Effect of inhaled corticosteroids on viral and bacterial infection in chronic obstructive pulmonary disease

A thesis submitted for the degree of

Doctor of Philosophy

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Abstract

Rhinovirus (RV) infections trigger exacerbations of chronic obstructive pulmonary disease (COPD) exacerbations and may precipitate secondary bacterial infections. Inhaled corticosteroids (ICS) are used commonly in COPD but are relatively ineffective in the context of virus-induced exacerbations and may also increase the risk of pneumonia. We hypothesised that, in a mouse model, ICS would suppress anti-viral and anti-bacterial immune responses leading to alteration of the airway microbiota and secondary bacterial infection following RV-induced exacerbation of COPD.

Despite extensive optimisation, we were unable to define a representative mouse model of the deficient anti-viral and anti-bacterial responses that are indicative of human COPD. For this reason, and because of difficulties in measuring the airway microbiota in mice, we employed models of primary RV1B and Streptococcus pneumoniae infection as surrogates for viral exacerbation and bacterial colonisation in COPD. Fluticasone propionate (FP) administration prior to RV1B infection suppressed innate and adaptive immune responses leading to impaired virus control, in a dose dependent manner. This effect was causally related to suppression of type I interferon (IFN) as administration of recombinant IFN-β reconstituted IFN-stimulated gene expression and restored virus control. FP suppressed RV-induced airway inflammation but led to enhanced airway mucin production, effects that were unaltered by recombinant IFN-β. FP administration also suppressed innate responses to S. pneumoniae including expression of anti-bacterial cytokines and cathelicidin-related anti-microbial peptide. High dose FP increased lung tissue bacterial loads with the opposite effect observed with lower dose FP despite similar anti-inflammatory effects.

Our findings demonstrate beneficial anti-inflammatory effects of ICS during virus-induced COPD exacerbations but reveal some previously unrecognised detrimental effects including increased virus replication and enhanced mucin production. Additionally, we show that high dose ICS administration may increase bacterial loads and thus increase pneumonia risk but lower doses may conversely reduce bacterial loads and therefore could be safer in COPD.
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<td>ACK</td>
<td>Ammonium-chloride-potassium</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyper-responsiveness</td>
</tr>
<tr>
<td>AMP</td>
<td>Anti-microbial peptide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
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<td>Cytopathic effect</td>
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<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GOLD</td>
<td>Global Initiative for Chronic Obstructive Lung Disease</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid response element</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDM</td>
<td>House dust mite</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidise</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule 1</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>Interferon alpha/beta receptor</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of NFkB</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INSPIRE</td>
<td>Investigating New Standards for Prophylaxis in Reduction of Exacerbations</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-gamma inducible protein 10kDa/CXCL10</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>LABA</td>
<td>Long acting β2 adrenoreceptor agonist</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MCH</td>
<td>Methacholine</td>
</tr>
<tr>
<td>MDA-5</td>
<td>Melanoma differentiation-associated protein-5</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MDC</td>
<td>Monocyte-derived chemokines/CCL22</td>
</tr>
<tr>
<td>MIG</td>
<td>Monokine induced by gamma interferon</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MKP</td>
<td>MAPK phosphatase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NACHT, LRR and PYD domains-containing protein</td>
</tr>
<tr>
<td>NOD2</td>
<td>Nucleotide-binding oligomerization domain-containing protein 2</td>
</tr>
<tr>
<td>OAS</td>
<td>Oligoadenylate synthetase</td>
</tr>
<tr>
<td>PAFR</td>
<td>Platelet activating factor receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEF</td>
<td>Peak expiratory flow</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly ethylene glycol</td>
</tr>
<tr>
<td>PenH</td>
<td>Enhanced pause</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin-chlorophyll proteins</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polynosinic-polycytidylicacid</td>
</tr>
<tr>
<td>PPM</td>
<td>Potentially pathogenic microorganism</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative (reverse transcriptase) polymerase chain reaction</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T cell expressed, and secreted/CCL5</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid inducible gene-I</td>
</tr>
</tbody>
</table>
RNA   Ribonucleic acid
ROS   Reactive oxygen species
RPMI  Roswell Park Memorial Institute
rRNA  ribosomal ribonucleic acid
RSV   Respiratory syncytial virus
RT-PCR Reverse transcription polymerase chain reaction
RV    Rhinovirus
SEM   Standard error of the mean
SLPI  Secretory leucocyte protease inhibitor
SP    Strepptococcus pneumoniae
STAT  Signal Transducer and Activator of Transcription
TARC  Thymus and activated-regulated chemokines /CCL17
T cell T lymphocyte
TCIDso Tissue culture infective dose
Th1/2  T helper lymphocyte type 1/2
TLR   Toll like receptor
TORCH Towards a Revolution in COPD Health
TMB   Tetramethyl benzidine chromogen
TNF   Tumour necrosis factor
UV    Ultraviolet
vs.   versus
Acknowledgements and Declaration of Originality

I declare that the work presented in this thesis was carried out by myself unless stated otherwise. The work presented was supported by a Wellcome Trust Clinical Research Training Fellowship [096382/Z/11/Z].

I would like to express particular gratitude to my supervisors at the Airway Disease Infection section, namely Professor Sebastian Johnston, Dr Nicholas Glanville and Dr Nathan Bartlett for their guidance and support throughout the project. I would particularly like to acknowledge Nick who has dedicated a lot of his time to teach me techniques in the laboratory and has also offered invaluable advice and guidance throughout this project.

