal cause of asthma exacerbations^{8,98}. It remains unclear how, in the atopic asthmatic lung, RV interacts with the pulmonary Th2 environment to trigger an exacerbation. In previous chapters, we have identified a pro-Th2 cytokine, IL-25, which is induced by RV infection *in vivo* and *in vitro*. In addition, allergen exposure and RV infection synergistically upregulated IL-25 and its receptor, IL-17RB, which was associated with disease severity as demonstrated by augmented AHR. To our knowledge, this is the first study to demonstrate the upregulation of IL-25 by RV, with or without exposure to allergen. In this chapter, we aimed to define the functional role of IL-25 in a model of RV-induced allergic airways disease by blocking the biological activity of RV-induced IL-25 using a neutralising antibody against IL-17RB.

Several studies have demonstrated the therapeutic benefit of blocking allergen-induced IL-25 by inhibiting Th2 inflammation in mice^{70,169,170}. Specifically, neutralising IL-25 before sensitisation in an allergen-induced asthma model prevented Th2 inflammation, including AHR, Th2 cytokine production and eosinophilia, with some of these inhibitory effects persisting even when the antibody was administered 4 hours before allergen challenge in previously sensitised mice¹⁷⁰. Furthermore, Camelo *et al.* demonstrated that blocking IL-25 signalling, using the same anti-IL-17RB antibody employed in this study, decreased nuocyte and NKT cell mucosal infiltration and decreased IL-13 in a type-2 model of colitis in mice¹⁷¹. These findings demonstrated that blocking IL-25 either directly or via receptor blocking was similarly effective at inhibiting biological activity of IL-25.

We aimed to investigate interactions between infection and allergic inflammation by transiently blocking RV-induced IL-25 signalling in OVA-sensitised and challenged mice and assess the effects on disease severity and immune response predominantly by measuring Th2-related outcomes. As we were specifically interested in the effect of IL-25 activity on RV-induced exacerbation of allergic airways inflammation we administered an anti-IL-17RB blocking antibody to allergen-sensitised and challenged mice at the time of RV infection and compared with responses in allergen-challenged mice alone as well as isotype-matched groups. Isotype antibody was also administered to all other treatment groups to control for

the effects of anti-IL-17RB while mice challenged with PBS and dosed with UV-inactivated RV (UV PBS) served as negative controls. Th2-related innate and adaptive responses were then measured in all groups by assessing early (8h and d1 p.i.) and later (d7 p.i.) responses after infection.

6.2 Hypotheses and Aims:

6.2.1 Hypotheses

- i.) Blocking IL-25 signalling prior to RV infection reduces early disease-associated end-points including:
 - Decreased Th2 cytokines in the airways
 - Decreased airway obstruction
 - Decreased Th2-associated innate cell populations
- ii.) Maintaining IL-17RB neutralisation post-RV infection reduces ongoing Th2-associated responses in an OVA-sensitised and challenged mouse, predominantly indicated by:
 - Decreased eosinophilia and lymphocytosis infiltration in the airways
 - Decreased Th2 cells in the lungs and airways

6.2.2 Aims

To investigate the functional role of RV-induced IL-25 and interactions between infection and allergic inflammation in a model of RV-induced allergic airway disease by transiently blocking IL-25 receptor signalling

- i.) To define the function of RV-induced IL-25 by neutralising IL-17RB in RV-infected and OVA-challenged mice and compare read-outs in Th2 inflammation to IL-17RB-treated OVA-challenged mice alone as well as identically treated isotype control groups. Any potential effects of IL-17RB neutralisation will be assessed by measuring:
 - Airway obstruction using whole-body plethysmography
 - Inflammatory airway leukocyte infiltration in the BAL via differential cell counts

- Th2-associated cytokine and chemokine proteins in the BAL and lung tissue by ELISA
 - IL-25 mRNA by qPCR and IL-25, IL-33 and TSLP protein by ELISA in the lung tissue
 - Respiratory mucin proteins including MUC5ac and MUC5b in the BAL by ELISA
 - Total and allergen-specific IgE in mouse serum by ELISA
- ii.) To examine the effect of blocking IL-25 signalling on innate and adaptive Th2-associated cell populations by flow cytometry analysis

6.3 Results

In this chapter, an IL-17RB neutralising antibody was administered to treatment groups in a model of RV-induced allergic airways disease (described in detail in section 2.2.5). The RV PBS treatment group was excluded due to the fact we were primarily interested in the effects of RV-induced IL-25 during allergic inflammation and the comparative differences between RV OVA and UV OVA groups and equivalent isotype-treated groups. An anti-IL-17RB antibody (Clone D9.2) was administered 4h pre-RV infection and at d3 and d5 p.i. and compared with identically treated groups administered isotype control antibody (anti-c-myc mouse IgG1 [Clone 9e10.2]). Treatment regime and nomenclature of all groups are summarised in the table below for clarification.

Experimental groups	Sensitisation (OVA/Alum i.p.)	Challenge (OVA i.n)	RV-1B infection (RV-1B i.n./UV-RV-1B)	Antibody treatment
RV OVA (anti-IL-17RB)	Yes	Yes	Yes	Anti-IL-17RB
UV OVA (anti-IL-17RB)	Yes	Yes	No	Anti-IL-17RB
UV PBS (anti-IL-17RB)	Yes	No	No	Anti-IL-17RB
RV OVA (ISO)	Yes	Yes	Yes	Isotype control
UV OVA (ISO)	Yes	Yes	No	Isotype control
UV PBS (ISO)	Yes	No	No	Isotype control

Table 6.1 Summarised treatment regime and nomenclature of antibody-treated groups (represented as 'anti-IL-17RB' and 'ISO') in model of RV-induced allergic airways disease.

6.3.1 Airway responses

We employed whole-body plethysmography to assess if blocking RV-induced IL-25 signalling affected airway obstruction in a model of RV-induced allergic airways disease. Enhanced pause (Penh) was measured in anti-IL-17RB and isotype-treated groups at d1 p.i. As previously observed, RV OVA (ISO) groups displayed significantly augmented Penh levels compared with UV PBS (ISO) controls at a dose of 30mg/ml ($P<0.01$) and 100mg/ml ($P<0.001$) of methacholine (Figure 6.2). Treatment of OVA-challenged mice with anti-IL-17RB resulted in equivalent Penh values in RV OVA (anti-IL-17RB) and UV OVA (anti-IL-17RB) mice, which were also similar in values in UV OVA (ISO) groups. However, comparison of anti-IL-17RB and isotype-treated RV OVA groups revealed no significant differences.

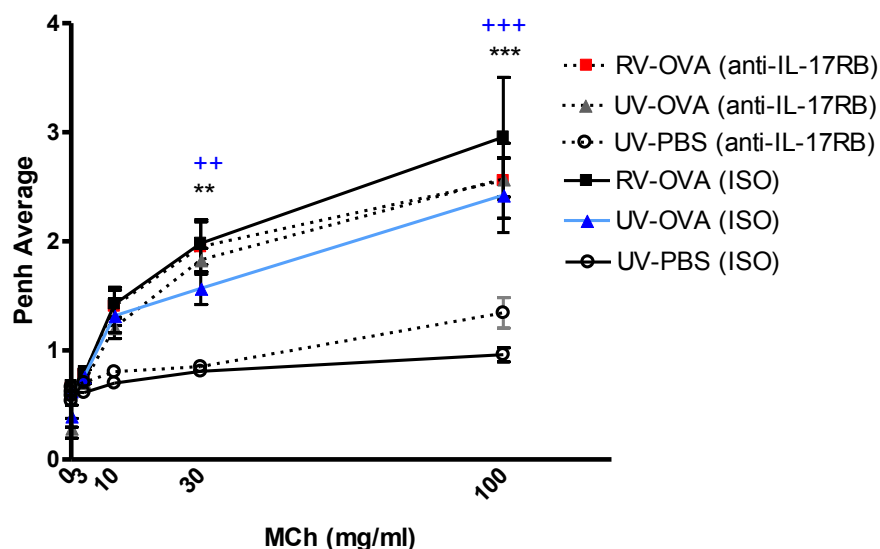


Figure 6.2 Effect of blocking IL-25 signalling on airway responses BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype antibody (groups represented as ‘ISO’) at 4h pre-infection as well as at d3 and d5 p.i. At d1 p.i., Penh was measured in response to increasing doses (in mg/ml) of aerosolised methacholine (MCh). Penh was measured over 5 minute periods after 1 minute aerosolisation challenge to methacholine. Aerosolised water was used to determine baseline values. Data is represented as the mean ($\pm$ -SEM) of 5 mice per treatment and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs UV PBS (anti-IL-17RB); + RV OVA (ISO) vs UV PBS (ISO) (**/++ $P<0.01$; ***/+++ $P<0.001$)

6.3.2 RV-1B viral load

To determine if neutralisation of IL-17RB affected viral replication, RV-1B RNA copies were measured in lung tissue. RV RNA levels were significantly reduced in RV OVA (anti-IL-17RB) mice compared with RV OVA (ISO) mice at 8h p.i. (Figure 6.3). At d1 p.i. RV RNA copies in RV OVA (ISO) mice were reduced to levels below those of RV OVA (anti-IL-17RB) mice, though these differences were not significant.

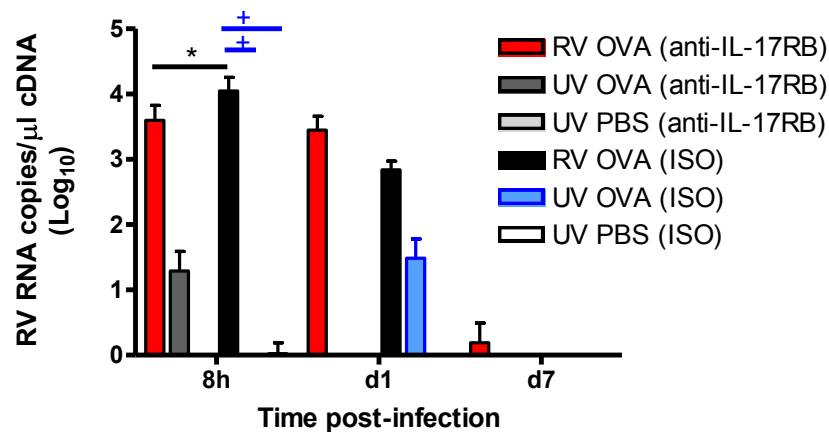


Figure 6.3 Effect of blocking IL-25 signalling on viral load BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as 'anti-IL-17RB') or isotype control antibody (groups represented as 'ISO') at 4h before infection as well as at d3 and d5 post-infection. Lungs were harvested at the indicated time-points and processed by extracting RNA and synthesizing cDNA to quantify viral RNA copies via qPCR. Data is expressed as the number of RNA copies per μl of cDNA added per qPCR reaction. Each group of mice (n=5) is represented as the mean (+/-SEM) and data is representative of 2 experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups (*/+ P<0.05).

6.3.3 Airway leukocyte infiltration

The effect of IL-17RB neutralisation on airway leukocyte infiltration was determined by differential staining of BAL cell cytopspins in our mouse model. In accordance with data from chapter 4, RV OVA (ISO) mice displayed significantly enhanced neutrophilia at d1 p.i. and delayed eosinophilia and lymphocytosis at d7 p.i. compared with all other isotype-treated groups.

In RV OVA (anti-IL-17RB) mice, airway neutrophilia was significantly decreased compared with RV OVA (ISO) ($P < 0.001$) and UV OVA (ISO) groups ($P < 0.001$) at d1 p.i. (Figure 6.4 A). Furthermore, RV OVA (anti-IL-17RB) mice had significantly reduced eosinophilia compared with RV OVA (ISO) groups ($P < 0.001$) at d7 p.i (Figure 6.4 B). Similarly, anti-IL-17RB treatment in RV OVA mice significantly decreased infiltration of lymphocytes into the airways compared with RV OVA (ISO) groups ($P < 0.001$) at d7 p.i (Figure 6.4 C). Analysis of UV OVA groups indicated that IL-17RB neutralisation had no significant effect on allergen-induced eosinophilia and lymphocytosis based on comparable levels in anti-IL-17RB and isotype-treated mice at d7 p.i.

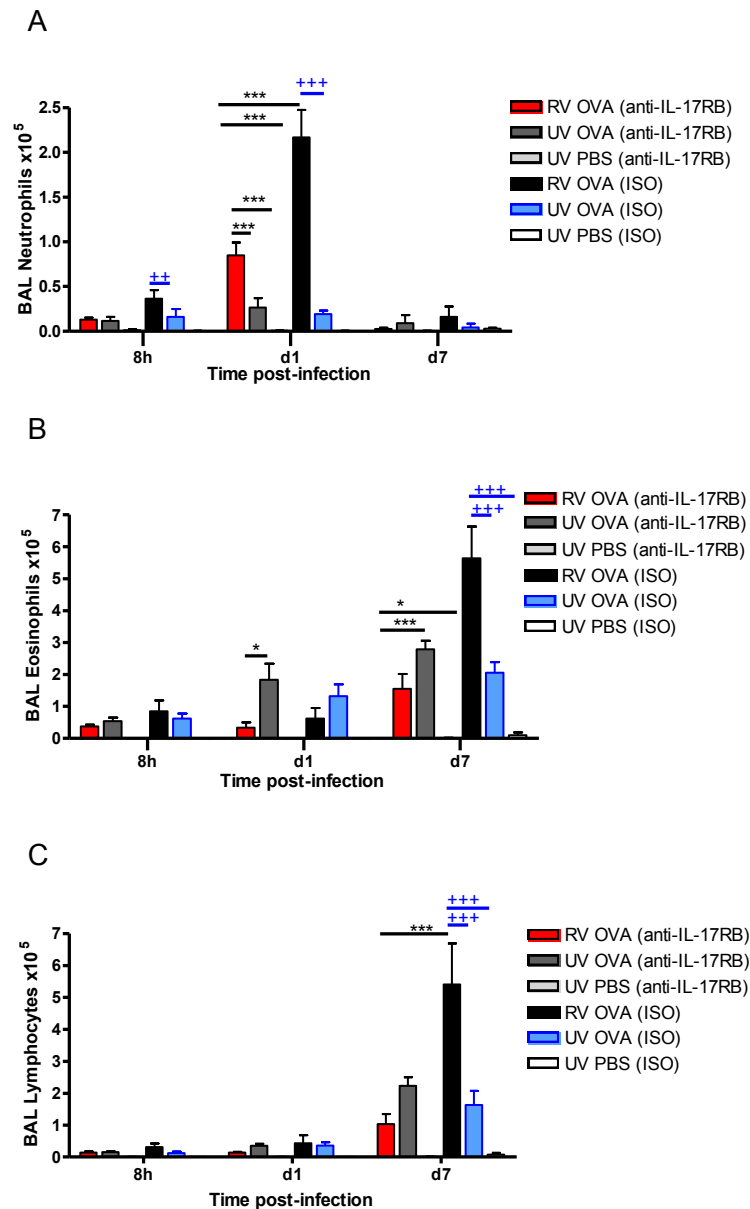


Figure 6.4 Effect of blocking IL-25 signalling on airway leukocyte infiltration BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. BAL cells from mice were fixed to cytospin slides and stained to quantify total numbers of neutrophils (A), eosinophils (B) and lymphocytes (C) at the indicated time-points p.i. All slides were blinded to experimental treatments. Data is represented as the mean (+/- SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all groups; + RV OVA (ISO) vs ISO groups (* $P < 0.05$; ++ $P < 0.01$; ***/+ $P < 0.001$).

6.3.4 BAL chemokines

Based on our findings that blocking IL-25 signalling decreased eosinophilic and lymphocytic infiltration in the airways of RV-infected and allergen-challenged mice, we sought to measure specific chemoattractants for these cell types. Specifically, the chemokines CCL17/TARC, CCL5/RANTES, CCL11/Eotaxin1 and CCL24/Eotaxin2 were measured in the BAL.

Comparing responses in isotype-treated groups confirmed the exacerbation phenotype represented in chapter 4; namely RV infection significantly induced chemokine expression in OVA-challenged mice compared with OVA-treated mice alone. In contrast, CCL5/RANTES, a potent chemoattractant of eosinophils and memory T cells, was significantly reduced in RV OVA (anti-IL-17RB) mice compared with RV OVA (ISO) mice ($P<0.001$) at d1 p.i (Figure 6.5 A). Blocking IL-17RB also resulted in lower levels of CCL17/TARC, which was significant compared with RV OVA (ISO) groups ($P<0.01$) at d7 p.i (Figure 6.5 B). In accordance with data displaying decreased eosinophilia, the eosinophil chemoattractants, CCL11/Eotaxin1 (Figure 6.5 C) and CCL24/Eotaxin2 (Figure 6.5 D), were significantly reduced in RV OVA (anti-IL-17RB) mice. Specifically, CCL11/Eotaxin1 protein was downregulated at 8h p.i. compared with RV OVA (ISO) groups ($P<0.05$) while CCL24/Eotaxin2 levels were significantly decreased compared with RV OVA (ISO) groups ($P<0.05$) at d1 p.i. IL-17RB neutralisation marginally downregulated allergen-induced CCL24/Eotaxin2 levels as indicated by decreased levels in UV OVA (anti-IL-17RB) mice compared with UV OVA (ISO) mice at d1 p.i., however these changes were not significant. Similar levels of chemokines and cytokines in OVA-challenged alone anti-IL-17RB and isotype-treated mice suggested that blocking the IL-25 receptor following allergen challenge had minimal effects on Th2 mediators in the airways.