All the experiments described were conceived and designed following discussion with my supervisors. Additionally, the animal work presented often required large harvests at individual timepoints, which would not have been possible to complete by myself without help from others. In these instances, I sought assistance from other members of the group and would therefore specifically like to thank Nathan Bartlett, Nicholas Glanville, Ross Walton and Yee-Man Ching. I also extend thanks to all members of the Johnston group for their assistance and advice throughout the course of the project. I would additionally like to thank my Supervisors at the Brompton Molecular Genetics and Genomics group including Professors William Cookson and Miriam Moffatt and the rest of their group, particularly Dr Michael Cox.

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Abstracts and presentations of work contained in Thesis

Some of the data reported in this thesis has been previously presented at the following conferences:


Chapter 1: Introduction

1.1 Chronic obstructive pulmonary disease

1.1.1 Epidemiology

Chronic obstructive pulmonary disease (COPD) is the most common chronic respiratory condition in adults, is a major cause of morbidity and is currently ranked as the 3rd leading cause of death worldwide.[1] COPD is also predicted to become the fifth leading cause of disability worldwide by 2020.[2]

Prevalence of COPD varies both within and between countries, which reflects differences in a number of factors including geographical and social factors, smoking patterns, environmental exposure to air pollution and access to healthcare.[3] According to the British Lung Foundation, an estimated 3.7 million people in the UK suffer from COPD [4] and the prevalence and burden of the disease are predicted to increase dramatically in the future due to persistent exposure to COPD risk factors and the increasingly ageing population. The financial burden to the UK National Health Service (NHS) of treating COPD was estimated to be over £800 million in 2011 with the total cost implications to the UK economy estimated to be around £2.7 billion per year when indirect costs such as working days lost are also considered.[5]

1.1.2 Aetiology

The development of COPD is multifactorial and related to a combination of genetic and environmental predisposing factors. There is overwhelming evidence that tobacco smoke is the main risk factor for COPD, especially in developed countries. In developing countries, outdoor air pollution and indoor smoke exposure from cooking and heating with organic fuels are also major causative contributors to development of COPD.[3, 6]

Genetic factors that predispose to abnormal lung responses to environmental exposures are also believed to play a central role in development of COPD. The most extensively studied genetic abnormality is a deficiency in alpha-1 antitrypsin but this abnormality only accounts for 1-3% of patients with COPD.[7] Numerous other genetic abnormalities have been implicated in increasing COPD susceptibility, including mutations in genes encoding transforming growth factor β (TGF-β), tumour necrosis factor-α (TNF-α), serpin peptidase inhibitor, clade A alpha-1 antiproteinase, antitrypsin member 1 (SERPINA 1), interleukin (IL)-13 and matrix metalloproteinase (MMP)-9, among others.[8]
1.1.3 Definition and Diagnosis

Definitions of COPD have evolved over time with the previous separately considered entities of chronic bronchitis (large airway inflammation and remodelling) and emphysema (destruction of the gas exchanging surfaces of the lung) more recently being replaced with an umbrella term of COPD. [9, 10] The diagnosis should be considered in patients with a suggestive medical history including dyspnoea, chronic cough and excessive sputum production and exposure to relevant risk factors for the disease. This clinical assessment should be combined with confirmation of post-bronchodilator FEV1/FVC (forced expiratory volume in 1 second/forced vital capacity) < 0.70. [9]

1.1.4 Immunology and Pathophysiology

The major pathologic processes involved in development of COPD include remodelling and narrowing of the small airways, destruction of the lung parenchyma with pulmonary emphysema and inflammation of the central airways with chronic bronchitis. [11]

1.1.4.1 Airway Inflammation

Exposure to cigarette smoke and other inhaled irritants activates a distinct inflammatory cascade inducing lung epithelial cells and resident alveolar macrophages to produce a number of inflammatory mediators including TNF-α, IL-1β, CXCL8/IL-8 and granulocyte-macrophage colony stimulating factor (GM-CSF). [11, 12] These cytokines and chemokines act in a coordinated fashion to recruit additional inflammatory cells into the lungs, leading to a state of chronic airway inflammation, a central feature of COPD that has been demonstrated in tissue biopsy specimens and sputum analyses. [13, 14]

Airway inflammation in COPD is characterised by an increase in a wide range of cell types including elevated macrophage numbers in the small airways and lung parenchyma [15] and increased T-lymphocyte numbers in central airways, small airways and lung parenchyma, with an increased ratio of cytotoxic cluster of differentiation (CD)8+ T lymphocytes to CD4+ T helper cells. [16] Increased numbers of activated neutrophils are also observed in sputum and bronchoalveolar lavage (BAL) of patients with COPD, although increases are not observed in airway wall and lung parenchyma, likely reflecting the rapid transit of these cells through lung tissue. [11] Activated neutrophils secrete proteases such as neutrophil elastase, proteinase-3 and MMP-8 and -9 which may further contribute to alveolar destruction and also promote mucus hypersecretion. [11]
Chapter 1: Introduction

1.1.4.2 Oxidative stress

Oxidative stress also plays an important role in COPD. Increased oxidant burden from cigarette smoking leads to production of reactive oxidant species (ROS) by inflammatory cells and epithelium, which have wide-ranging effects in the airways leading to activation of multiple inflammatory genes and further amplification of the inflammatory response.[17]

1.1.4.3 Mucus hypersecretion

The inflammatory cell infiltration into lung tissue in COPD leads to abnormal tissue repair and remodelling that induces large airway mucous gland hyperplasia and goblet cell metaplasia in the small airways. This leads to a mucus hyper-secretory phenotype with increases in the amount and viscosity of mucus production. These processes may worsen airway obstruction [18] and lead to impairment of mucociliary clearance with retention of particulate matter and pathogens increasing susceptibility to respiratory infections.[19]

1.1.4.4 Systemic Inflammation

In addition to heightened airway inflammation, there is increasing recognition of the importance of systemic inflammation in COPD with reported increases in circulating levels of cytokines, chemokines and inflammatory cells.[20] This may contribute to disease systemic manifestations including skeletal muscle weakness, cardiovascular and metabolic disease and may also impact upon other comorbid diseases.[11]