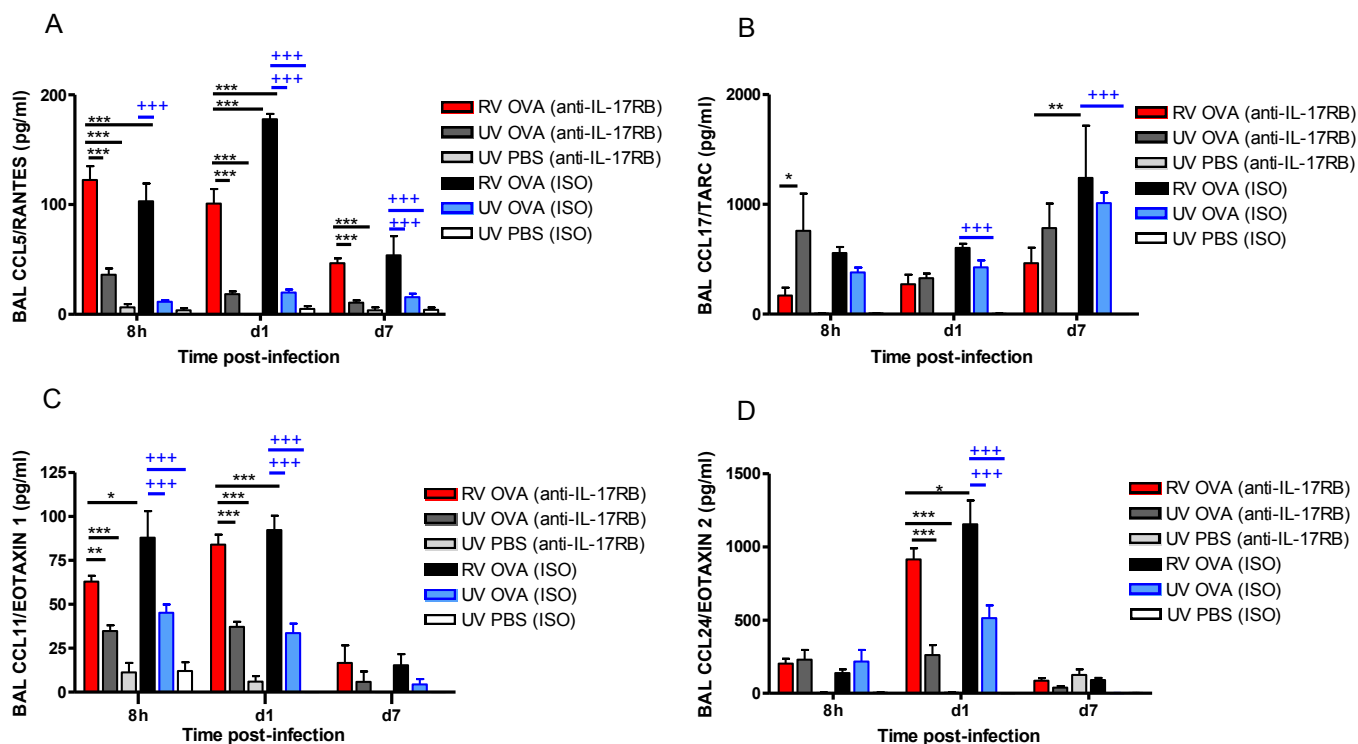


Figure 6.5 Effect of blocking IL-25 signalling on BAL chemokines BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. BAL from mice was collected at the indicated time-points and BAL supernatant was used to quantify CCL5/RANTES (A), TARC/CCL17 (B), CCL11/Eotaxin1 (C) and CCL12/Eotaxin2 (D) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups (* $P < 0.05$; ***/+++ $P < 0.001$).

6.3.5 BAL IL-6

As previously stated, RV infection of human epithelial cells induces the proinflammatory cytokine IL-6. Although this pleiotropic cytokine has a broad range of effects including immunomodulatory properties, whether it is beneficial or harmful during infection remains unclear²¹⁷. Results from isotype-treated groups confirmed previous finding that RV infection significantly enhanced early IL-6 protein production at 8h p.i. in OVA-challenged mice compared with allergen challenge alone ($P < 0.001$). We found that neutralising IL-17RB, in contrast to Th2-promoting chemokines, resulted in a profound enhancement of BAL IL-6 protein in RV OVA (anti-IL-17RB) mice which was significant compared with all groups of anti-IL-17RB and isotype-treated RV and/or OVA-challenged mice at 8h p.i. (RV OVA (anti-IL-17RB) vs RV OVA (ISO) $P < 0.001$; RV OVA (anti-IL-17RB) vs UV OVA (ISO) $P < 0.001$) (Figure 6.6).

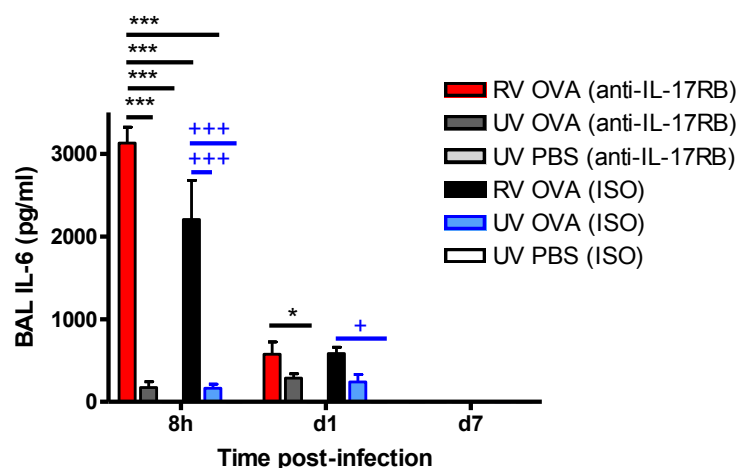


Figure 6.6 Effect of blocking IL-25 signalling on BAL IL-6 BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. BAL from mice was collected at the indicated time-points and BAL supernatant was used to quantify IL-6 by ELISA. Data is represented as the mean ($\pm$ SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups (*/+ $P < 0.05$; ***/+++ $P < 0.001$).

6.3.6 Th2 cytokine expression

6.3.6.1 BAL Th2 cytokines

Due to their central role in mediating asthma pathogenesis, we sought to investigate whether RV-induced enhancement of Th2 cytokines in OVA-challenged mice (reported in chapter 4) was affected by blocking IL-25 signalling. Therefore, BAL IL-4, IL-5 and IL-13 were measured in our mouse model of disease. Th2 cytokine responses in isotype-treated groups were consistent with previous data and confirmed an early exacerbation at 8h p.i. of Th2 cytokines in RV OVA mice compared with UV OVA mice (IL-4 $P<0.001$; IL-5 $P<0.01$; IL-13 $P<0.05$). Strikingly, levels of IL-4 (Figure 6.7 A), IL-5 (Figure 6.7 B) and IL-13 (Figure 6.7 C) protein in the airways were significantly reduced early on (8h p.i.) in RV OVA (anti-IL-17RB) mice compared with RV OVA (ISO) mice (IL-4 $P<0.001$; IL-5 $P<0.05$; IL-13 $P<0.05$). At d1 p.i., IL-5 was the only Th2 cytokine to remain significantly reduced in RV OVA (anti-IL-17RB) mice compared with isotype-matched groups.

By blocking IL-25 signalling, BAL Th2 cytokines levels in RV OVA (anti-IL-17RB) mice were reduced to levels similar to that in UV OVA (ISO) groups at 8h p.i. indicating specific inhibition of RV-induced Th2 inflammation. A modest decrease in IL-13 levels in UV OVA (anti-IL-17RB) groups compared with UV OVA (ISO) groups at 8h p.i. was observed, however these differences were not significant. The minimal decreases in Th2 cytokines in UV OVA (anti-IL-17RB) compared with isotype-matched groups further indicate that blocking the IL-25 receptor after allergen challenge had minimal effect on allergic inflammation.

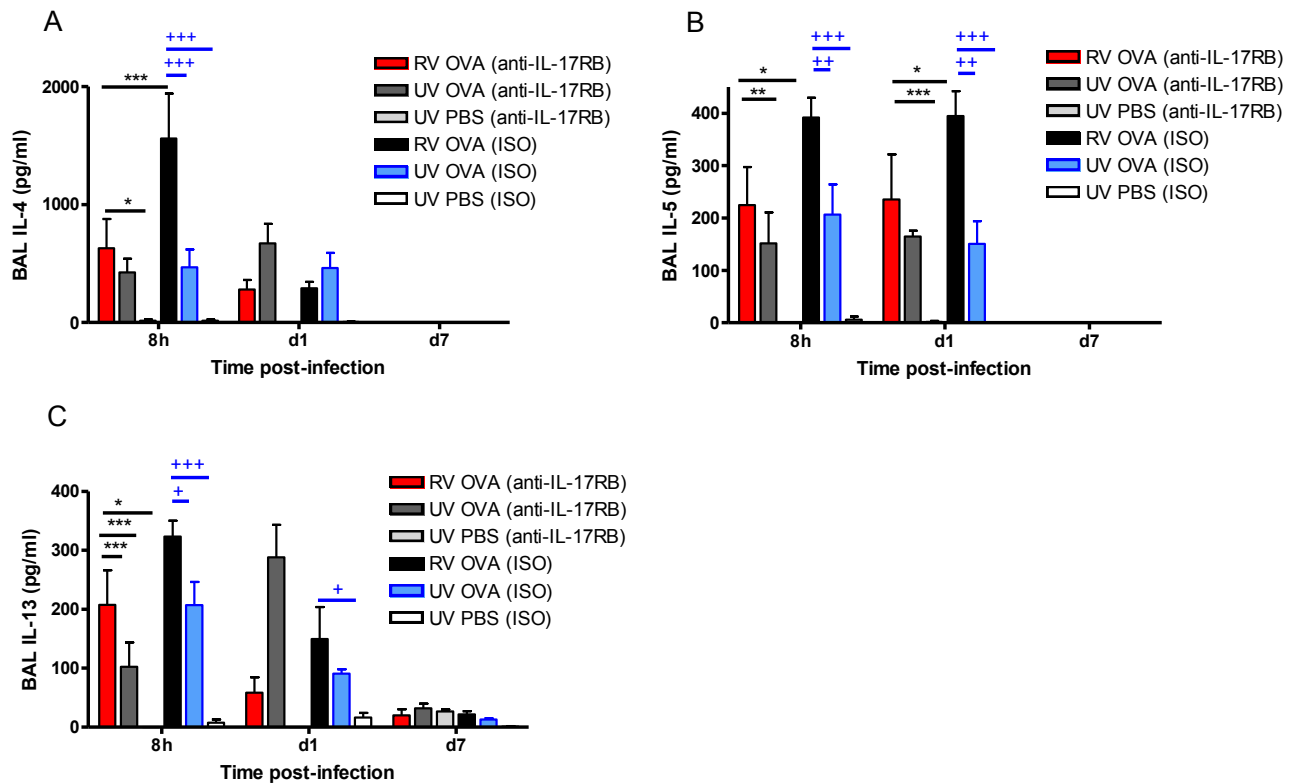


Figure 6.7 Effect of blocking IL-25 signalling on BAL Th2 cytokines BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. BAL from mice was collected at the indicated time-points and BAL supernatant was used to measure IL-4 (A), IL-5 (B) and IL-13 (C) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups (*/+ $P < 0.05$; ++/** $P < 0.01$; ***/+++ $P < 0.001$).

6.3.6.2 Th2 cytokines in lung homogenate

To assess if Th2 cytokine profiles in the BAL reflected those in the lung parenchyma, IL-4, IL-5 and IL-13 were also measured in lung homogenate from anti-IL-17RB and isotype-treated mice by ELISA. In contrast with IL-4 and IL-5 measurements in the BAL, there were no significant differences in IL-4 (Figure 6.8 A) and IL-5 (Figure 6.8 B) expression in lung homogenate between RV OVA (anti-IL-17RB) and RV OVA (ISO) groups at any time-points p.i. However, IL-13 protein expression in lung homogenate closely matched early IL-13

expression in the BAL and resultant blocking of IL-25 signalling significantly reduced protein in RV OVA (anti-IL-17RB) at all time-points p.i. compared with isotype-matched groups (Figure 6.8 C). Furthermore, blocking allergen-induced IL-25 signalling in UV OVA (anti-IL-17RB) groups decreased IL-13 level to those measured in RV OVA (anti-IL-17RB) mice at all time-points p.i.

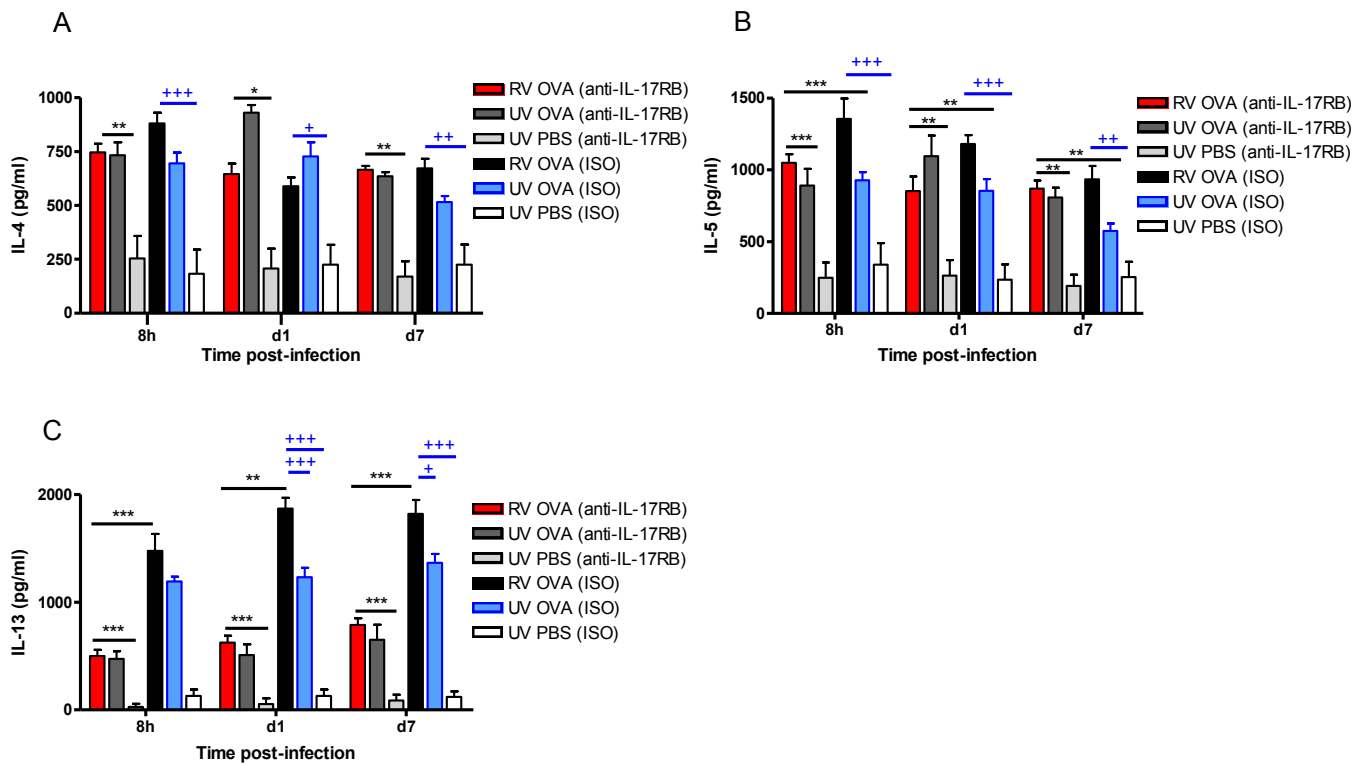


Figure 6.8 Effect of blocking IL-25 signalling on Th2 cytokines in lung tissue BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as 'anti-IL-17RB') or isotype control antibody (groups represented as 'ISO') at 4h before infection as well as at d3 and d5 post-infection. Lung tissue from mice was collected at the indicated time-points and processed lung homogenate was used to measure IL-4 (A), IL-5 (B) and IL-13 (C) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups (*/+ P<0.05; ++/** P<0.01; ***/+++ P < 0.001).

6.3.7 IL-25 expression

The effect of IL-17RB neutralisation on IL-25 expression was investigated in all antibody-treated groups. As IL-25 protein was undetectable in the BAL, lung homogenate was used to detect protein by ELISA while mRNA was quantified by qPCR in lung tissue. The early effects of receptor neutralisation did not significantly alter IL-25 mRNA (Figure 6.9 A) and protein expression (Figure 6.9 B) in any treatment groups at 8h p.i., indicated by similarities between anti-IL-17RB and isotype-matched mice. However, gene and protein levels were significantly reduced in RV OVA (anti-IL-17RB) mice compared with RV OVA (ISO) groups at d1 p.i. (mRNA $P<0.05$; protein $P<0.05$). In both instances, expression was reduced to similar quantities in UV OVA (ISO) and UV OVA (anti-IL-17RB) groups. Though differences in mRNA between RV OVA groups were no longer significant at d7 p.i., protein levels remained lower in anti-IL-17RB-treated mice compared with isotype-treated groups while mRNA levels in all treatment groups were sharply decreased. Allergen-induced IL-25 was not significantly altered by IL-17RB neutralisation indicated by similar levels in UV OVA (anti-IL-17RB) and UV OVA (ISO) groups at all time-points.