1.2 COPD exacerbations

The natural course of COPD is punctuated by acute exacerbations which are described as episodes of symptomatic worsening that are beyond the normal day to day variations and require a change in medication.[9] Exacerbations are a major cause of morbidity, mortality and healthcare costs [21] and lead to accelerated lung function decline [22, 23] and reduced quality of life.[24] Data from the ECLIPSE (Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints) study demonstrated the presence of a frequent exacerbation phenotype which is more common as severity of airflow obstruction increases, with a history of prior exacerbations being the major factor associated with this phenotype.[25]
1.2.1 Aetiology of exacerbations

COPD exacerbations have been associated with a number of aetiological factors including respiratory tract infection by bacteria and/or viruses [26, 27] and non-infectious aetiologies including air pollution [28], climatic factors such as air temperature [29] and cardiovascular events, particularly pulmonary embolism.[30]

1.2.1.1 Viruses

Respiratory viruses (most commonly rhinoviruses (RV)), are frequently detected in naturally occurring COPD exacerbations. Using molecular identification techniques, respiratory viruses have been identified in ~ 48-64% of naturally occurring exacerbations.[31-33] Picornaviruses including rhinovirus are the most frequently detected pathogen.[34] Experimental infection studies have further confirmed a direct causal relationship between RV infection and COPD exacerbation with >90% of RV infected subjects experiencing an exacerbation of disease.[35]

1.2.1.2 Bacteria

Bacteria are also frequently detected during COPD exacerbations but a causal relationship between bacteria and exacerbation was previously debated when longitudinal studies showed no differences between rates of isolation of S. pneumoniae and H. influenzae by sputum culture in acute exacerbations and in stable disease.[36, 37] However, more recent culture based studies have reported greater bacterial detection rates during exacerbation compared to stable state [31, 38, 39] and molecular typing has shown that acquisition of new bacterial strains may have a role in triggering exacerbations in COPD.[40]

1.2.2 Human rhinoviruses

1.2.2.1 Classification

Rhinoviruses are positive-sense, single-stranded-RNA (ssRNA) viruses that are members of the family Picornaviridae and the genus Enterovirus.[41] RVs are classified according to serotype or, broadly, as ‘major’ and ‘minor’ groups based on the receptor type used to gain entry into host cells. Major group RVs utilise the intracellular adhesion molecule 1 (ICAM-1) and minor group RVs utilise the low-density lipoprotein receptor (LDL-R).[42, 43]
1.2.2.2 Epidemiology

RVs are the most frequent cause of the common cold and were first isolated from nasal samples of subjects with upper respiratory tract symptoms in the early 1960s.[44] RV infections are believed to account for ~70% of infectious agents detected in individuals displaying cold symptoms.[45] Other viral aetiopathological agents implicated in the common cold include influenza viruses A and B, coronaviruses, parainfluenza and respiratory syncytial viruses (RSV).[45]

Previous studies have reported that adults are infected with RVs around 2-4 times per year with rates as high as 8-12 times per year observed in pre-school age children. Symptomatic infection rates decrease with older age, which is believed to be due to development of immune memory.[46] RV infections can occur throughout the year but peak during spring and early autumn in temperate climates.[47] Transmission of RV is likely to be secondary to both aerosol inhalation and spread via contaminated surfaces.[48, 49]

1.2.2.3 Innate immune response to rhinovirus infection

RVs enter and replicate in epithelial cells lining the respiratory tract and trigger a cascade of distinct immune responses. Following entry of RV into epithelial cells, uncoating of the virus leads to release of viral RNA which is recognised by pattern recognition receptors (PRRs) including endosomal toll-like receptors (TLR) 3, 7 and 8 and the intracellular RNA helicases retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated protein-5 (MDA5).[50-52] Following viral recognition, TLRs interact with adaptor molecules MyD88, TIR-domain containing adaptor-inducing interferon (IFN)-β (TRIF) and Mal and TRIF related adaptor molecule (TRAM) and RIG-I/MDA5 interact with mitochondrial anti-viral signalling protein (MAVS).[53, 54] This triggers a signalling cascade that leads to activation of several key transcription factors including nuclear factor (NF)-κB, interferon regulatory factor (IRF)-3 and IRF-7 and activating transcription factor (ATF)-2 [55, 56] These activated transcription factors then translocate to the nucleus and induce transcription of type I IFNs –α and –β.[57, 58]

The type I IFN response to RV infection is biphasic with initial IFN release triggered by detection of invading virus, through processes described above. The secondary step occurs where secreted type I IFNs bind to the type I IFN receptor complex (IFNAR-1 and -2), leading to activation of janus kinase (JAK) and tyrosine kinase proteins (TYK). These proteins then recruit and activate signal transducer and activator of transcription (STAT) proteins.[55] This leads to formation of a heterotrimeric complex containing IRF-9, known as interferon-stimulated gene factor-3 (ISGF3) which binds to
promoters of interferon-stimulated genes (ISGs) leading to their transcriptional activation.[59] ISGs encode a range of proteins that selectively interfere with virus replication, protein synthesis or protein trafficking.[57] In addition to type I IFNs, the more recently described type III IFNs—λ1, -2 and -3 (IL-29, IL-28A and IL-28B respectively) may also have important roles in the innate anti-viral response to RV.[60]

Type I IFN signalling is important for regulating function of natural killer (NK) cells which also participate in the innate anti-viral response by directly eliminating virally infected cells.[56, 61] Other innate components that have been shown to be important for the control of other respiratory viruses include macrophage function [62] and TNF-α expression [63] but whether these effects are directly relevant to control of RVs is unclear.