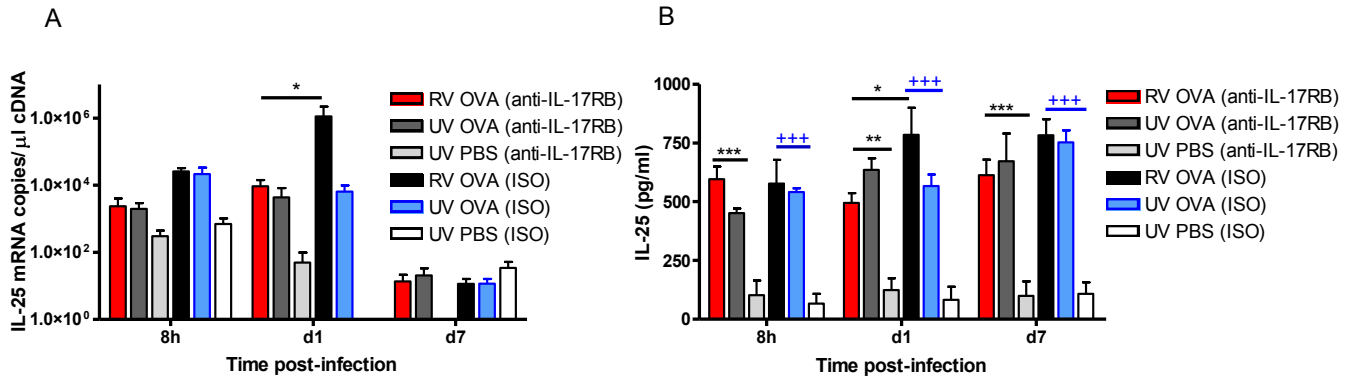


Figure 6.9 Effect of blocking IL-25 signalling on IL-25 expression in lung tissue BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. Lung tissue from mice was collected at the indicated time-points and used to measure IL-25 mRNA (A) and protein expression (B). Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups (* $P<0.05$; ** $P<0.01$; ***/+ P<0.001).

6.3.8 IL-33 and TSLP expression

Due to the related roles that IL-33 and TSLP have with IL-25 in the innate Th2 response, protein levels of IL-33 and TSLP were measured in lung homogenate from anti-IL-17RB and isotype-treated mice by ELISA. By d1 p.i., IL-33 (Figure 6.10 A) and TSLP levels (Figure 6.10 B) in RV OVA (anti-IL-17RB) groups were significantly reduced compared with RV OVA (ISO) groups. By d7 p.i., IL-33 levels in anti-IL-17RB and isotype-treated mice were still decreased while TSLP protein in RV OVA (anti-IL-17RB) groups was significantly upregulated compared to UV OVA (anti-IL-17RB) groups and matched levels in RV OVA (ISO) and UV OVA (ISO) groups. Notably, allergen-induced IL-33 and TSLP protein was downregulated by neutralising IL-17RB in UV OVA groups at d1 as well as d7 p.i. indicated by reduced amounts in UV OVA (anti-IL-17RB) mice compared with UV OVA (ISO) mice.

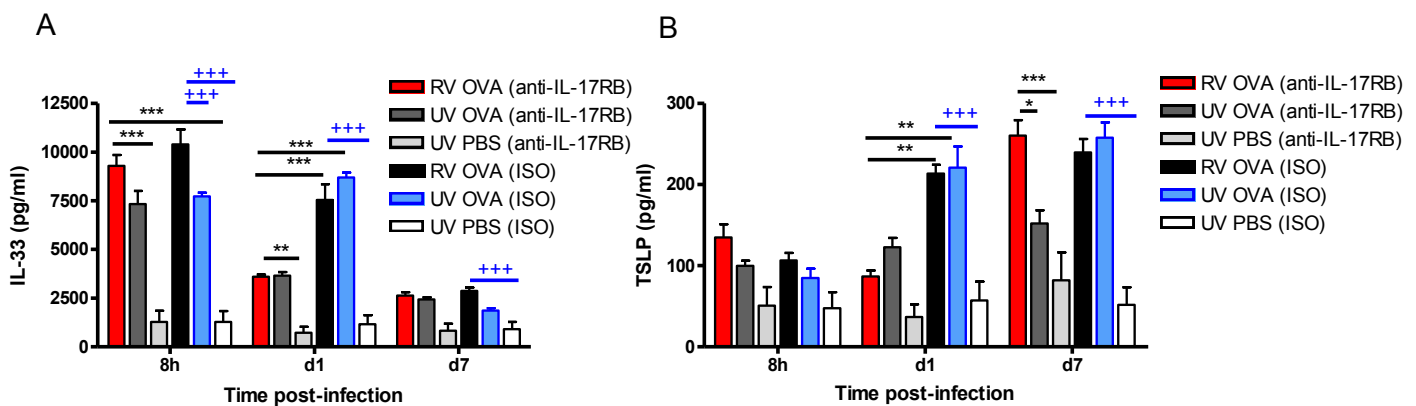


Figure 6.10 Effect of blocking IL-25 signalling on IL-33 and TSLP protein expression in lung tissue
BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as 'anti-IL-17RB') or isotype control antibody (groups represented as 'ISO') at 4h before infection as well as at d3 and d5 post-infection. Lung tissue from mice was collected at the indicated time-points and processed lung homogenate was used to measure IL-33 (A) and TSLP (B) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups; (* P < 0.05; ** P < 0.01; ***/+ P < 0.001)

6.3.9 BAL mucins

Levels in the BAL of the two major respiratory mucins, MUC5ac and MUC5b, were measured to assess the effects of blocking IL-25 signalling. Results recorded in isotype-treated groups substantiated data from chapter 4; namely, MUC5ac was significantly enhanced in RV OVA mice compared with OVA-challenged mice alone at d7 p.i. ($P<0.001$) (Figure 6.11 A), whereas there were no differences in MUC5b protein levels between OVA-challenged groups at any time-points (Figure 6.11 B). IL-17RB neutralisation caused a significant reduction in MUC5ac protein in RV OVA (anti-IL-17RB) mice compared with RV OVA (ISO) groups ($P<0.001$) at d7 p.i. In contrast, MUC5b levels between both groups of antibody-treated OVA-challenged mice remained largely unchanged at all time-points p.i.

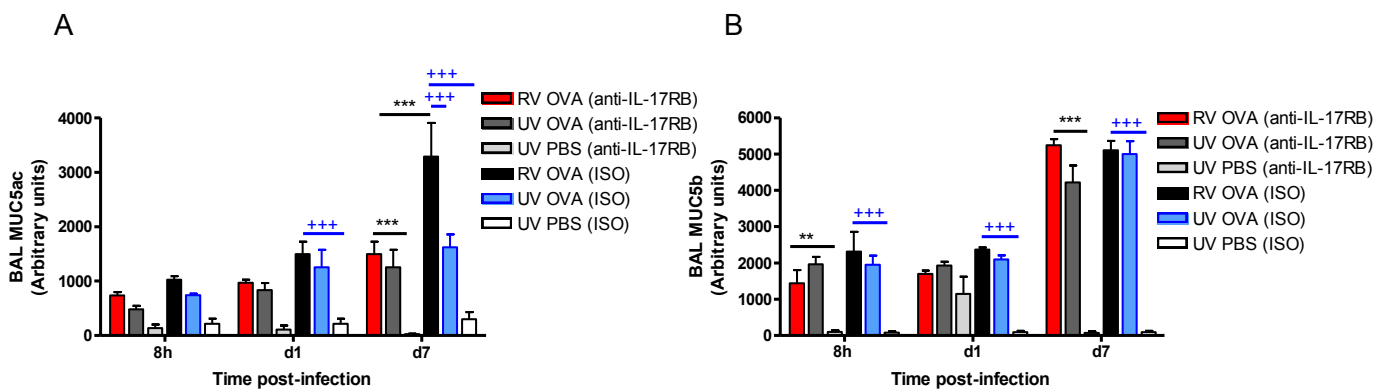


Figure 6.11 Effect of blocking IL-25 signalling on BAL mucins BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. BAL from mice was collected at the indicated time-points and BAL supernatant was used to quantify MUC5ac (A) and MUC5b (B) by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups (** $P<0.01$; ***/+ P<0.001).

6.3.10 Measurement of serum IgE

Total and OVA-specific IgE in serum from both antibody-treated groups at d7 p.i. was measured by ELISA. Neutralisation of IL-17RB caused a trend towards decreased total serum IgE levels in RV OVA (anti-IL-17RB) groups compared with RV OVA (ISO) groups though these differences were not significant (Figure 6.12 A). No clear differences in OVA-specific IgE levels were observed between anti-IL-17RB and isotype-treated groups (Figure 6.12 B). Allergen-induced IgE was largely unaltered by anti-IL-17RB treatment indicated by similar OVA-specific and total IgE levels in UV OVA (anti-IL-17RB) and UV OVA (ISO) groups.

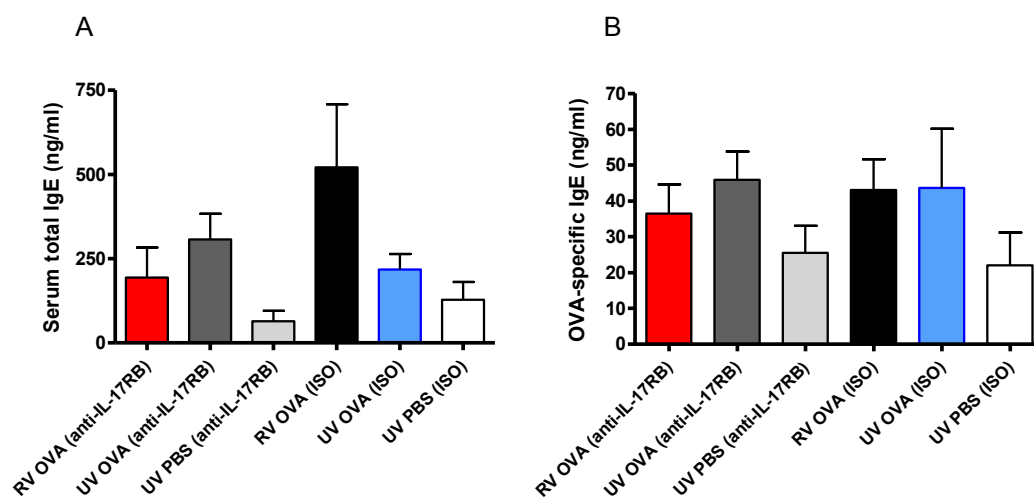


Figure 6.12 Effect of blocking IL-25 signalling on total and OVA-specific IgE BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as 'anti-IL-17RB') or isotype control antibody (groups represented as 'ISO') at 4h before infection as well as at d3 and d5 post-infection. Blood was collected from the carotid artery of mice at d7 p.i. and serum was separated to measure total (A) and OVA-specific (B) IgE by ELISA. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments.

6.3.11 Effect of blocking IL-25 signalling on innate (non-T) and adaptive CD4+ T cell populations

Flow cytometry was used to characterise the effects of IL-17RB neutralisation on the magnitude and kinetics of non-T and T cell populations associated with Th2 immunity in a model of RV-induced allergic airways disease. An identical panel of markers (as described in chapter 5 and table 2.10) was used to identify cell populations. Due to the fact that the same antibody clone (Clone D9.2) was used for *in vivo* neutralisation and for previous identification of IL-17RB+ cells, flow cytometry detection of IL-17RB expressing populations could not be carried out in blocking antibody experiments.

6.3.11.1 Innate cell populations

i.) Innate lymphoid cells (ILC) 2

Based on an enhanced infiltration of ILC2s into the airways of RV OVA mice at d1 p.i. reported in chapter 5, we sought to investigate the effects of blocking IL-25 signalling on this IL-25-responsive cell population in our model of RV-induced allergic airways disease. Furthermore, due to the fact that ILC2s are reported as an early source of IL-5 and IL-13 in the airways of allergen-sensitised and challenged mice, we hypothesized that reduced BAL IL-5 and IL-13 levels demonstrated in section 6.3.7.1 may be caused by inhibition of cytokine release due to IL-17RB neutralisation.

A population of T1/ST2+ICOS+IL-4-Fc ϵ RI-CD49b-CD3-CD4- cells was characterised in the lung and BAL of IL-17RB-neutralised mice and isotype-treated controls, which matched the surface phenotype of previously identified ILC2s (predominantly indicated by the fact that identified populations were non T cells yet expressed ICOS). There was a trend towards a reduction in cell populations in the airways of RV OVA (anti-IL-17RB) groups compared with RV OVA (ISO) groups at all time-points p.i.; however analysis revealed no significant differences between groups (Figure 6.13 A). With regard to whole lung tissue, cell numbers in both antibody-treated groups were similar with no significant variations at any time-point p.i. (Figure 6.13 B).

Cell populations in the airways of isotype-treated OVA-challenged mice displayed similar results to those recorded in chapter 5; namely a peak in number at d1 p.i. that was

significantly enhanced in RV OVA groups compared with UV PBS groups. In addition, cells in whole lung tissue from OVA-challenged isotype-treated mice peaked at 8h and d7 p.i. with a sharp decrease between time-points at d1 p.i. which was also consistent with findings in chapter 5.

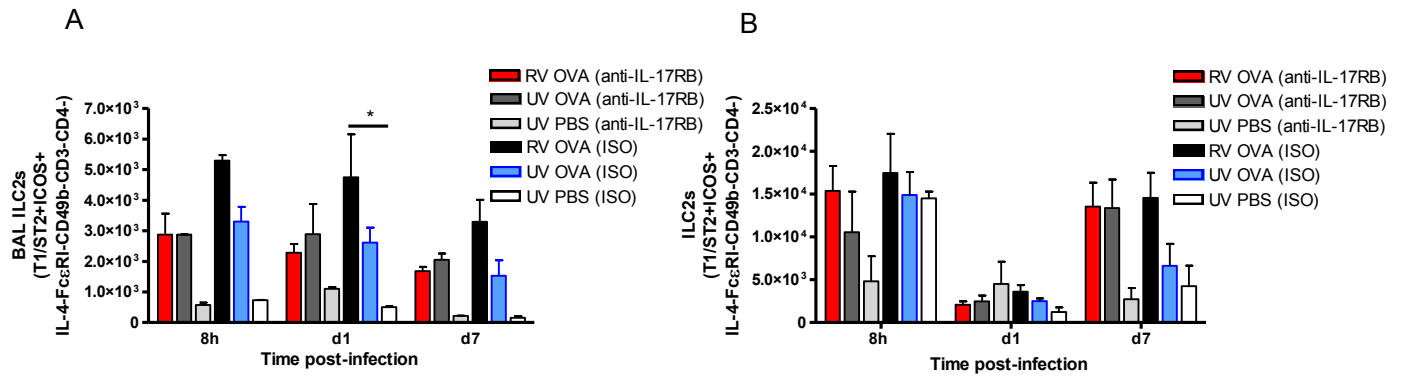


Figure 6.13 Effect of blocking IL-25 signalling on ILC2s BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. The left lung lobe was harvested at the indicated time-points p.i. (after collecting the BAL) and lung cells were processed as previously described. Cells were stained for CD3, CD4, T1/ST2, ICOS, FcεRI, CD49b and intracellular IL-4. Total BAL ILC2s (T1/ST2+ICOS+IL-4-FcεRI-CD49b-CD3-CD4-) (A) and total cells numbers per whole mouse lung (B) are represented. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments.

ii.) IL-4+ basophils

Due to a significant early decrease in BAL IL-4 in anti-IL-17RB-treated RV OVA mice, we also investigated intracellular sources of IL-4 and the effects of blocking IL-25 signalling by flow cytometer analysis.

Analysis of the IL-4+ basophils (FSC^{lo}/SSC^{lo}CD3-CD4-FcεRI+T1/ST2+CD49b+) in anti-IL-17RB and isotype-treated mice revealed varied profiles in the airways and in whole lung tissue. Specifically, RV OVA (anti-IL-17RB) groups had significantly reduced BAL IL-4+ basophils compared with RV OVA (ISO) at d1 p.i (Figure 6.14 A and 6.15). In contrast, cell numbers measured in whole lung tissue from RV OVA (anti-IL-17RB) and RV OVA (ISO) groups were

similar at 8h and d1 p.i. (Figure 6.14 B) with no significant differences between any other treatment groups.

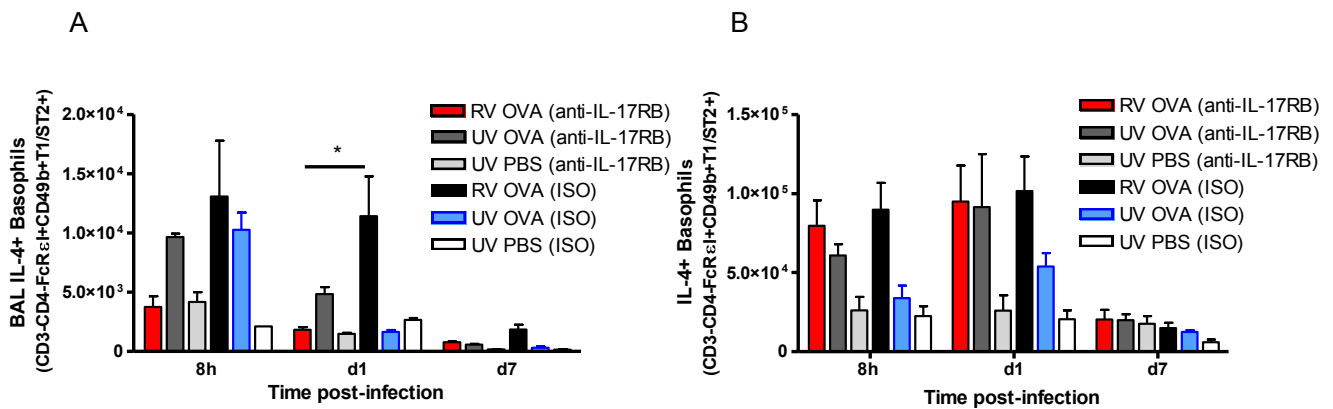


Figure 6.14 Effect of blocking IL-25 signalling on basophils BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. The left lung lobe was harvested at the indicated time-points p.i. (after collecting the BAL) and lung cells were processed as previously described. Cells were stained for CD3, CD4, FcεRI, CD49b, T1/ST2, ICOS and intracellular IL-4. The total IL-4+ basophils (CD3-CD4-FcεRI+CD49b+T1/ST2+) in BAL lung cells (A) and total cells numbers per whole mouse lung (B) are represented. Data is represented as the mean (+/-SEM) of 5 mice per group and is representative of 2 independent experiments.