1.2.2.4 Adaptive immune response to rhinovirus infection

Adaptive immune responses are also an important component of host defence against respiratory virus infections. CD4+ and CD8+ T lymphocytes possess cytotoxic effector functions and several studies have shown that these cells have direct roles in the control of a number of viruses including influenza and RSV.[64-66] T cell responses in RV infections have not been extensively studied but a few studies have reported increased T cell recruitment to the airways in response to experimental RV infection in human and mouse models.[67-69] CD4+ T cells also provide help to B cells and are thus indirectly involved in production of antibody.[70] Pre-existing neutralising antibodies to RVs provide protection against infection and reduce symptoms in humans and may also provide protection against future RV infections.[71, 72]

1.2.2.5 Disease

Experimental RV infection studies in humans have been informative in defining the relationship between virus infection, inflammatory responses and symptoms. In healthy individuals, RV infections cause only mild upper respiratory tract symptoms including sore throat, rhinorrhea, cough and headache. Symptoms generally correlate with virus loads and begin around 12 hours (h) post-challenge, peaking at day (d) 2-3 with resolution within 7-10d.[73-75]

RV infection induces nasal mucus hypersecretion and plasma leakage and is associated with the production of pro-inflammatory cytokines including TNF-α IL-1β, IL-6 and chemokines CXCL8/IL-8, CCL3/macrophage inflammatory protein (MIP)-1α and CCL5/regulated on activation, normal T cell expressed and secreted (RANTES) in the upper airways, leading to a predominantly T helper-cell type 1 (Th1) profile which limits virus shedding and symptoms.[76] Neutrophilia represents the major
cellular inflammatory component in RV infections and upper airway neutrophilia or neutrophil chemokine levels have been shown to correlate with symptom severity in several previous studies.[77-79]

1.2.3 Mechanisms of rhinovirus-induced exacerbation in COPD

1.2.3.1 Anti-viral immune responses

Although RVs cause a mild self-limiting condition of the upper respiratory tract in healthy individuals, they can also infect the lower airways [80] and thus can precipitate acute disease exacerbations in patients with underlying COPD. As discussed, rhinoviruses are implicated in a large number of COPD exacerbations and the recently described human experimental model of RV infection [35] has allowed characterisation of disease-specific alterations in these mechanisms to be studied in COPD.

As discussed, a robust IFN response is essential for resolution of RV infection in the airways and there is emerging evidence that these responses may be impaired in COPD. In vitro, cigarette smoke extract has been shown to impair RV-induction of IFNs –α and –β and ISGs in airway epithelial cells.[81, 82] BAL cells from patients with moderate COPD infected with RV ex vivo had impaired induction of IFN-β and a trend towards impaired IFN-α and –λ associated with impaired induction of ISG CXCL10/Interferon-gamma inducible protein 10kDa (IP-10).[35] Conversely, it has been reported that epithelial cells from COPD patients actually show enhanced type III IFN expression in response to RV infection.[83] Impairment of innate anti-viral immune responses may increase the susceptibility of patients with COPD to rhinovirus infections and/or lead to delayed RV clearance from the airways during established infection. This is supported by a study which reported that patients with COPD who were experimentally infected with RV16 had increased virus loads compared to healthy controls [35] and another in vitro study where airway epithelial cells from patients with COPD infected with RV29 had increased virus titres compared to cells from control subjects.[83]

Very few studies have characterised the adaptive immune response to RV in COPD. Following experimental RV infection in COPD, CD8+ T cells were increased in BAL compared to baseline, an effect that was not observed in control subjects.[84] Reduced levels of immunoglobulin (Ig)G1 antibody to the viral capsid protein of RV have also been shown to correlate with increased risk of acute exacerbations of COPD requiring hospitalisation.[85]
1.2.3.2 Airway inflammation

Recognition of RV by PRRs also leads to upregulation of pro-inflammatory mediators and cytokines that promote chemoattraction of inflammatory cells into the airways.[86] A number of studies assessing naturally occurring exacerbations in COPD have provided insight into the nature of airways inflammation during COPD exacerbations but few have examined specific inflammatory responses to viruses such as RV. COPD exacerbations represent a further amplification of the inflammatory process in the lungs from stable state. Regardless of aetiological cause, in comparison to stable state, exacerbations are associated with increases in cellular airways inflammation including neutrophilic and lymphocytic inflammation in sputum [31, 87, 88], increased neutrophil chemokine CXCL8/IL-8 [87, 89] and lymphocyte chemokines CXCL10/IP-10 [90] and CCL5/RANTES [87, 91] and exaggerated production of pro-inflammatory cytokines such as IL-6 [35, 91-93] and TNF-α in the airways.[89, 91]

Studies that have assessed the presence of viruses during exacerbations have reported that increased sputum IL-6 [93] and eosinophils [31, 91, 94] may specifically correlate with exacerbations in which a virus is present. Elevated levels of CXCL10/IP-10 in serum have also been identified as a marker of RV positive exacerbations in COPD but whether concurrent increases are observed in the airways is unknown.[95] The human model of experimental RV infection in COPD discussed above reported increased BAL neutrophil and lymphocyte numbers and increased sputum CXCL8/IL-8 protein in patients with COPD compared to healthy controls. Additionally, virus loads also correlated with a number of sputum inflammatory indices in COPD including IL-6, CXCL8/IL-8, TNF-α and neutrophil numbers.[35] Some of the inflammatory mediators that have been shown to be increased during acute exacerbation of COPD compared to stable state or compared to infection in healthy controls are summarised in table 1.1.
Increased compared to stable COPD | Increased compared to infection in healthy patients
---|---
**Inflammatory cells** | Neutrophils \([31, 35, 87, 96]\) | Neutrophils [35]  
| Lymphocytes \([35, 87, 96]\) | Lymphocytes [35]  
| Eosinophils \([31, 87, 94, 96]\) |  |