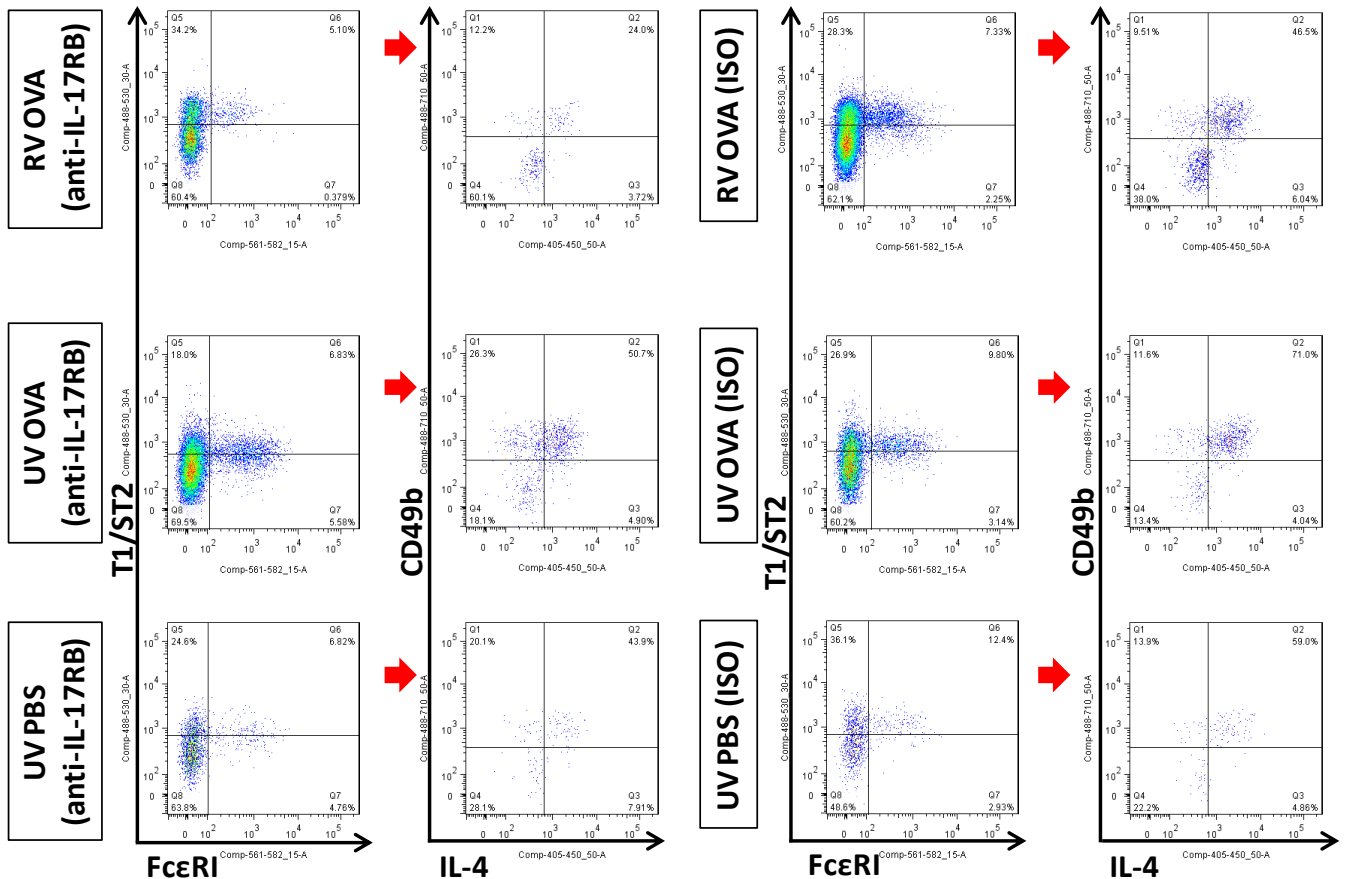


Figure 6.15 Representative flow plots of BAL IL-4+ basophils in a model of RV-induced allergic airways disease at d1 p.i. Flow plots represent gated T1/ST2+/FcεRI+ and CD49b+/IL-4+ BAL cells in anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’)-treated RV OVA, UV OVA and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 2 independent studies.

6.3.11.2 Adaptive Th2 cell populations

i.) IL-4+ CD4+ T cells

Based on the effects of RV-induced enhancement of Th2 cells in OVA-challenged mice reported in chapter 5, we aimed to investigate the effect of blocking IL-25 signalling in our mouse model of disease.

In isotype-treated mice, RV infection in OVA-challenged and sensitised groups significantly enhanced IL-4+CD4+ T cells compared with OVA-challenge alone at d7 p.i. as demonstrated in chapter 5. Analysis of the effects of blocking IL-25 signalling demonstrated that IL-17RB neutralisation had a striking effect on IL-4+CD4+ T cells in the airways and in whole lung cell

preparations of RV-infected and OVA-challenged mice. Specifically, levels of BAL (Figure 6.16 A and 6.17.1) and lung IL-4+ CD4+ T cells (Figure 6.16 B and 6.17.2) in RV OVA (anti-IL-17RB) mice were significantly reduced compared with RV OVA (ISO) groups (BAL $P<0.01$; Lung $P<0.001$) at d7 p.i. These levels were reduced to quantities observed in UV OVA (ISO) groups and were also similar to amounts in UV OVA (anti-IL-17RB) groups, indicating that IL-17RB neutralisation had minimal effect on allergen-induced IL-4+ CD4+ T cells.

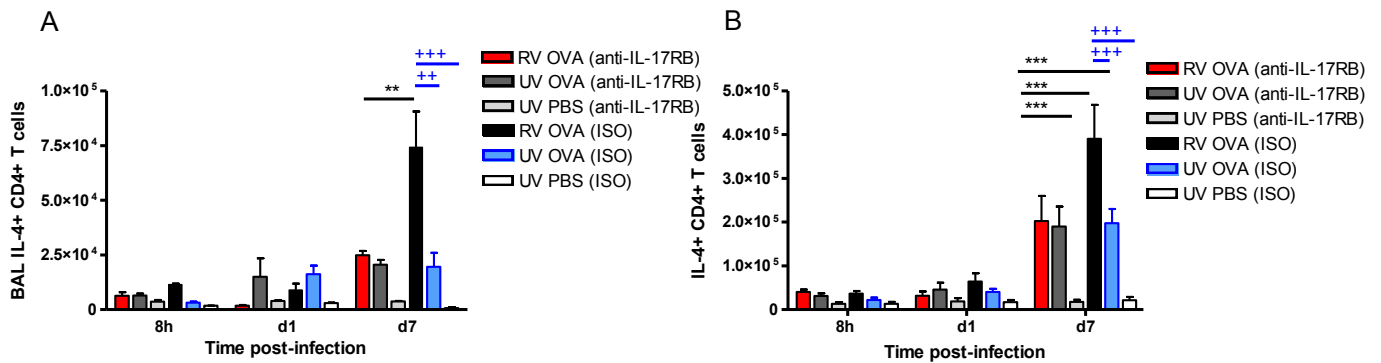


Figure 6.16 Effect of blocking IL-25 signalling on IL-4+ CD4+ T cells BALB/c mice were treated as described in section 2.2.5 and administered anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’) at 4h before infection as well as at d3 and d5 post-infection. The left lung lobe was harvested at the indicated time-points p.i. (after collecting the BAL) and lung cells were processed as previously described. Cells were stained for CD3, CD4, FcεRI, CD49b, T1/ST2, ICOS and intracellular IL-4. Total IL-4+ CD4+ T cells in the BAL (A) and total cells numbers per whole mouse lung (B) are represented. Data is represented as the mean (+/- SEM) of 5 mice per group and is representative of 2 independent experiments. * RV OVA (anti-IL-17RB) vs all other groups; + RV OVA (ISO) vs ISO groups (** $P<0.01$; ***/+ P<0.001).

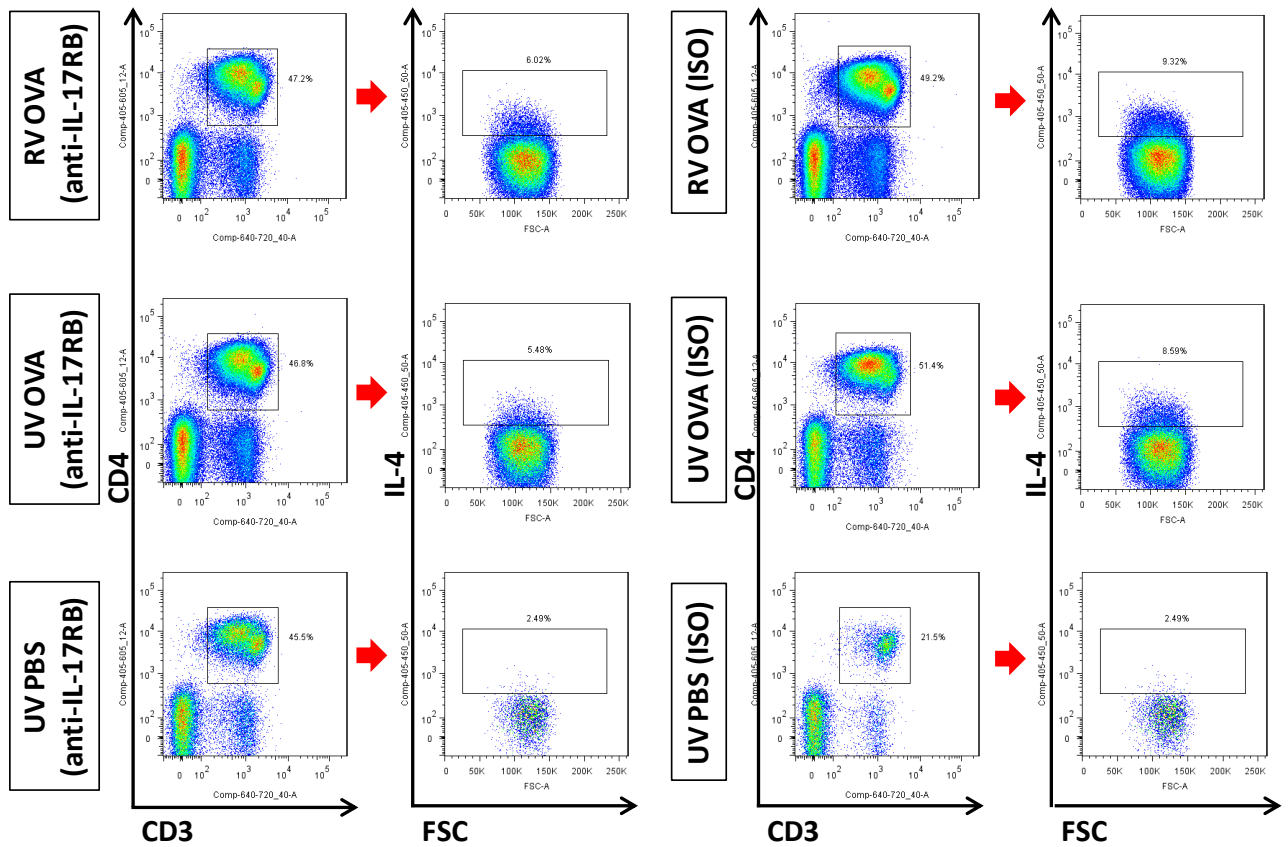


Figure 6.17.1 Representative flow plots of BAL IL-4+ CD4+ T cells in a model of RV-induced allergic airways disease at d7 p.i. Flow plots represent gated CD3+/CD4+ and IL-4+ BAL cells in anti-IL-17RB antibody (groups represented as ‘anti-IL-17RB’) or isotype control antibody (groups represented as ‘ISO’)-treated RV OVA, UV OVA and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 2 independent studies.

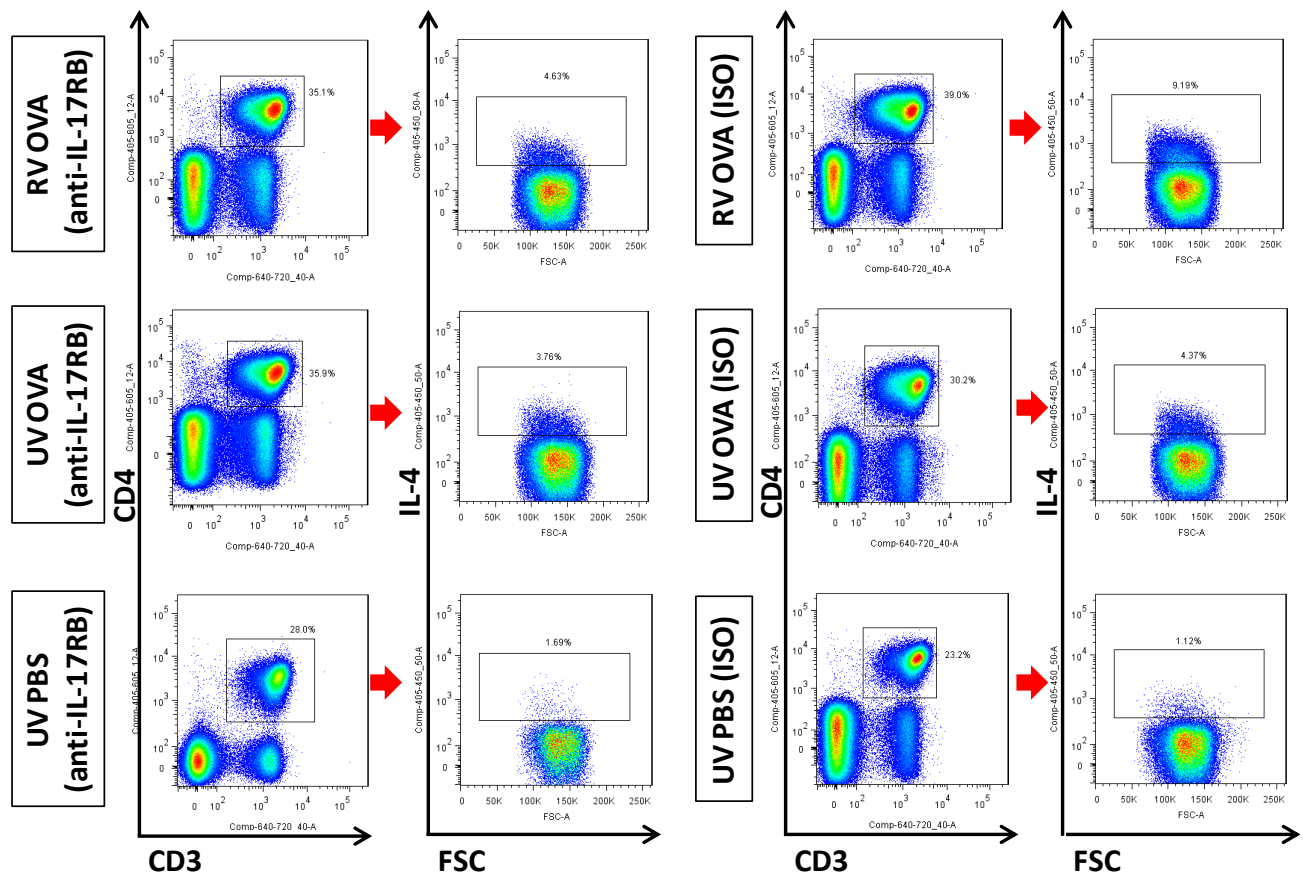


Figure 6.17.2 Representative flow plots of IL-4+ CD4+ T cells in whole lung tissue in a model of RV-induced allergic airways disease at d7 p.i. Flow plots represent gated CD3+/CD4+ and IL-4+ cells in anti-IL-17RB antibody (groups represented as 'anti-IL-17RB') or isotype control antibody (groups represented as 'ISO')-treated RV OVA, UV OVA and UV PBS mice. Numbers indicated on the plots reflect the percentage of cells that are present with the gated areas. Results are representative of 2 independent studies.

	Early/innate effects of IL-17RB neutralisation in RV OVA mice		Later/adaptive effects of sustained IL-17RB neutralisation in RV OVA mice
	8h p.i.	d1 p.i.	d7 p.i.
Airway obstruction	-	-	-
RV viral load	↓ (*)	-	-
Airway neutrophilia	-	↓ (***)	-
Airway eosinophilia & lymphocytosis	-	-	↓ (***)
BAL IL-4 protein	↓ (***)	-	-
BAL IL-5 protein	↓ (*)	↓ (*)	-
BAL IL-13 protein	↓ (*)	-	-
Lung IL-4 protein	-	-	-
Lung IL-5 protein	-	-	-
Lung IL-13 protein	↓ (***)	↓ (***)	↓ (***)
Lung IL-25 protein and mRNA	-	↓ (*)	-
Lung IL-33 protein	-	↓ (***)	-
Lung TSLP protein	-	↓ (**)	-
BAL CCL5/RANTES protein	-	↓ (***)	-
BAL CCL17/TARC protein	-	-	↓ (**)
BAL CCL11/Eotaxin 1	↓ (*)	-	-
BAL CCL24/Eotaxin 2	-	↓ (*)	-
BAL MUC5ac protein	-	-	↓ (***)
BAL MUC5b protein	-	-	-
BAL IL-6 protein	↑ (***)	-	-
Total and OVA-specific IgE	-	-	-
BAL ILC2s	-	-	-
Lung ILC2s	-	-	-
BAL basophil	-	↓ (*)	-
Lung basophil	-	-	-
BAL IL-4+ CD4+ T cells	-	-	↓ (**)
Lung IL-4+ CD4+ T cells	-	-	↓ (***)

Table 6.2 A summary of relative statistically significant increases and decreases for RV OVA (anti-IL-17RB) vs RV OVA (ISO). *P<0.05; **P<0.01; ***P<0.001; - no significant changes

6.4 Summary

By blocking IL-25 signalling, RV-mediated enhancement of several key Th2-associated allergic responses were inhibited as indicated by reductions in anti-IL-17RB-treated RV OVA groups compared with isotype-treated RV OVA groups. In some instances, these responses were reduced to levels measured in allergen-treated mice alone therefore indicating a reversal of the exacerbating effect of RV infection. The most striking examples of abrogated Th2 inflammation was a dramatic early reduction in airway IL-4, IL-5 and IL-13 protein levels and a later decrease in BAL and lung IL-4+ CD4+ T cells in RV OVA mice administered anti-IL-17RB antibody. In contrast, blocking allergen-induced IL-25 signalling had a minimal effect on several key features of Th2 inflammation in established allergic airways disease specifically indicating a role for RV-induced IL-25 in the exacerbation phenotype.

Additional early reductions in Th2-related mediators in anti-IL-17RB-treated RV OVA mice included CCL5/RANTES, CCL11/Eotaxin 1 and CCL24/Eotaxin 2 in the airways as well as decreased IL-25, IL-33, TSLP and IL-13 in lung tissue. All these read-outs were significantly reduced at 8h and/or d1 p.i. compared with isotype-matched groups. Notably, neutrophilic inflammation, which has previously been associated with symptom severity²⁰⁵, was also significantly diminished in the airways of RV OVA (anti-IL-17RB) mice at d1 p.i.

Repeated doses of anti-IL-17RB antibody administered at d3 and d5 p.i. sustained inhibition in RV OVA mice resulting in reduced airway eosinophilia and lymphocytosis at d7 p.i., which coincided with decreased levels in the Th2 cell chemokine, CCL17/TARC. Later effects of IL-17RB neutralisation also included reduced levels of respiratory mucin, MUC5ac, in RV OVA mice. Again, these observations were all significantly decreased compared with isotype-treated RV OVA mice.

Reduction of early BAL IL-5 and IL-13 observed in RV OVA (anti-IL-17RB) mice coincided with a trend towards decreased ILC2 numbers in the airways. However, future work will focus on measurement of intracellular expression of IL-5 and IL-13 by flow cytometry to establish an association between cell numbers and cytokine expression. We were able to demonstrate that reduced IL-4 in the airways was partly due to a lack of infiltrating IL-4+ basophils as cell numbers were reduced in the airways of RV OVA (anti-IL-17RB) mice at 8h and d1 p.i. In contrast, IL-4+ basophils in the whole lung tissue were similar in anti-IL-17RB and isotype-

treated groups potentially indicating that blocking IL-17RB resulted in a decrease in IL-4-expressing basophil recruitment into the airways.

Despite a predicted decrease in a number of Th2-associated read-outs in anti-IL-17RB-treated RV OVA groups, some results were in contradiction with our original hypotheses. For example, despite significantly reduced levels of IL-13 and airway leukocyte infiltration in the lungs and airways in anti-IL-17RB-treated mice, airway responses as measured by whole-body plethysmography were not inhibited. An intriguing finding was that blocking IL-25 signalling significantly reduced viral load at 8h p.i. which coincided with upregulated BAL IL-6 levels in RV OVA mice, potentially indicating an increased Th1 response as a result of a dampened Th2 responses caused by IL-17RB neutralisation.

In summary, blocking IL-25 signalling negated RV-induced enhancement of Th2 immunity suggesting that RV-induced IL-25 plays a role in enhancement of Th2 inflammation in a mouse model of RV-induced allergic airways disease.

Chapter 7: Discussion and future work

RV infections are the principal cause of asthma exacerbations. While Th2-mediated inflammation is clearly implicated in the asthmatic response, it is unknown how the immune response to RV infection interacts with Th2 immunity to enhance disease pathogenesis^{92,95,218}. Epithelial-derived IL-25 has been identified in several studies as an initiator and regulator of Th2 immunity and plays a role in asthma pathogenesis^{70,127,170}. Given that bronchial epithelial cells are the primary site of RV infection, it may be hypothesised that RV infection of the airway epithelium induces the pro-Th2 cytokine, IL-25, providing a link between infection and Th2 driven allergic inflammation.

In this study, we aimed to:

- investigate RV-induced IL-25 expression in human airway epithelial cell lines and HBECs obtained from bronchoscopic brushings from atopic asthmatics and healthy patients
- examine induction of IL-25 to RV infection in the otherwise untreated mouse airway
- employ a mouse model of RV-induced allergic airways disease to assess the effects of infection and allergen on IL-25, Th2-associated mediators and IL-17RB-expressing cell populations
- define a mechanistic role for RV-induced IL-25 by blocking signalling mediated by IL-25 in our model of disease and measure key Th2 mediators associated with the exacerbation phenotype

These objectives were carried out to facilitate the characterisation of the role of IL-25 in RV-induced asthma exacerbations.

7.1 RV-induced IL-25 expression *in vitro*

Based on the pivotal role the airway epithelium plays in shaping immune responses to various stimuli including RV infection, we investigated RV-induced expression of IL-25 in human airway epithelial cell lines and HBECs obtained from atopic asthmatics and non-asthmatic healthy controls.

Of major significance was the finding that RV infection induced epithelial-derived IL-25 mRNA and protein *in vitro*. Furthermore, HBECs from asthmatics exhibited a greater

capacity to produce IL-25 than cells from healthy controls. The finding that RV upregulates a driver of Th2 inflammation represents a mechanistic link between the antiviral response and Th2 induction and potentially a role for IL-25 in the initiation of RV-induced exacerbation of allergic inflammation. Previous *in vitro* studies have reported IL-25 gene expression in response to the allergens, *Aspergillus oryzae* and ragweed, in the human alveolar basal epithelial cells, A549, and mouse primary lung epithelial cells and cell lines⁷⁰. Xu *et al.* also demonstrated IL-25 induction in cultured HNECs in response to the dsRNA analogue, poly I:C, mimicking viral infection¹⁶⁰. To our knowledge this is the first study to demonstrate RV-induced IL-25 from HBECs *in vitro*.

The differential response of HBECs from asthmatics and healthy controls in response to RV infection also highlights intrinsic differences in the bronchial epithelium between groups. Employing similar conditions for *ex vivo* culturing of HBECs from asthmatic and healthy patients, a separate study demonstrated that RV-infected cells from asthmatics produced less type I and type III IFNs which was associated with enhanced viral load^{102,103}; therefore innate deficiencies may result in increased viral replication accounting for increased proinflammatory mediators including upregulated IL-25 production in the asthmatic epithelium. Similar findings were reported relating to the Th2 promoting cytokine, TSLP; HBECs from asthmatic children were shown to express more TSLP in response to RSV infection compared with cells from healthy children¹³⁷. These results also suggest a greater propensity of the asthmatic epithelium to drive Th2 inflammation via the augmented release of virus-induced mediators.

Enhanced IL-25 protein expression was also associated with clinical outcomes of disease; increased protein levels at 24h p.i. significantly correlated with sensitisation to a greater number of common environmental allergens in asthmatic patients while there was also an association with increased numbers of recorded asthma exacerbations per year. This is intriguing given that clinical studies indicate that the combination of allergic sensitisation, exposure to high levels of allergen and viral infections are strongly associated with the risk of hospital admittance with acute asthma^{92,95}. The fact that atopy (as indicated by the presence of allergen-specific IgE) was associated with a tendency to produce more RV-induced IL-25 in atopic asthmatics further suggests that RV interacts with the allergic

phenotype potentially increasing susceptibility to initiation of acute asthma mediated by IL-25.

RV-induced IL-25 gene expression was also detected in the human bronchial epithelial cell line, BEAS-2B. Based on an absence of IL-25 mRNA in response to UV-inactivated virus and a robust infection-dependant induction of IL-6 protein, IL-25 expression was dependant on viral replication. However, gene expression levels in RV-infected BEAS-2B cells were comparatively low compared with HBECs infected *ex vivo* as indicated by undetectable IL-25 protein levels. Furthermore, additional airway epithelial cell lines (including A549s and H292s) were a poor model for RV-induced IL-25 compared with robust expression in HBECs from asthmatics and, to a certain extent, healthy controls. Varying responses between primary cells and cell lines is not an uncommon phenomenon and disparities in responses to stimuli between primary HBECs and airway epithelial cell lines have been reported²¹⁹. Modifications of cell lines via transformation or derivation from human lung carcinoma to allow unlimited cell division indicate a tendency for genetic drift and therefore models are less physiologically relevant compared with primary cells²¹⁹. *Ex vivo* models of HBECs from asthmatics and healthy controls represent not only the most accurate physiological conditions, but also enable direct comparisons of varying responses to stimuli in the diseased and healthy state.

In our study, submerged cultures were employed on the basis that this enabled measurement of IL-25 expression after 2-3 passages as opposed to air-liquid interface cultures where cells are continuously cultured for a number of weeks. This was important for our purposes as it was theorised that extended culture may result in loss of any intrinsic variations between asthmatic and healthy cells. In addition, the fact that cell lines as well as commercially purchased HBECs (which were frozen down and passaged 5-6 times before harvest) expressed very limited amounts of IL-25 gene may be as a result of multiple passages/extended time in culture. As a result, cells from bronchial brushings were grown as submerged monolayer cultures.

7.2 RV-induced IL-25 expression *in vivo*

Based on the findings that RV induced IL-25 *in vitro*, we sought to characterise IL-25 expression in a mouse model of RV infection. Using this model, we confirmed a release of mediators in BALB/c mice that have been measured in patients experimentally infected with RV as well as *in vitro* studies of RV-infected HBECs^{40,205}. These read-outs included an early robust neutrophilic response at 10h p.i. followed by lymphocytic inflammation as well as strong induction of the mouse CXCR2-specific chemokine, CXCL1/KC, the proinflammatory cytokines, IL-6, IFN- λ and CCL5/RANTES in the airways of RV-infected mice which were consistent with published results for this model²⁰².

In accordance with findings *in vitro*, RV infection significantly upregulated IL-25 gene and protein expression in the otherwise untreated mouse airway. Increased levels of IL-25 in RV-infected mice demonstrated that production was predominantly virus replication dependent. However, mice treated with UV-inactivated virus also expressed comparatively reduced levels which mimicked patterns of IL-25 expression observed in RV-infected mice indicating that a component of IL-25 induction was also dependant on virus-receptor binding²²⁰. The induction of IL-25 in response to viral infection in naive BALB/c mice has also been demonstrated by Siegle *et al.* However, this study employed a chronic asthma model in neonatal mice infected with a species-specific form of RSV. In this instance, measurement of IL-25 gene was limited to two time-points after infection only, namely 2 weeks and 4 weeks p.i.¹⁷⁷. In contrast, we investigated the acute effects of RV infection on IL-25 gene and protein over several time-points up until d7 p.i. We reported a significant early increase in IL-25 gene expression in the lungs which decreased steadily while protein levels in RV-infected mice remained elevated till d7 p.i. Based on these findings, future assessment of the duration that IL-25 protein levels remain upregulated after infection as well as what effects re-infection may have on further IL-25 enhancement may be noteworthy.

IL-25 gene expression was also significantly upregulated at 10h p.i. in the BAL cells of RV-infected mice and, like total gene expression levels, gradually decreased till d7 p.i. The fact that neutrophils comprised over 80% of the total BAL cells early after infection may implicate these cells as a source of IL-25. In support of this, we identified immunoreactive IL-25 cells in the airway lamina propria that were morphologically similar to neutrophils.

Previous identification of neutrophils as IL-25 producers has not been reported; however it is pertinent that IL-25 has been shown to enhance neutrophil infiltration via upregulation of the neutrophil chemoattractant, CXCL8/IL-8, possibly indicating a positive self-feedback loop²²⁵. Furthermore, IL-25-induced CXCL8/IL-8 production was shown to be mediated by activation of NF- κ B *in vitro*, representing a common transcription factor activated in response to RV and IL-25 following CXCL8/IL-8 production¹⁵². Accordingly, IL-25 in conjunction with RV infection may have an additive effect on neutrophil recruitment via upregulated CXCL8/IL-8 levels. This is of relevance as RV-induced neutrophilia has been associated with disease severity while neutrophil deficiency in mice was related to decreased AHR and BAL mucins²²¹. An observed decrease in neutrophilia in the airways of IL-17RB-neutralised RV-infected and OVA-challenged mice supports this premise which will be discussed in greater detail in section 7.5.

IL-25 immunoreactivity in the bronchial epithelium of RV-infected mice confirmed *in vitro* findings, demonstrating the epithelium to be a major source of early RV-induced IL-25 expression. Comparable with findings in HBECs from patients, epithelial-derived IL-25 protein levels were highest at 24h p.i. Similarly, Kaiko *et al.* demonstrated RSV-induced IL-25 in the respiratory epithelium of NK cell/IFN- γ -depleted mice by IHC analysis at 4 days p.i. In this instance, IL-25 upregulation was attributed to a deficiency in IFN- γ which was demonstrated to have a suppressive effect on IL-25 production¹⁵⁷. In contrast, here we report the acute induction of IL-25 gene and protein by virus in the bronchial epithelium of a naive BALB/c mouse which, to our knowledge, has not previously been demonstrated.

Kinetic expression of IL-25 as measured by IHC indicated initial secretion by the bronchial epithelium which was followed by infiltration of IL-25-expressing inflammatory cells. Specifically, preliminary analysis of IL-25+ cells in the airway lamina propria identified macrophages (based on cellular morphology) as a potential source of IL-25 in RV-infected mice. Using the environmental irritant, titanium dioxide, Kang *et al.* validated alveolar macrophages to be a source of IL-25 in rats¹⁶⁵. In addition, immunostaining indicated the presence of RV-induced infiltrating IL-25-producing lymphocytes in the airway lamina propria. A mixed profile of CD4+ and CD8+ T cells have been reported as the main lymphocyte populations in the airways of patients experimentally infected with RV^{83,89};

therefore it is of interest that Owyang *et al.* demonstrated that IL-25 was constitutively expressed by CD4⁺ and CD8⁺ T cells¹⁶³. It was this constitutive expression in naive mice which imparted immunity to *Trichuris* infection; when tested in strains deficient in IL-25, mice failed to clear the infection and developed severe intestinal inflammation. *Trichuris* infection studies were also carried out in mice deficient in NF- κ B1 (p50) which developed very similar symptoms of disease¹⁶³.

In light of similarities between NF- κ B and IL-25 deficiencies in relation to disease symptoms, it is also of interest that RV, in addition to propagating in the airway epithelium, has also been reported to replicate in macrophages and that both cell types require the transcription factor, NF- κ B, for RV-induced release of proinflammatory mediators^{37,38,222}. Specifically, RV infection of epithelial cells was shown to increase NF- κ B activation required for CXCL8/IL-8, IL-6, ICAM-1 and MMP-9 expression^{37,38,220,223,224} while TNF- α production by macrophages was reported to be dependent on RV-induced NF- κ B signalling²²². Based on the findings that IL-25 induction was largely dependent on virus replication as well as the presence of an NF- κ B binding site upstream of the IL-25 encoding region, investigation of a potential role for this transcription factor in RV-induced IL-25 regulation is noteworthy¹⁵⁶. Analysis would also be valuable based on the fact that very little is known about regulation of IL-25 gene expression.

It is of importance that despite expression of IL-25, Th2 inflammation (including IL-4, IL-5 and IL-13 and airway eosinophilia) was absent in our model of RV infection. By contrast, the intranasal and intraperitoneal administration of recombinant IL-25 has been reported to induce allergic inflammation marked by IL-4, IL-5 and IL-13, eosinophilia and epithelial cell hyperplasia in the lungs and digestive tract of mice^{153,225}. Additional studies have also shown that overexpressing IL-25 in the lung epithelium of transgenic mice displayed increased CCL11/eotaxin-1, CCL24/eotaxin-2, CCL22/MDC and MUC5ac gene expression in lung tissue as well as increased epithelial hyperplasia and eosinophils in the lung parenchyma⁷⁰. Comparison of the amount of recombinant protein used to initiate allergic inflammation in these instances indicated that the smallest dose used was in the order of a 1000 fold more than the amount of IL-25 induced by RV infection in this study^{70,77,78,153,225}. Furthermore, these studies administered repeated treatments on consecutive days, and in some cases,

mice were dosed daily for up to ten days, clearly not reflecting accurate physiological conditions⁷⁰. Similarly, measurement of IL-25 in the BAL of transgenic mice indicated amounts approximately 10 fold more than IL-25 levels upregulated in RV-infected mice¹⁶⁹. Based on these findings, we suggest that the amount of IL-25 induced by RV infection was insufficient to initiate Th2 inflammation in the pulmonary environment.