**Chemokines** | CCL5/RANTES \([87, 91, 94]\) | Serum CXCL10/IP-10 [95]  
| CCL4/MIP-1β \([91]\) | CXCL8/IL-8 [35]  
| CXCL8/IL-8 \([35, 87, 89, 97]\) |  
| CXCL10/IP-10 \([90]\) |  
| CCL2/monocyte chemotactic protein (MCP)-1 \([96]\) |  |

**Cytokines** | IL-6 \([32, 91, 93, 94]\) |  
| TNF-α \([89, 91]\) |  
| IL-1β \([91]\) |  |

**Other mediators** | Leukotriene B4 \([96]\) | Neutrophil elastase [35]  
| Neutrophil elastase \([35, 87]\) |  
| Eosinophilic cationic protein \([87, 96]\) |  
| Myeloperoxidase \([38, 97]\) |  |

Table 1.1 Inflammatory mediators increased during COPD exacerbations in comparison to stable state or in comparison to infection in healthy controls.
1.2.4 Mechanisms of bacterial exacerbation in COPD

1.2.4.1 Bacterial colonisation

Culture based studies have revealed the presence of bacteria in the lower respiratory tract of patients with COPD during clinical stability, an entity that has been previously termed ‘colonisation’. [98] Isolated species can be broadly divided into two groups: potentially pathogenic microorganisms (PPMs) such as *Haemophilus* species, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* and non-potentially pathogenic microorganisms (non-PPMs) such as components of the oropharyngeal and gastrointestinal flora including *Corynebacterium*, *enterococci* and *Neisseria* species. [99]

The term ‘colonisation’ implies the presence of bacteria with no or minimal pathological significance. However, recent studies have shown that the presence of bacteria in the lungs is associated with host immune responses including increased neutrophilic airway inflammation [100, 101] and exaggerated production of inflammatory mediators such as TNF-α [101], IL-6 [102], IL-1β [102, 103], CXCL8/IL-8 [100, 102, 104-106], leukotriene-B4 [104, 106] and myeloperoxidase [104]. Bacterial infection in stable state is also associated with adverse clinical outcomes including increased risk of exacerbation [107, 108], impaired health status [102, 106] and accelerated lung function decline. [109, 110] This has led to postulation that ‘colonisation’ may represent a misnomer and alternative terms such as ‘chronic bronchial infection’ have subsequently been proposed. [98]

1.2.4.2 Increased bacterial load

Since COPD is associated with the presence of PPMs in the airways during clinical stability, studies have assessed the hypothesis that the occurrence of exacerbations may be related to increases in concentrations of existing PPMs, the ‘fall and rise’ or quantitative hypothesis of exacerbation pathogenesis. [111] This hypothesis is the subject of debate, as some studies have shown increased bacterial loads at exacerbation compared to stable state [38, 39] but one study conversely showed no difference or reduced concentrations of a number of PPMs at exacerbation. [112]

1.2.4.3. Acquisition of new bacterial strain

Studies that have employed molecular typing of bacterial isolates during stable state and exacerbation have led to development of the ‘new bacterial strain’ model of exacerbation pathogenesis. This states that acquisition of new strains of bacteria or antigenic change in a pre-existing strain directly triggers acute exacerbations of COPD. [98] Sethi *et al* showed a two-fold
increase in exacerbation frequency in patients who had a new strain of one of four major PPMs (S. pneumoniae, H. influenzae, M. catarrhalis or P. aeruginosa) compared to patients in whom no new strains were isolated.[40] Furthermore, new strain exacerbations have also been shown to be associated with greater changes in airway inflammation and increased clinical symptom scores in a separate study by the same group.[113]

1.2.4.4 Inflammatory responses during bacterial exacerbation

Similar to virus-induced episodes, exacerbations associated with bacterial triggers also lead to enhanced airway inflammatory indices compared to stable state including increases in cellular airways inflammation [38, 91] and exaggerated production of chemokines and cytokines including TNF-α [96, 113], IL-6 [96], CXCL8/IL-8 [96], IL-1β [31] and leukotriene B4 [96]. Mechanisms of susceptibility to bacterial infection in COPD are discussed in section 1.3.2.

1.2.4.5 The lower respiratory microbiome in COPD

As discussed previously, our understanding of the role of bacteria in pathogenesis of exacerbations to date has been predominantly based on studies that use classical microbiological culture techniques or molecular typing of specific pre-determined bacterial strains. However, new culture-independent molecular methods have revealed a diverse microbiota in the lower airways of healthy subjects and patients with chronic lung disease including the presence of bacteria that were not previously amenable to culture.[114-117] These culture-independent techniques are based on PCR amplification of the 16S rRNA gene, a highly conserved component of the bacterial genome. The gene consists of conserved and variable regions and, by using universal primers targeted at the conserved regions, the entire spectrum of microorganisms within a sample can be amplified with the variable regions allowing discrimination between different microorganisms.[118, 119]

Studies using 16S rRNA sequencing to assess the lower respiratory microbiota in stable COPD have yielded conflicting results with one study showing increased community diversity in moderate to severe COPD vs. healthy controls [120] and other studies showing reduced or no change in diversity.[115, 116] Another study which assessed microbiota in endotracheal aspirates from intubated patients with COPD identified two distinct sub-groups, one group with a loss of bacterial community diversity and a second with increased diversity.[117] Studies have reported increased representation of Firmicutes phylum in severe COPD vs. smokers or healthy controls [115] and in severe vs. moderate COPD.[120] Proteobacteria have been reported to be more frequent in COPD
patients compared to healthy controls in one study [114] but less frequent in severe vs. moderate COPD in another study [120].