In addition to insufficient levels, it may be that in a skewed Th1 environment, demonstrated by a robust induction of antiviral and proinflammatory cytokines in our model of RV infection, the absence of Th2-associated cell populations could account for a lack of IL-25-induced allergic inflammation. Numerous studies have indicated that IL-25 has the potential to act directly on both innate and adaptive Th2 cytokine-producing cells in the lung^{77,127,172}. Hence, in a Th2 pulmonary environment (such as in the allergic asthmatic lung), RV-induced IL-25 may activate recruited cells resulting in Th2 cytokine release and enhanced allergic inflammation. In support of this, Tamachi *et al.* reported that transgenic overexpression of IL-25 in the lung failed to initiate a Th2 response in mice. However, following OVA sensitisation and challenge which induced recruitment of OVA-activated CD4+ T cells to the lung, IL-25 induced significant enhancement of allergic airways disease¹⁶⁹.

Therefore, after confirming the suitability of this mouse model for the detection of RV-induced IL-25, we aimed to investigate the effect of RV infection on IL-25 in a Th2 allergic environment shaped by allergen sensitisation and challenge.

7.3 IL-25 induction in response to allergic and viral interactions in a model of RV-induced allergic airways disease

To investigate mechanisms of interplay between allergen exposure and RV infection in relation to IL-25 expression, a mouse model of RV-induced allergic airways disease was developed in our laboratory and employed in these studies²⁰². One of our primary objectives was to investigate potential synergistic effects of allergen in conjunction with RV infection on IL-25 expression. We first confirmed the suitability of our model by comparing results to published findings as well as comparison with clinical aspects of acute asthma. We also carried out a thorough analysis of RV-induced enhancement of Th2 responses to allergen to define end-points potentially regulated by IL-25 which could be measured

subsequent to blocking IL-25 signalling. These findings will be discussed below before examining the combined effect of RV infection and allergen treatment on IL-25 gene and protein expression.

Infiltration of a mixed profile including early neutrophilia and later eosinophilic inflammation was significantly enhanced in RV-infected and allergen-challenged mice which corresponded with cellular profiles measured in induced sputum samples in patients with acute asthma⁸⁸. In accordance with previously published results of this model, RV infection also enhanced lymphocytic airway inflammation in allergen-sensitised and challenged mice in addition to increasing cholinergic hyperresponsiveness indicating increased disease symptoms^{89,202}. We also confirmed that augmented airway inflammation coincided with upregulated T cell and eosinophil chemoattractants; specifically, the T cell chemoattractant, CCL5/RANTES, as well as CCL11/Eotaxin 1 and CCL24/Eotaxin 2 were upregulated in the airways of RV OVA mice early after infection which corresponded with recently published results of this model²²⁶. Furthermore, RV OVA treatment resulted in an increase of the Th2 cell chemoattractant, CCL17/TARC, which corresponded with upregulated Th2 (IL-4+CD4+) cells observed at d7 p.i. Also in accordance with published results from this model, RV-infected and allergen-treated mice exhibited enhanced expression of the major respiratory mucin, MUC5ac²⁰². Although the mucin, MUC5b, was not significantly upregulated above other treated groups, RV-infected and allergen-treated groups had the greatest accumulative mucus secretion at d7 p.i. This indicates an additional hallmark of acute exacerbation recorded in patients where mucus plugging of the airways has been demonstrated in fatal asthma cases^{65,83}.

A primary aim was to carry out a thorough analysis of Th2 cytokines at various time-points after infection and compare expression with IL-25 induction in this model of disease. We were particularly interested in investigating early events after infection with the aim of characterising the innate effects of virus and allergen on all cytokines; therefore, an earlier time-point at 10h p.i. was incorporated which has not previously been studied in this model. Analysis of the BAL indicated RV infection induced an early source of IL-4 and IL-13 in OVA-sensitised and challenged mice at 10h p.i. and IL-5 at d1 p.i. that was significantly upregulated compared with mice treated with OVA or RV alone. By d2 p.i. levels of IL-4, IL-5 and IL-13 were low or undetectable in all treated groups. In stark contrast, analysis of

cytokines in harvested lobes from the right lung (excluding the apical lobe) indicated sustained expression of IL-4, IL-5 and IL-13 until d4 p.i. in both groups of OVA-challenged mice. Analysis of cytokine expression in lung tissue indicated that RV infection enhanced all Th2 cytokines in allergen-treated mice at d1 p.i. above OVA-treated mice alone; this was followed by a later peak in expression at d4 p.i. that was predominantly allergen-driven. Both peaks may suggest an early and late phase response to RV infection and OVA treatment which has been well characterised in allergen challenge studies in mouse models and humans^{69,73,74}. In line with this theory, preliminary analysis of IL-4-producing cells by flow cytometry indicated that basophils accounted for the majority of early IL-4 expression at d1 p.i. while an enhanced population of Th2 cells were present in the lung tissue of RV OVA mice at d4 and d7 p.i.^{66,73}. With regard to differences recorded in the airways and lung tissue, several reports have demonstrated that cellular profiles in the BAL fluid do not always reflect cell populations in the lung parenchyma and that variable cytokine expression can occur in different lung compartments^{227 228-230}. Although previously published results from this model validated sharp reductions in BAL Th2 mediators at d1 p.i. to d2 p.i.²⁰², comparison of cytokine expression from different lung compartments has not previously been investigated. Furthermore, the robust induction of IL-4 and IL-13 in response to RV infection and OVA treatment as early as 10h p.i. has not previously been reported. The fact that the predominant source of IL-4 was from basophils and not Th2 cells suggests the presence of innate cellular sources of Th2 cytokines in the airways early after infection. Particularly striking were the large quantities of IL-4 levels in the airways of RV OVA mice at 10h p.i.; not only were amounts in RV OVA mice ~2 fold more than OVA-treated mice alone but were also ~2 fold higher than those measured in lung tissue. Given its integral role in inducing differentiation towards the Th2 phenotype as well as its function in isotype switching of B cells to IgE synthesis⁷³, investigation of innate sources and the potential effects of upregulated IL-4 levels in RV-infected and OVA-challenged mice in the airways was noteworthy and will be discussed in detail in section 7.4 and 7.5.

Due to the fact that the epithelial-derived Th2 cytokines, TSLP and IL-33, have been shown to interact with IL-25 predominantly in the innate Th2 response, protein expression was measured in lung tissue in our mouse model of disease¹³⁰. Both cytokines displayed

different kinetic protein expression compared with other measured Th2 cytokine profiles; though an early peak in IL-33 expression was observed at d1 p.i., a later characteristic peak at d4 p.i. was absent in both IL-33 and TSLP.

In line with published findings which demonstrated that allergen treatment in the form of HDM upregulated IL-33 in mice, we showed that OVA induced IL-33 expression in our model⁶⁷. Though RV infection enhanced expression in OVA-treated mice in our studies, there were no significant differences between OVA-challenged groups. In contrast with reports that have documented upregulation of IL-33 in response to viruses including influenza A in the mouse lung¹²², RV infection failed to significantly induce IL-33 above negative controls. Of interest was the fact that, compared with other Th2 mediators measured in lung tissue, IL-33 levels remained comparatively low in OVA-challenged groups after d2 p.i., indicating a more predominant role in the early allergic response. Recently, several papers have highlighted an important role for IL-33 as a crucial amplifier of innate Th2 responses in the lung and gut as opposed to adaptive immunity^{231 232}. Namely, a prominent role in the proliferation and activation of basophils and mast cells has been demonstrated in mouse and human studies^{233 131,234}. An early peak in expression and absence of a later peak at d4 p.i. may support these findings as well as the fact that both innate cell populations characterised by flow cytometry in this mouse model expressed the IL-33 receptor, T1/ST2.

In contrast, TSLP expression was predominantly driven by allergen exposure until d4 p.i. after which levels in RV OVA groups were upregulated above levels in UV OVA groups at d7 p.i. A recent study demonstrated that TSLP played a prominent role in the adaptive response by promoting virus-specific CD8⁺ T-cells in response to influenza A infection; levels in this model were found to peak at d5 p.i.²³⁵. Therefore, characterisation of a potential adaptive function for TSLP in RV OVA mice after d7 p.i. would be of interest. However, contrasting with these results, though the combination of RV infection and allergen sensitisation in RV PBS groups enhanced TSLP levels above UV PBS controls, viral infection alone, as demonstrated in our model of RV infection, failed to induce TSLP expression (data not shown). Human *in vitro* studies have demonstrated that TSLP was upregulated in normal HBECs infected with RV and that supplementing the cell media with IL-4 was shown to enhance RV-induced TSLP¹²¹. These results are in conflict with observations in RV-infected

and OVA-challenged mice i.e. in an IL-4-enriched milieu, RV infection failed to upregulate TSLP levels compared with OVA-treated mice alone.

In accordance with our hypothesis, allergen challenge and RV infection following sensitisation enhanced IL-25 production compared to induction by allergen or RV alone. This was confirmed by significantly enhanced mRNA and protein at 10h and d1 p.i. respectively in RV OVA mice. As demonstrated in our mouse model of RV infection, induction of IL-25 in response to infection in RV PBS groups was also confirmed. Comparison of IL-25 protein expression with IL-4, IL-5 and IL-13 measured in lung tissue from RV OVA mice revealed a similar trend; namely, an early and late response that was most pronounced in OVA-treated mice infected with RV at d1 p.i. Again, this suggests an early source of IL-25 as well as a later, adaptive cellular source. In contrast with all other measured Th2 cytokines, amounts of IL-25 induced by RV alone and OVA alone were equivalent at d1 p.i. and were significantly upregulated above double-negative controls. These novel findings indicate that both RV and allergen are capable of independently inducing IL-25, a property unique to this Th2 cytokine.

As previously discussed, several reports have demonstrated upregulation of IL-25 in response to allergen in the lungs of mice and humans^{71,158,169}. In line with findings from our study, Tamachi *et al.* reported that OVA sensitisation and challenge induced IL-25 gene expression which was measured at 4h, 8h and d1 after the final OVA challenge. Expression was slightly elevated at 8h p.i. compared with d1 p.i. which was similar to our observations. However based on a thorough kinetic analysis of gene expression up until d7 p.i., we were also able to identify a second higher peak in expression at d2 p.i. Furthermore, as opposed to measurement of protein expression at a single time-point at d1 as reported by others, we demonstrated that induction by allergen was most pronounced at d4 p.i.¹⁶⁹. This detailed kinetic analysis of IL-25 expression in response to OVA treatment has not previously been demonstrated and allowed us to draw parallels between peaks in allergen-induced IL-25 compared to IL-4, IL-5 and IL-13 expression in lung tissue.

Although a detailed analysis of the antiviral response was not characterised in our study, enhanced viral load was found to coincide with augmented Th2 cytokines, increased airway inflammation and enhanced AHR in RV-infected and OVA-treated mice. In accordance with these findings, Message *et al.* demonstrated that RV load in asthmatics was related to enhanced lower airway inflammation and increased bronchial hyperreactivity while

augmented Th2 cytokines were associated with greater disease severity⁹³. Therefore, these clinical findings draw parallels with findings in our mouse model of disease. In addition, a strong induction of IL-6 was also demonstrated in RV-infected and allergen-treated mice. In line with these results, elevated IL-6 levels in sputum have been reported in atopic asthmatic patients experimentally infected with RV²⁰⁵. Similar to BAL Th2 cytokines, the highest levels of IL-6 in the airways were measured at 10h p.i. in RV OVA mice, which was ~2.5 fold greater than amounts observed at d1 p.i., again suggestive of an early cellular source. This is a striking example of the enhancing effects of RV infection and allergic inflammation; virus and allergen alone induced ~8 fold less IL-6 than when both components were administered, highlighting the complex interactions between the antiviral and allergic response. Possible implications for increased IL-6 levels as well as the fact that blocking IL-25 signalling further enhanced levels are discussed further in section 7.5.

In summary, RV infection of an allergen-sensitised and challenged mouse predominantly enhanced Th2 inflammation as well as viral load and disease severity as demonstrated by augmented AHR. The interaction of virus and allergen resulted in the greatest induction of IL-25 gene and protein expression compared with either factor alone verifying our hypothesis. We then investigated whether IL-25 levels measured in RV-infected and allergen-treated mice corresponded with enhanced recruitment of IL-17RB-expressing cells to the lung in addition to characterising cell populations potentially activated by enhanced IL-25 in our mouse model of disease.

7.4 IL-17RB-expressing cell populations in a model of RV-induced allergic airways disease

On the basis that IL-25 has been demonstrated to mediate its biological effects through a multitude of innate and adaptive cell populations^{77,78,127,187,193}, we employed several Th2-associated markers to characterise IL-17RB-expressing non-T and T cell populations recruited to the lung in our mouse model of disease. We also investigated the effects of virus and/or allergen on the magnitude and kinetics of total IL-17RB-expressing cells in the lungs and airways of treated mice hypothesising that enhanced levels of IL-25 in RV OVA mice correlated with increased receptor expression.

7.4.1 Total IL-17RB+ cell populations

In line with previous findings, we observed that allergen exposure increased pulmonary IL-17RB expression in the lung^{71,236}. Specifically, Petersen *et al.* have demonstrated that repeated treatment with cockroach allergen in mice upregulated IL-17RB and reciprocally increased IL-25 gene expression⁷¹. Other studies reported that *ex vivo* PBMCs from patients with allergic rhinitis challenged with allergen extract for a week expressed significantly enhanced IL-17RB after 4 days of culture as determined by flow cytometry²³⁶. In addition to confirming these findings, we demonstrated that RV infection enhanced allergen-induced IL-17RB-expressing cells in lung tissue. In line with our hypothesis, this coincided with the greatest induction of IL-25 in RV OVA groups, indicating that IL-25 may serve as an amplification factor in Th2 inflammation. The greatest number of IL-17RB-expressing cells, which comprised approximately 14% of the total lung lymphocyte population, was observed in RV OVA mice at d7 p.i. Further analysis revealed that ~40% of IL-17RB+ cells at this time-point were IL-4+ Th2 cells. Analysis of flow cytometry plots indicated that a large population of IL-17RB+ CD4+ T cells did not express IL-4. These cells may represent IL-5 or IL-13-expressing Th2 cells or, alternatively, Th9 cells which have also been shown to express IL-17RB¹⁸⁷. In addition, a population of cells which we classified as ILC2s comprised a further ~3% of IL-17RB-expressing cells at d7 p.i. Future flow cytometry analysis of this model will aim to incorporate intracellular staining (ICS) for IL-5, IL-9 and IL-13 as well as employ reporter mouse strains expressing GFP at the IL-4 and IL-13 loci to gain detailed information on potential IL-25-responsive Th2 cytokine-secreting cells.

In contrast to results in lung tissue, interaction of RV infection and OVA treatment in mice induced an early infiltration of IL-17RB+ cells into the airways representing ~20% of ILC2s and ~10% IL-4+Th2 cells at d1 p.i. However, IL-17RB+ cells in the airways at d7 p.i. exhibited no discernable differences between OVA-challenged groups or RV-infected mice alone. The majority of IL-17RB+ cells in OVA-challenged groups at this time-point were again identified as IL-4+ Th2 cells which accounted for ~25% while ILC2s made up ~9%.

7.4.2 IL-17RB+ ILC2s

Due to the large variety of IL-25-responsive innate cell populations that have been characterised and the vast range of markers used to phenotype them^{68,71,77,78,192,193,237}, we employed a selective panel that was phenotypical of innate lymphoid cells. Using these markers, we identified an early IL-17RB+T1/ST2+ICOS+IL-4-FcεRI-CD49b-CD3-CD4- population in OVA-challenged mice which we termed ILC2s, representing the newly grouped subset made up of nuocytes, lh2 and NH cells which share expression of IL-17RB, T1/ST2 and ICOS, in addition to exhibiting a lin- phenotype⁸¹. Other IL-17RB-expressing innate cells that did not fall within this subset were excluded based on a process of elimination of published findings; specifically, the IL-25-responsive T2M cells, which have been demonstrated to be induced in the lungs of allergen-challenged mice and in response to IL-25 administration, were excluded based on the fact that they do not express T1/ST2 and are representative of a myeloid subset⁷¹. Similarly, IL-17RB+ MPP^{type2} cells, reported to confer immunity to *T. muris* infection in *Il-25*^{-/-} mice, were also previously characterised as T1/ST2⁻⁷⁷.

Based on published findings of ILC2 subsets, nuocytes represented the most likely candidate for IL-17RB+ innate cells identified in our model of disease. Namely, nuocytes are the only group of innate lymphoid cells reported to infiltrate the lung in response to OVA challenge in a mouse model of allergic airways disease where they provided a major source of innate IL-13 upon IL-25 activation. Furthermore, using a day 12 and day 25 OVA-induced lung allergy model, nuocytes were not only detected in the BAL at the start of the allergic response but also persisted through the adaptive response with greater numbers detected at day 25 post-sensitisation⁷⁶. In line with these findings, OVA-challenge induced ILC2s at 6h and later at d7 p.i. in lung tissue in our model. A significant early induction at d1 p.i. was also measured in the airways of RV-infected and OVA-challenged mice; however all other time-points measured in the BAL and lung p.i. revealed no enhancing effect of RV infection in OVA-challenged mice indicating that this cell population may be predominantly allergen-driven. Of note, the amount of ILC2 numbers recruited into the airways in response to OVA-challenge in the early response at d1 p.i. and the later response at d7 p.i. were very similar to those measured in the day 12 and day 25 OVA-induced lung allergy model i.e. ~2000 cells per mouse in the early response and ~4000 cells enumerated at the later time-point post-allergen challenge⁷⁶. Future studies will aim to confirm these preliminary findings by

confirming a lin⁻ phenotype in cells as well as ascertain whether this cell population is a contributing source of IL-13 expression as previously demonstrated by nuocytes in the lung⁷⁶.