Only two studies to date have assessed the changes that occur in the lung microbiome from stable state to exacerbation in COPD. Huang et al assessed temporal changes in the airway microbiome of sequential sputum samples taken from patients with COPD at clinical stability and during exacerbation. An increase in *Proteobacteria* at exacerbation was observed that decreased with antibiotic therapy.[121] Similar findings were reported by a study that assessed the microbiota in sputum samples taken from subjects experimentally infected with RV16, where an increase in *Proteobacteria*, specifically *H. influenzae* was observed in patients with COPD but not in healthy controls.[122]

### 1.3 Pneumonia in COPD

Patients with COPD are known to be at increased risk of developing bacterial pneumonia.[123-126] The distinction between bacterial exacerbation and pneumonia in COPD is based on the anatomical site of infection with exacerbations caused by infection of the larger airways and pneumonia affecting the lung parenchyma and smaller airways.[127, 128] Clinically, pneumonia can be identified by the presence of new consolidative changes on chest imaging. In reality, it can often be difficult to distinguish between a bacterial exacerbation and pneumonia in COPD and there is likely to be considerable overlap between these syndromes, especially since many studies of exacerbations in COPD do not routinely include chest imaging in the protocol.[98] The distinction between these two entities is clinically important because studies that have used radiographic definitions have reported worse outcomes for patients with COPD who present with pneumonia compared to non-pneumonic exacerbation.[129, 130]

#### 1.3.1 Aetiology of pneumonia in COPD

Bacterial pathogens are the most common aetiological cause of pneumonia, although viruses have also been identified as potential causative agents.[131] The spectrum of microbial aetiology associated with pneumonia in COPD is similar to patients without chronic lung disease with *S. pneumoniae* frequently identified as the most common cause.[132, 133] However, *H. influenzae* and *P. aeruginosa* have been reported to be more common in COPD.[132-134]
1.3.2 Innate immune responses to *Streptococcus pneumoniae*

Innate immune responses to bacterial respiratory pathogens have been most extensively studied in *S. pneumoniae* infection. *S. pneumoniae* is an encapsulated gram-positive diplococcus that belongs to the group *α*-*Streptococci*. S. pneumoniae frequently colonises the upper respiratory tract in humans and, although most episodes of carriage are benign, the anatomical continuity of the nasopharynx with the lower airways makes pneumonia an ever-present risk. S. pneumoniae is an encapsulated gram-positive diplococcus that belongs to the group *α*-S. pneumoniae. S. pneumoniae frequently colonises the upper respiratory tract in humans and, although most episodes of carriage are benign, the anatomical continuity of the nasopharynx with the lower airways makes pneumonia an ever-present risk. The precise mechanisms that determine progression from nasopharyngeal colonisation to pneumonia are poorly understood, although factors such as human immunodeficiency virus (HIV) may predispose to increased risk. As with RV infection, the first recognition of *S. pneumoniae* by the host is mediated by PRRs including TLRs and NOD-like receptors (NLRs) which recognise specific bacterial pathogen associated molecular patterns (PAMPs). Components of the pneumococcal wall including lipoteichoic acid and lipoproteins are recognised by TLR2 which has been shown to be important in mouse models of disease as TLR2 deficient mice show reduced bacterial clearance following challenge with *S. pneumoniae*. TLR4 was previously thought to be activated exclusively by products of Gram-negative bacteria only but more recent data now suggests that TLR4 may be activated by pneumolysin, a pneumococcal virulence factor. TLR9 is also believed to play a role in host recognition of *S. pneumoniae* and TLR9 deficient mice also have increased bacterial loads and reduced survival in response to pneumococcal challenge.

NLRs are the other major type of PRRs that are involved in bacterial recognition. Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) recognises bacterial peptidoglycan components and has been shown to promote macrophage recruitment and clearance of *S. pneumoniae* colonisation in mice. NLR family, pyrin domain containing 3 (NLRP3) is another member of the NLR family that forms protein complexes called inflammasomes which are involved in post-translational regulation of IL-1β production. NLRP3 -/- mice have been shown to be more susceptible to pneumococcal pneumonia.

Recognition of *S. pneumoniae* by PRRs triggers intracellular signaling cascades that lead to activation of transcription factors including NFκB, STATs and IRF-3 and -7. NFκB, in particular, plays an important role in pneumococcal infection and triggers expression of a number of proinflammatory genes. Targeted disruption of the P50 subunit of NFκB in mice increases susceptibility to pneumococcal infection and polymorphisms of inhibitor of NF-κB (IκB) gene correlate with protection against invasive pneumococcal disease in humans. Transcription factor activation
culminates in increased expression of proinflammatory mediators including cytokines such as IL-6, TNF-α and neutrophil chemokines such as CXCL8/IL-8 which all have important anti-bacterial roles. Anti-TNF-α antibody administration in a mouse model of pneumococcal pneumonia has been shown to increase systemic dissemination and accelerate mortality.\[147\] IL6 -/- mice show increased lung bacterial loads and mortality following pneumococcal infection [148] and selective depletion of neutrophils also increased bacterial lung loads in mice [149].

Alveolar macrophages play a key role in initial phagocytosis of bacteria in the airways and also in coordinating the innate immune response to infection including amplification of TNF-α production.[150, 151] As infection progresses, macrophages cease to be involved in phagocytosis as neutrophils become the major phagocytosing cell.[151] Neutrophil action is crucial for S. pneumoniae clearance through a number of specific functions including chemotaxis, phagocytosis and direct microbial killing and selective depletion of neutrophils in mice leads to reduction in clearance of S. pneumoniae.[149] Other host defence mechanisms that have been shown to have important roles in bacterial lung infection include mucociliary clearance [152], complement [153], type I and II IFNs [154, 155], IL-17 [156] and anti-microbial peptides (AMPs) such as cathelicidin [157], β defensin [158, 159] and surfactant protein-A [160].

### 1.3.3 Impairment of anti-bacterial host defence in COPD

The precise underlying pathophysiology of pneumonia in COPD has not been extensively studied. Therefore, whether similar mechanisms to the pathogenesis of bacterial exacerbations, such as increases in bacterial load of colonising species and/or new strain acquisition are also involved development of pneumonia in COPD is unclear. However, a number of studies have assessed some components of anti-bacterial innate responses in COPD and some of the reported impairments may potentially lead to increased risk of pneumonia.

#### 1.3.3.1 Pattern recognition receptor expression

Conflicting data exists on the expression of bacterial PRRs TLR2 and TLR4 in COPD. Studies have reported decreased TLR2 expression on macrophages [161] and neutrophils [162] from patients with COPD and TLR4 has also been shown to be down-regulated on nasal epithelial cells in severe COPD [163]. These effects would potentially increase risk of pneumonia in COPD due to reduced bacterial recognition and suppressed initiation of anti-bacterial immune responses. However, other studies have shown increased expression of TLR2 on monocytes and sputum from patients with COPD.[164, 165] TLR2 and TLR4 polymorphisms have additionally been shown to be associated with accelerated...
lung function decline in COPD and this further suggests that these PRRs may play a role in disease progression, perhaps by increasing risk of bacterial infection.[166] There are no reported data suggesting that expression of NLRs is impaired in COPD, although polymorphisms of NOD2 have been shown to be associated with lower FEV1 % predicted values.[167]

1.3.3.2 Macrophage and neutrophil function

As discussed in section 1.1.4.1, stable COPD is associated with increased absolute numbers of macrophages and neutrophils in the airways. Since these cells are responsible for phagocytic clearance of bacterial pathogens, it might be expected that phagocytosis would be enhanced rather than suppressed in COPD. However, accumulating evidence suggests that, although numbers of phagocytic cells are increased, function is markedly impaired in COPD.[168] Phagocytosis of potentially pathogenic species including S. pneumoniae and H. influenzae has been shown to be impaired in alveolar and monocyte-derived macrophages from patients with COPD.[169, 170] These defects appear to be specific to bacteria, since alveolar macrophages from patients with COPD have preserved capacity to phagocytose inert beads.[170-172] Additionally, defective efferocytosis has been shown in alveolar macrophages from patients with COPD compared to healthy controls.[171]

Relatively few studies have assessed the phagocytic ability of neutrophils in COPD. Some studies have reported impaired phagocytosis of bacteria by peripheral blood neutrophils in COPD [173, 174] but these findings have not been replicated in other studies [175, 176]. Sapey et al reported specific functional defects in neutrophils from patients with COPD including reduced chemotaxis and poor migratory accuracy towards inflammatory sources.[177]

1.3.3.3 Mucociliary clearance

Optimal airway defence relies on an adequate balance between mucus production and clearance. As discussed in section 1.1.4.3, COPD is associated with defective mucus secretion with increased mucus viscosity and mucus retention. Tracheobronchial clearance following inhalation of radioaerosol has been shown to be impaired in COPD.[19] Impaired clearance may lead to accumulation of bacteria and thus increase susceptibility to exacerbations and pneumonia in COPD. Bacteria such as S. pneumoniae have also been shown to directly upregulate the airway mucin MUC5AC [178, 179] and thus lower airways colonisation may directly perpetuate mucus hypersecretion. In addition to altered quantity and quality of mucus in COPD, there is also evidence of impaired ciliary function in COPD which may further contribute to particle and pathogen retention and increased risk of bacterial infection.[180, 181]
1.3.3 Anti-microbial peptides

AMPs are soluble anti-bacterial factors that are present in airway surface fluid and are secreted by epithelium and/or inflammatory cells.[182] Studies evaluating the production of AMPs in COPD have reported varying patterns of expression. During clinical stability, lower levels of salivary lysozyme [183] have been shown to correlate with increased risk of COPD exacerbation and sputum levels also fall with acquisition of new bacterial strains.[184] In contrast, lactoferrin levels appear to remain constant between stable state and exacerbation.[184] Secretory leucocyte protease inhibitor (SLPI) protein levels have been shown to be elevated in stable COPD [185] but decrease following bacterial infection [184] with lower levels reported to correlate with more frequent exacerbations in one study.[186] Both increased [187] and decreased [185, 188] expression of human β-defensins have been reported in stable COPD. Cathelicidin/LL37 levels have been shown to be increased in the airways of patients with mild obstruction but reduced with more severe COPD.[189] Increased cathelicidin/LL37 levels have also been reported with colonisation or bacterial infection compared to stable state.[184]

1.3.4 Coinfection and virus-induced secondary bacterial infection

As discussed previously, both viruses and bacterial pathogens can trigger acute exacerbations of COPD. Dual virus and bacterial infection is reported in up to 25% of COPD exacerbations and this has led to the hypothesis that one infection may follow another so that they are infrequently detected together.[31, 190] A large body of evidence supports an association between influenza infection and secondary bacterial pneumonia triggered by S. pneumoniae, S. aureus or H. influenzae and several molecular mechanisms for these associations have been proposed.[191, 192] However, influenza is a lytic virus which causes significant necrosis to the airway epithelium [193] and therefore, mechanisms involved in influenza-induced secondary bacterial infection may not be directly applicable to rhinoviruses which do not cause similar epithelial damage.[194]

1.3.4.1 Clinical studies

The majority of studies assessing pathogens in naturally occurring infections in COPD have tested for viruses and bacteria in single samples obtained during exacerbation and rates of dual infection reported in these studies range from 8-25%.[31, 91, 195] Hutchinson et al performed sequential sampling at exacerbation onset and then 5-7 days later and found that, in 36% of exacerbations where a virus was detected, a bacterial species was identified in the second sample.[196] In another study by George et al, sequential sputum samples taken from patients with COPD during
exacerbation were analysed by PCR for rhinoviruses and bacterial pathogens *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. In patients with RV positive exacerbations but no bacteria detected at exacerbation onset, 73% were found to be subsequently positive for bacteria at d14 following exacerbation onset.[197] In the human experimental RV infection model, 60% of patients with COPD were shown to develop secondary bacterial infection identified by positive sputum culture, an effect that was not observed in healthy controls.[190]