7.4.3 IL-4+ basophils

A striking effect of RV infection in the allergic lung was a very robust induction of IL-4 in the airways early after infection. Based on these findings, we sought to identify potential IL-17RB-expressing cellular sources that co-expressed IL-4 by flow cytometry analysis. Though no IL-4+IL-17RB+ cells were observed early after infection, we did identify an FSC^{lo}/SSC^{lo}FcεRI+T1/ST2+CD49b+CD3-CD4- cell population in whole lung cell preparations and BAL leukocytes which produced IL-4. While there exists divided opinion as to a definitive panel of markers for basophil characterisation, analysis of the literature revealed that these cells fitted the basophil phenotype most accurately^{214,215,134,216,238}. Namely, mast cells were discounted based on the fact they do not express CD49b while eosinophils were also excluded based on SSC^{high}FcεRI^{lo} properties²³⁸. Other studies have recently characterised FcεRI-expressing DCs which were demonstrated to infiltrate the mouse lung in response to viral infection as well as inhaled allergen^{238,239}. Specifically, paramyxoviral infection of mice induced high expression of FcεRI on resident lung DCs which were reported to be regulated by type I IFN receptor and not IgE. However, identification of these myeloid cells was based on isolation of SSC^{high}/FSC^{high} populations which excluded FSC^{lo}/SSC^{lo} FcεRI+ cells identified in our model²³⁹. In addition, IL-4-expressing FcεRI+ DCs, characterised by Hammad *et al.*, were reported to infiltrate the mouse lung in response to HDM allergen. Based on the fact this population exhibited a varied forward/side scatter profile and did not express CD49b, these cells were also excluded as potential candidates²³⁸. As a result, we concluded that basophils most accurately represented characterised IL-4-producing non-T cells in our mouse model of disease.

Others have demonstrated that in a model of OVA-induced allergic airways inflammation, basophils comprised the largest proportion of non-T IL-4-producing cells which were shown to infiltrate the lung after allergen challenge²⁴⁰. Our findings demonstrated similar results with IL-4-expressing basophils accounting for ~85% of innate IL-4 producers at d1 p.i. in both OVA-challenged groups suggesting that they were the principal early source of IL-4 in the airways of RV OVA mice. In line with their role in the innate allergic response, basophils

peaked at d1 p.i. in the airways and lung tissue of OVA-challenged mice and rapidly declined after this time-point. Peak levels in OVA-challenged mice accounted for ~0.5% of total cells from lung tissue within the 'lymphocyte' gate. These findings are in accordance with other studies which have reported that basophils represent a relatively small population of leukocytes during the allergic response; however they have also been demonstrated to generate greater amounts of IL-4 on a per-cell basis than Th2 cells implicating them as an important source of IL-4^{241, 242}.

Studies of respiratory viruses and basophil activation have demonstrated that RSV induced basophil accumulation in the lungs of BALB/c mice and that these cells were the primary early source of IL-4²¹⁵. *In vitro* studies have also reported that RV-infected cell lines from human mast cells and basophils can enhance IgE/anti-IgE-induced histamine release in both cell types as well as IL-4 and IL-6 protein in basophil supernatant¹⁰⁶. Demonstrating interaction between virus and allergic inflammation, RV infection augmented total serum IgE in asthmatic patients which correlated with reduced lung function while RV-infected allergic rhinitis patients displayed elevated total serum IgE and histamine compared with infected non-allergic controls^{107,108}. Due to the fact that serum was taken at a single time-point at d7 p.i. in our study, we could not demonstrate a temporal relationship between IgE levels and FcεRI-expressing cells. In addition to allergen-induced cross-linking of IgE, activation of basophils can occur via stimulation with IL-33 together with IL-3 which has been shown to directly induce IL-4 production^{243, 244}. Though amounts were not significantly elevated above OVA-challenged mice alone, the greatest levels of IL-33 at d1 p.i. were measured in RV OVA mice which coincided with peak basophil levels in the lung tissue and the airways. Confirmation of T1/ST2 expression on cells was also consistent with a potential effector function of IL-33 in the induction of basophil activation²⁴⁵.

In summary, we identified IL-17RB-expressing Th2-associated cell populations as well as an early source of IL-4-producing basophils in our mouse model of disease which have not previously been characterised. Finally, to define a role for IL-25 in RV-induced allergic airways disease, RV-induced IL-25 signalling was blocked to investigate potential outcomes on identified cell populations as well as on RV-enhanced Th2 responses.

7.5 Blocking IL-25 signalling in a model of RV-induced allergic airways disease

It is unclear how, in the asthmatic lung, RV infection interacts with the pulmonary Th2 environment to trigger an exacerbation. We have demonstrated that IL-25 is induced by RV infection *in vivo* and *in vitro*. In addition, the combination of allergen exposure and RV infection enhanced expression of IL-25 and its receptor, IL-17RB, which was associated with disease severity as measured by AHR. To define a functional role of RV-induced IL-25 in a model of allergic airways disease we systemically blocked the biological activity of RV-induced IL-25 using a neutralising antibody against IL-17RB, which was previously employed in flow cytometry analysis. To achieve these aims, anti-IL-17RB antibody was administered to treatment groups pre-RV infection. An additional 2 doses were administered to mice at d3 and d5 p.i. and groups compared with isotype-matched controls.

Using the same antibody clone utilised in this study, Camelo *et al.* demonstrated its efficacy in decreasing Th2 inflammation in a type 2 model of colitis. By blocking IL-25 signalling, mice displayed decreased IL-13 levels, blood eosinophilia and IgE which correlated with reduced IL-4, IL-5 and IL-13 production by cultured mesenteric lymph node (MLN) cells¹⁷¹. Furthermore, symptoms of disease were lessened and mucosal infiltration of NKT cells and neutrophils was reduced. Antibody binding was shown to be specific for mouse and human IL-17RB and exhibited no cross-reactivity with other members of the IL-17 receptor family¹⁷¹. Using an identical dose of blocking antibody administered i.p. (as reported by Camelo *et al.*) we also observed reduced Th2 inflammation in our mouse model of disease, which has not previously been demonstrated in the lung.

Blocking IL-25 signalling attenuated RV-mediated enhancement of airway inflammation and granulocyte numbers in OVA-sensitised and challenged mice, a key feature of acute asthma in human disease^{83,88}. Administration of anti-IL-17RB antibody in RV OVA mice significantly reduced airway neutrophilia at d1 p.i. and eosinophilia and lymphocytosis at d7 p.i. compared with isotype-treated RV OVA mice, clearly implicating a role for RV-induced IL-25 in airway leukocyte infiltration. As previously stated, this significant decrease in airway neutrophilia in anti-IL-17RB-treated RV OVA mice may be explained by the inability of IL-25 to induce the neutrophil chemoattractant, CXCL1/KC, in accordance with studies of human renal cells which produced CXCL8/IL-8 in response to IL-25 stimulation¹⁵². Furthermore,

human eosinophils treated with IL-25 were reported to secrete CXCL8/IL-8 in an NF- κ B-dependant manner *in vitro*⁸⁴. Therefore, a significant reduction of total eosinophil numbers as well as the inability of IL-25 to act on potential responsive cells may account for a CXCL1/KC-dependent decrease in neutrophilic inflammation mediated by IL-25. As preliminary characterisation of the effects of IL-17RB neutralisation on this mouse model of disease was predominantly focused on Th2-associated read-outs, future studies will center on evaluation of Th1 immune responses including measurement of CXCL1/KC as a mechanism for reduced neutrophilia. The fact that IL-25 may also mediate virus-associated inflammatory responses such as neutrophil recruitment implicates IL-25 receptor neutralisation as a promising therapeutic target for virus-induced allergic disease, particularly in light of the fact RV-induced neutrophilia has been associated with disease severity^{88,221}.

Eosinophilic inflammation was also reduced in the airways of anti-IL-17RB-treated RV OVA mice at d7 p.i. Previous studies reported that IL-25 can act indirectly and directly on eosinophils to increase viability and induce infiltration into the lung respectively^{70,188,199}. Angkasekwinai *et al.* demonstrated that treatment of mouse lung epithelial cells with IL-25 strongly induced expression of CCL11/Eotaxin-1 and CCL5/RANTES as well as TSLP and MUC5ac gene expression⁷⁰. In line with these findings, the eosinophil chemoattractants, CCL11/Eotaxin-1 and CCL5/RANTES, as well as CCL24/Eotaxin-2 were significantly downregulated in anti-IL-17RB-treated RV OVA mice compared with isotype-matched groups, while reduced levels of BAL IL-5 in the airways at 8h and d1 p.i. may also have contributed to decreased eosinophil recruitment⁷³. However, eosinophil chemoattractants in OVA-challenged mice alone were lower than anti-IL-17RB-treated RV OVA groups even though their total BAL eosinophil numbers were higher, indicating an additional pathway contributing to reduced eosinophilia. IL-25 has also been reported to bind directly to eosinophils enhancing cell viability and therefore promoting eosinophilia in allergic inflammation¹⁹⁹. Therefore, it is plausible that blocking RV-induced IL-25 signalling decreased eosinophilic survival in the late-phase allergic response.

Lymphocytic inflammation was also dramatically reduced in anti-IL-17RB-treated RV OVA groups; this was in line with results demonstrating decreased levels of the memory T cell chemoattractant, CCL5/RANTES, as well as the Th2 cell chemoattractant, CCL17/TARC. In

addition, flow cytometry data corroborated results demonstrating a striking decrease in Th2 cells in anti-IL-17RB-treated RV OVA.

In contrast with these results, the anti-IL-17RB dosing regime used in this study had minimal effects on allergen-induced eosinophilia and lymphocytosis. Other studies have demonstrated that blocking IL-25 reduced recruitment of eosinophils and CD4⁺ T cells into the airways as well as decreased Th2 cytokines and IgE in OVA-sensitised and challenged mice. In these instances, antibody was administered before allergen sensitisation or before mice were challenged^{169,170}. Therefore, it is most probable that because anti-IL-17RB antibody was administered after 2 allergen challenges in our model, treatment was unable to suppress ongoing inflammatory responses. The fact that other read-outs of Th2 inflammation, including BAL Th2 cytokines, chemokines, mucin proteins and Th2 cells, were also largely unchanged by blocking IL-17RB in UV OVA mice further indicates that once the allergic response was initiated, IL-25 may not be required for propagation of allergen-induced Th2 inflammation.

One of the most striking examples of abrogated Th2 inflammation was a dramatic early reduction in BAL IL-4, IL-5 and IL-13 in RV OVA mice administered anti-IL-17RB antibody. Based on the fundamental role these mediators play in Th2 cell differentiation, IgE synthesis, mucus secretion, bronchioconstriction and cellular recruitment, this finding was of great relevance⁷³. These results further indicated that RV-induced IL-25 was implicated in augmentation of allergic airways disease as blocking allergen-induced IL-25 signalling again had minimal effects. In contrast, analysis of IL-4 and IL-5 expression in the lung homogenate of all treated mice revealed no significant differences between anti-IL-17RB and isotype-matched groups indicating IL-25-mediated signalling had negligible effects in total lung tissue. These differences between cytokine expression in the lung parenchyma and BAL may suggest that blocking IL-17RB resulted in a direct or indirect decrease in IL-4 and IL-5-expressing cells recruited into the airways. For example, IL-4⁺ basophil levels in the lung tissue of RV OVA and UV OVA mice were not significantly altered by IL-17RB neutralisation compared with isotype-matched groups. However, infiltrating IL-4⁺ basophils were significantly reduced in the airways of RV OVA (anti-IL-17RB) mice at d1 p.i compared with isotype-treated RV OVA mice. Several chemokines, including CCL5/RANTES, have been reported to promote basophil chemotaxis to the site of allergic inflammation²⁴⁶. The fact

that others demonstrated that CCL5/RANTES was induced by the epithelium in response to IL-25⁷⁰ as well as findings from our study that showed levels were significantly decreased in the BAL of IL-17RB-treated RV OVA mice at d1 p.i. may represent an indirect mechanism of decreased basophil infiltration in the airways. Furthermore, additional basophil chemoattractants, including CCL2/MCP-1 and CCL3/MIP-1 α , have also been reported to be secreted by IL-25-stimulated eosinophils^{188,247}.

The reduction of early BAL IL-5 and IL-13 observed in RV OVA (anti-IL-17RB) mice coincided with a reduced trend in ILC2 numbers in the airways. However, future studies will focus on employing reporter mouse strains expressing GFP at the IL-13 loci as well as ICS staining for IL-5 to explore if identified ILC2s represent an IL-25-responsive source of Th2 cytokines that may be reduced as a result of neutralising IL-17RB. Alternatively, cytokine profiles of ILC2s harvested from treated groups in our model could be measured after *ex vivo* culturing in the presence of anti-IL-17RB or isotype controls to assess potential abrogation of cytokine release. Further characterisation of ILC2s would be of value based on findings from Camelo *et al.* who demonstrated that blocking IL-25 signalling in a model of type 2 colitis resulted in a significant reduction in infiltrating nuocytes and NKT cells into the mucosa which correlated with decreased IL-5 and IL-13 levels¹⁷¹.

Blocking IL-25 signalling resulted in a significant reduction of IL-13 in the BAL and lung tissue in both groups of anti-IL-17RB-treated OVA-challenged mice compared with isotype-matched controls. Barlow *et al.* recently demonstrated that by inhibiting IL-25 before allergen challenge in mice, IL-13 levels were significantly decreased with a reciprocal increase in IL-17A that was shown to be important for protection against Th2 inflammation¹⁷². Furthermore, transgenic overexpression of IL-13 in mice that were administered IL-25 blocking antibody failed to upregulate IL-17A. This suggests that IL-13, acting downstream of IL-25, plays a role in suppressing IL-17A and that IL-25 acts as a key regulator of this IL-13/IL-17A axis. Pertinent to our study was the fact that although IL-17A has been documented to induce neutrophilia, the reciprocal elevation of IL-17A in anti-IL-25-treated mice did not induce significant neutrophil infiltration in the blood or BAL of mice¹⁷². It is also interesting to speculate that, based on findings that IL-13 blocks *in vitro* differentiation of Th17 cells by inhibiting IL-6, IL-1 β and IL-23, the large increase in IL-6 observed in anti-IL-17RB-treated RV OVA mice may be as a result of enhanced IL-17A

production¹⁶⁶. Corroborating a beneficial role for IL-17A in allergic disease, a separate study demonstrated that, when administered to allergen-sensitised mice, IL-17A could suppress allergic inflammation and AHR²⁴⁸.

Based on these findings, IL-25 may well act as a regulator of the IL-13/IL-17A axis in RV-induced exacerbation of allergic airways disease and future studies will focus on measuring IL-17A and related Th17-promoting cytokines; however, certain read-outs in our model were in contradiction with published findings. For example, Barlow *et al.* reported that blocking IL-25 resulted in complete abrogation of AHR which was dependent on IL-17A production¹⁷². We found that although blocking IL-25 signalling reduced numerous mediators of Th2 inflammation including IL-13 and granulocytes in the airways of anti-IL-17RB-treated RV OVA mice, airway obstruction as measured by whole-body plethysmography (WBP) was not significantly reduced. Future studies will aim to corroborate airway obstruction data with measurement of AHR by double-chamber plethysmography (DCP) as previously carried out in our model of RV-induced allergic airways disease. Although experiments to investigate the effects of blocking IL-25 signalling were repeated twice, due to the amount of techniques employed to measure read-outs at each time-point and the large number of mice per study, WBP was considered the most practical system for measurement of airway responses. This is largely due to the fact that DCP is more time-consuming, requiring several rounds of acclimation to familiarise mice with the restraining neck seals to prevent confounding reading as a result of stress²⁴⁹. However, DCP permits measurement of sRaw (indicating the sum of lung volume and upper airway or nasal resistance), a more relevant read-out of airway mechanics²⁴⁹. This fact together with the finding that employing DCP in our previous study showed significantly greater AHR in RV OVA mice compared with OVA treatment alone (a difference that was not reflected in WBP), represents a rationale for repeating these studies with a specific focus on measuring this important read-out of disease severity via DCP.

Gregory *et al.* recently demonstrated that blocking IL-25 in mice exposed to HDM completely abrogated IL-33 and TSLP production suggesting that IL-25 drives both IL-33 and TSLP in the lung²⁰¹. In contrast, we reported that blocking IL-25 signalling had no effect on TSLP and IL-33 at 8h p.i., indicating induction of both mediators in response to RV infection and/or OVA exposure by the epithelium. However, IL-17RB neutralisation in both OVA-

challenged groups resulted in a significant decrease in IL-33 and TSLP at d1 p.i. indicating a later IL-25-regulated cellular source. It has been reported that stimulation of the mouse lung epithelium with IL-25 induced TSLP expression⁷⁰; therefore, IL-25 may activate a later source of epithelial-derived TSLP as indicated by reduced expression in IL-17RB-neutralised mice at d1 p.i. In a similar way, IL-33 is produced by structural cells including airway smooth muscle cells, endothelial cells and lung fibroblasts which have been reported to express the IL-25 receptor; therefore these structural cells may represent a source of IL-33 regulated by IL-25^{126,142,189-191}. While levels of IL-33 were reduced in all treated groups by d7 p.i., TSLP levels were in fact elevated in IL-17RB-neutralised RV OVA mice. These results indicated that adaptive sources of TSLP were not regulated by RV-induced IL-25. In contrast, a reduction in allergen-driven TSLP was indicative of a cellular source dependent on IL-25 activation.

Based on the fundamental role that Th2 cells play in mediating asthma pathogenesis, it was of major relevance that blocking RV-induced IL-25 resulted in a significant reduction in cells in the airways and lung of RV OVA mice. This was apparent from cell numbers in IL-17RB-neutralised RV OVA mice that were restored to levels measured in UV OVA mice and the fact that Th2 cells were largely unchanged in anti-IL-17RB and isotype-treated UV OVA groups. At this preliminary stage of characterising IL-25 signalling blockade in our disease model, there are numerous mechanisms that could be postulated to describe this striking reduction. For example, significant decreases in the Th2 chemoattractant, CCL17/TARC, which were exclusively reduced in RV OVA groups at d7 p.i. could have contributed to decreased recruitment of Th2 cells expressing CCR4; this is in accordance with other studies that demonstrated blocking CCL17/TARC in a mouse model of allergic disease reduced CD4⁺ T cell infiltration and Th2 cytokines²⁵⁰. Furthermore, the absence of an early source of IL-4 as measured in the airways of RV OVA mice may play a role in reduced Th2 cell differentiation contributing to decreased total Th2 cell numbers²⁵¹. In a previous study, IL-25 was shown to induce early IL-4 expression in naive CD4⁺ T cells from mice via upregulation of NFATc1 and JunB which resulted in increased GATA3 expression and Th2 cell differentiation *in vitro*. Therefore, the lack of IL-4 observed in RV OVA mice may be directly related to blocking RV-induced IL-25 signalling which may result in reduced Th2 cell differentiation⁷⁰. IL-25 has also been demonstrated to enhance proliferation and induce activated IL-17RB-expressing Th2 memory cells to acquire an effector-memory phenotype

by directly binding to its receptor. Therefore blocking RV-induced IL-25 signalling may directly reduce Th2 cell proliferation and activation. Our observation of decreased Th2 cells in RV OVA mice is in support of this¹²⁷.

In view of the fact that Kaiko *et al.* demonstrated a reciprocal relationship between IL-25 and IFN- γ , it may be suggested that blocking IL-25 signalling increased levels of IFN- γ resulting in an augmented antiviral response as indicated by reduced viral load in IL-17RB-neutralised RV OVA mice at 8h p.i. However, measurement of IFN- γ in our model of disease revealed no significant differences between treatment groups (data not shown). Prospective measurement of the type I (IFN- β and IFN- α) and type III (IFN- λ) IFNs in our disease model would be relevant based on their ability to disrupt viral replication resulting in reduced viral load in the innate immune response. Furthermore, the fact that enhanced viral shedding due to deficiencies in IFN- β and IFN- λ were highlighted as potential mechanisms of exacerbation of disease in HBECs from atopic asthmatics indicates characterisation of these innate responses are of particular importance^{102,103}.

Of interest was the fact that decreased viral load in IL-17RB-neutralised RV OVA mice coincided with significantly upregulated levels of BAL IL-6 early after infection. Whether this pleiotropic cytokine is beneficial or harmful in respiratory disease is largely unknown. Its ability to activate B cells and proliferate cytotoxic T cells indicates a potential antiviral role²⁵²; of interest, mice deficient in IL-6 that were exposed to aerosolised endotoxin displayed enhanced levels of airway neutrophilia, TNF α and MIP-2²⁵³. Therefore, further characterisation of this cytokine and its ability to upregulate Th1 as well as Th17 responses will be carried out in future studies.

7.6 Summary of findings

RV infection of HBECs induced IL-25 gene and protein expression. Importantly, HBECs from atopic asthmatics demonstrated a greater propensity to express IL-25 which may represent a mechanism whereby RV interacts with the allergic phenotype to drive Th2 inflammation in RV-induced asthma exacerbation. In a similar way, RV infection of the untreated mouse airway induced IL-25 expression in the airway epithelium as well as in inflammatory cells in the airway lamina propria. However this expression was not sufficient to initiate Th2 inflammation. It may be this system models RV infection in an otherwise healthy individual. That is, despite the fact that IL-25 is upregulated, a beneficial antiviral response (as indicated by increased type III IFN production) is induced and Th2 inflammation is not initiated due to the absence of allergen sensitisation and challenge. Based on clinical studies that have demonstrated that allergen exposure increases the risk of acute asthma in conjunction with viral infection in sensitised asthmatics, our primary aim was to investigate the role of RV-induced IL-25 in a model of RV-induced allergic airways disease. Using this model, we first demonstrated that RV infection enhanced allergen-induced IL-25 levels as well as key features of Th2 inflammation and pathogenesis in the lung (Figure 7.1). We also showed that enhanced IL-25 in RV-infected and allergen-treated mice corresponded with increased recruitment of IL-17RB-expressing non-T (ILC2s) and T cells (Th2 cells) to the lung suggesting that IL-25 may serve as an amplification factor in RV-induced Th2 inflammation. Finally, to investigate the role of RV-induced IL-25 on enhancement of Th2 inflammation, we blocked RV-induced IL-25 signalling in our mouse model of disease. Neutralising IL-17RB reduced several key features of the exacerbation phenotype in RV OVA mice including airway leukocyte infiltration, BAL Th2 cytokines and chemokines, Th2 cells and total mucin production. In contrast, Th2-associated responses were largely unchanged by blocking IL-25 signalling in established allergic inflammation. These findings suggest that RV-induced IL-25 plays a role in augmenting Th2 inflammation associated with the exacerbation phenotype which it mediates through binding to its receptor, IL-17RB.

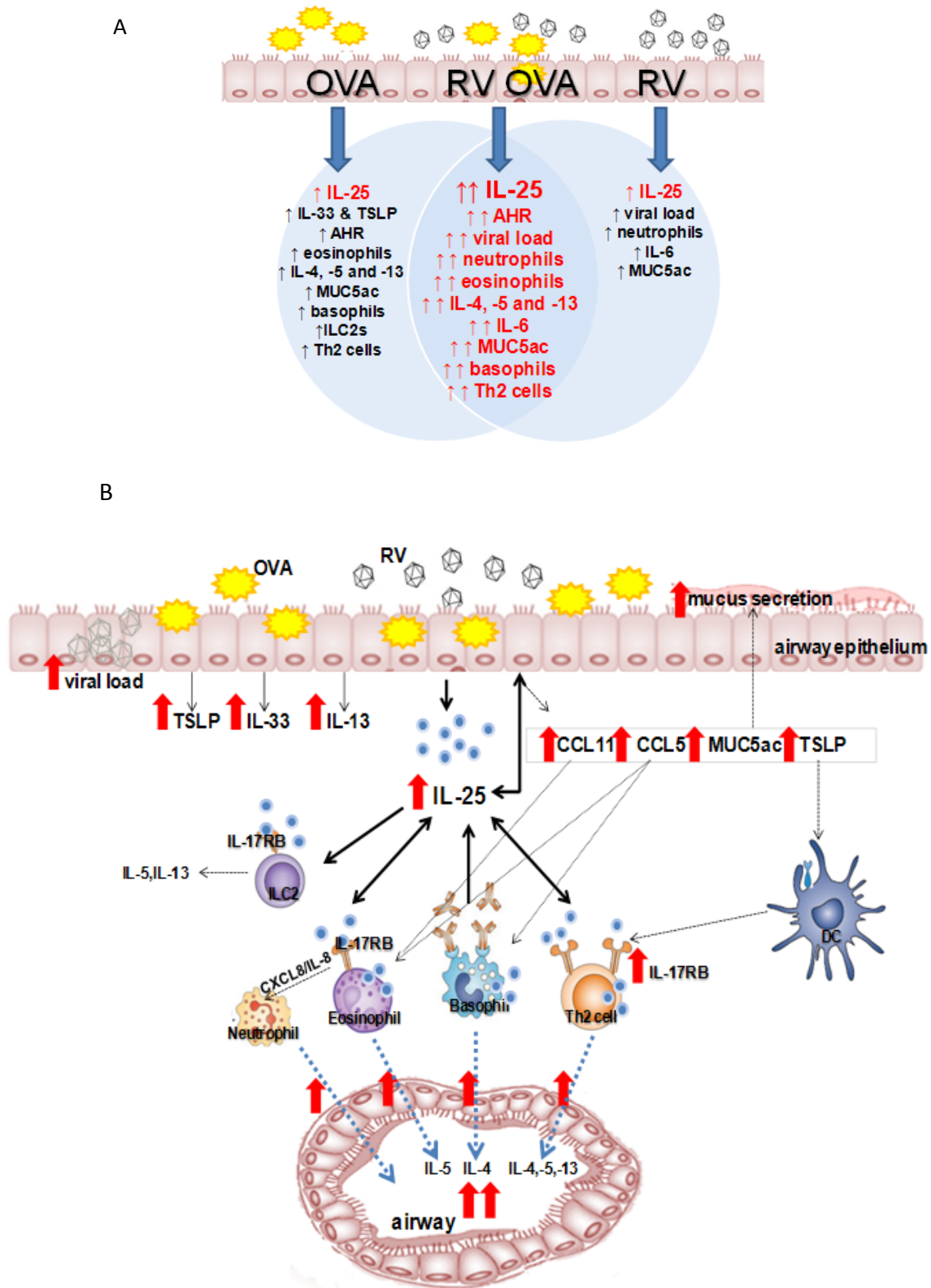


Figure 7.1 (A) A Venn diagram illustrating the enhancing effects of OVA and RV infection (overlapping space between both circles) as opposed to characteristic responses of each factor alone. (B) Representation of the role of RV-induced IL-25 in our model of RV-induced allergic airways disease based on findings from blocking IL-25 signalling. RV-induced IL-25 increased key aspects of the exacerbation phenotype including

neutrophils, eosinophils, Th2 cells and basophils into the airways. Furthermore, RV-induced IL-25 upregulated Th2 cytokines and chemokines as well as mucus secretion and possibly viral load.

7.7 Potential therapeutic applications

The findings that blocking IL-25 signalling in a system that models RV-induced asthma exacerbations not only dramatically reduced several key symptoms of allergic inflammation in the lung but also, surprisingly, decreased early viral load as well as virus-induced airway neutrophilia highlights a strong therapeutic potential. The benefits of blocking IL-25 to reduce disease severity in a model of allergic airways disease have been previously demonstrated^{70,169,170}. However, this is the first study that indicates that neutralising IL-17RB is able to reduce immunopathogenic mechanisms relating to both virus and allergen in a model of RV-induced allergic airways disease. Therapies for viral-induced asthma are largely limited due to its mixed Th1/Th2 phenotype characterised by neutrophilic inflammation that has been demonstrated to be resistant to corticosteroid treatment^{83,90}. Moreover, therapeutic targeting of allergic inflammation using oral corticosteroids has, in some instances, proven detrimental due to reciprocal interference of antiviral immunity resulting in increases in viral titre⁹¹. Therefore the observation that blocking the IL-25 receptor resulted in reduced viral load and virus-driven inflammation (neutrophilia) as well as reduced Th2 inflammation is novel. Furthermore, findings from our study as well as others show that IL-25 is produced by the respiratory epithelium suggesting that it acts upstream of Th2 inflammation and that targeting this cytokine may be more efficacious in reducing disease progression than inhibiting specific cytokines further downstream of the Th2 immune cascade^{70,157}. However, it will also be necessary to comprehensively assess what effects blocking IL-25 signalling may have on immune homeostasis in the respiratory mucosa. Based on the findings that a deficiency in IL-25 resulted in autoimmune disorders including inflammatory bowel disease as a result of a reciprocal increase in Th17-associated immunity, a thorough investigation of potential secondary effects in other organs will be necessary^{130,166}. The results obtained in this study are promising and indicate that transiently blocking IL-25 signalling is beneficial in reducing the exacerbation phenotype and may enhance antiviral responses in a model of RV-induced allergic airways disease.

7.8 Future work

7.8.1 Cellular sources of IL-25 protein in the lung

As our findings are the first to detect RV-induced IL-25, we included IHC to measure IL-25 immunoreactivity to corroborate protein data as measured by ELISA. Preliminary analysis of IL-25 immunoreactive cells in our model of RV infection demonstrated novel findings; in particular, the fact that neutrophils are a potential source of IL-25. Future work will focus on a more detailed analysis of cellular sources using double staining IHC for phenotypic markers such as ly-6G and ly-6C to identify neutrophils. Alternatively, flow cytometry may be employed to characterise cells expressing IL-25 via intracellular staining as well as co-expression of additional surface phenotypes.

Furthermore, a primary aim of future studies will be to assess IL-25-producing cells in our mouse model of RV-induced allergic airways disease again either employing double staining IHC or flow cytometry.

7.8.2 Characterisation of regulation of IL-25 expression

Very little is known about the regulation of IL-25 gene expression. Based on the presence of a putative NF- κ B binding site upstream of the IL-25 encoding region as well as the fact that RV infection of epithelial cells was shown to increase NF- κ B activation³⁷, we have carried out preliminary analysis of the role of NF- κ B in IL-25 regulation by pharmacological inhibition of the I κ B kinase complex (I κ K2) (a crucial signalling component of the NF- κ B pathway) in RV-infected mice. Future studies will aim to confirm these findings, however initial results are promising indicating decreased IL-25 gene expression in I κ K2-treated mice infected with RV.

7.8.3 Characterisation of Th2 cytokine expression and confirmation of cell populations

Preliminary flow analyses demonstrated the presence of IL-17RB-expressing ILC2s and Th2 cells in the lung and BAL of mice in our model of RV-induced allergic airways disease. To explore a mechanistic function for these cells via the secretion of Th2 cytokines, we aim to employ reporter mouse strains expressing GFP or alternatively the Tomato fluorescent gene

at the IL-4 and IL-13 loci to determine cytokine expression together with our panel of selective surface markers in mice treated with anti-IL-17RB and isotype controls.

In addition, investigation of the effects of IL-25 stimulation as well as anti-IL-17RB inhibition on relevant cell populations in *in vitro* cultures harvested from our mouse model would also allow us to gain a greater understanding of what cell types are responsive to IL-25. Of particular interest, IL-17RB-expressing ILC2s and Th2 cells will be assessed for their ability to secrete Th2 cytokines in response to IL-25 stimulation and similarly the ability of anti-IL-17RB to inhibit cytokine release.

Future work will also involve a thorough characterisation of identified IL-17RB-expressing cells. Specifically, a lineage negative panel to confirm the absence of T and B cell, macrophage, DC, neutrophil, eosinophil, mast cell, basophil, NKT cell or NK cell markers will be assembled to definitively classify ILC2s in our disease model.

7.8.4 Development of RV-induced allergic airways disease model

OVA, which does not contain protease activity, largely induces Th2 inflammation via interaction with DCs and subsequent presentation to T cells. The fact that a large proportion of allergens shown to induce IL-25 from the respiratory epithelium have been reported to possess proteolytic activity^{67,70}, indicates that the investigation of concomitant treatment with RV together with a more complex physiologically relevant allergen is noteworthy. For example, employing the common human aeroallergen HDM together with RV infection would be of interest given that HDM was demonstrated to trigger TLR4 on lung structural cells in mice inducing IL-25 expression⁶⁷. As carried out in our mouse model of disease in relation to OVA challenge, a thorough titration of the allergen dose will be required to optimise treatment regime. Based on a demonstrated role for IL-25 in airway remodelling, it would also be of relevance to carry out a chronic allergen challenge protocol over a number of weeks and assess the effects on IL-25 expression over an extended duration.

7.8.5 Variation of treatment regime for blocking IL-25 signalling

Given that we were primarily focussed on investigating the role of RV-induced IL-25 in our model of disease, we administered a blocking antibody 4 hours before infection. Future studies will investigate varied treatment regimes by administering the blocking antibody at different points before and after infection. For example, administering the blocking antibody prior to sensitisation and assessing the effect on allergen-induced and RV-induced IL-25 would be of great interest.

7.8.6 Investigation of mechanisms of suppression of Th2 responses by blocking IL-25 signalling

A primary aim of future work will be to characterise a mechanism for reduced Th2 inflammation as well as reduced viral load and neutrophilia measured as a result of blocking IL-25 signalling in RV-infected and OVA-challenged mice. This will include analysis of potentially augmented Th1 and Th17-associated mediators. Specifically, the innate type I and type III interferons will be measured as a possible mechanism for reduced viral load on the basis of their ability to enhance antiviral responses. Previous studies have reported that IL-25 induced CXCL8/IL-8 in human renal cells via NF- κ B activation¹⁵². Therefore, measurement of neutrophil chemoattractants including CXCL1/KC will be carried out in our disease model. This would be of particular interest since enhanced neutrophilia in the airways is a hallmark of RV-induced asthma in patients which has been associated with augmented disease severity; therefore a reduction in neutrophilic inflammation via IL-17RB neutralisation may be of therapeutic benefit.

In addition, IL-17A will be measured to ascertain whether IL-25 plays a role in regulating the IL-13/IL-17A axis and if reduced IL-13 levels measured in our model of disease are a result of reciprocally increased IL-17A production. Additional Th17 associated mediators, including the Th17-promoting cytokines IL-1 β and IL23, will also be measured to assess if they coincide with upregulated IL-6 and whether they play a role in inducing Th17 cell differentiation. Also of interest is whether these Th17-associated factors are beneficial to the exacerbation phenotype which could be investigated by antibody-mediated blocking of relevant cytokines and measuring read-outs of Th2 inflammation.

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