### 1.3.4.2 Alteration of the respiratory microbiome by virus infection

Emerging evidence suggests that secondary bacterial infection may be precipitated by respiratory viruses through alterations in the respiratory microbiome. 16S rRNA sequencing of sputum samples from the human model of experimental RV infection in COPD showed a sixfold increase in 16S bacterial copy number at d15 post RV infection in COPD with a greater expansion of *H. influenzae* in patients with COPD compared to healthy controls.[122] A study by Leung *et al* reported that the oropharyngeal microbiota of patients coinfected with influenza and pneumonia had increased *Pseudomonas* representation compared to patients with pneumonia alone.[198] Studies have also shown alteration in the respiratory microbiota in response to influenza challenge in mice with concentration in two dominant communities containing *Staphylococcus*, *Prevotella*, *Acinetobacter* and *Moraxella* bacteria genera.[199]

As discussed in section 1.2.4.5, there is evidence that the lower respiratory microbiota may be altered in stable COPD with increased representation of phyla such as *Proteobacteria*. Therefore, virus infection in patients with COPD may potentially lead to further dysregulation of the microbiota with expansion of existing species and increased risk of secondary bacterial infections and pneumonia.

### 1.3.4.3 Mechanisms of rhinovirus-induced secondary bacterial infection

A number of potential mechanisms for influenza-induced secondary bacterial infection have been identified but much less is known about the effect of rhinoviruses on bacterial infection. *In vitro* studies have reported that RV infection leads to increased adherence of *S. pneumoniae*, *S. aureus* and *H. influenzae* to nasal epithelial cells [200] and increased adherence of *S. pneumoniae* to tracheal epithelial cells [201]. Increased adherence may be mediated by upregulation of surface molecules such as platelet activating factor receptor (PAFR) or carcinoemryonic antigen-related cell adhesion molecule (CEACAM) facilitating increased binding of bacteria.[200, 201] Additionally,
studies have shown that RV can disrupt the barrier function of airway epithelial cells and directly increase transmigration of bacteria through cell layers in vitro.[202, 203]

Impaired macrophage and neutrophil recruitment and function have been postulated as potential mechanisms underlying the increased risk of secondary bacterial infection following influenza infection.[204-207] Some of these effects may also be relevant to RVs, which have been shown to impair responses to bacterial products by alveolar macrophages in vitro [208] and a recent study also reported that RV1B suppressed neutrophil recruitment in response to secondary H. influenzae challenge in mice leading to impaired bacterial clearance.[209]

Other potential mechanisms of RV-induced secondary bacterial infection in COPD include induction of proteases such as neutrophil elastase which can cleave AMPs such as SLPI and elafin, an effect that was suggested by data from the human experimental RV infection model where patients with COPD who developed bacterial infection had higher sputum levels of neutrophil elastase but lower levels of SLPI and elafin than bacteria-negative patients.[190] Several other mechanisms have been identified by studies of influenza-induced secondary bacterial infection including upregulation of IL-10 [210], type I [211, 212] and type II [213] IFN signalling. Whether any of these mechanisms are relevant to RV-induced secondary bacterial infection is, to date, unclear.

1.4 Management of COPD

1.4.1 Overview of management in stable COPD

Effective management of COPD should be individualised and multi-faceted with specific goals targeted towards symptomatic relief, improvement of exercise tolerance and health status, reduction of exacerbations and prevention of disease progression.[9] Management algorithms for COPD are evolving and the previous recommendations of treatment based solely on spirometric parameters are now recognised to be over-simplistic and potentially ineffective.[214] Recent guidelines from the Global Initiative in Obstructive Lung Disease (GOLD) recommend treatment based on stratification of patients according to a combination of clinically relevant factors including spirometry, frequency of exacerbations and breathlessness as assessed by the Medical Research Council (MRC) dyspnoea score or the COPD assessment test (CAT).[9]

Broadly, management can be subdivided into nonpharmacologic and pharmacologic treatment. Nonpharmacologic approaches include smoking cessation (which can include pharmacologic
Pharmacologic strategies include bronchodilators (β2 adrenoceptor agonists and anticholinergics), inhaled corticosteroids (ICS), phosphodiesterase inhibitors and oxygen therapy. For very severe COPD, surgical options including lung volume reduction and lung transplantation can be considered in selected patients.

1.4.2 Inhaled corticosteroids

1.4.2.1 Indications

ICS are frequently used as therapeutic agents in COPD and are recommended in patients with FEV1<50% predicted and/or frequent exacerbations that are not adequately controlled by use of long acting β2 adrenoceptor agonists (LABAs) alone. Monotherapy with ICS is not recommended in COPD as it has been shown to be less effective than ICS/LABA combined. Despite these specific recommendations, there is evidence of more widespread use of ICS in patients with milder disease. The most widely used ICS compounds in COPD are fluticasone propionate (FP) and budesonide with other compounds such as beclomethasone and ciclesonide infrequently used.

1.4.2.2 Mechanisms of action

The molecular mechanisms underlying suppression of inflammation by ICS in airways diseases have been well characterised. The pharmacological actions of ICS are mediated by binding of the drug to the glucocorticoid receptor (GR) in the cell cytoplasm, forming a complex that subsequently translocates to the nucleus to interact with glucocorticoid response elements (GRE) located in the promoter region of target genes. Binding to GREs leads to an activation of the transcription of anti-inflammatory proteins (transactivation) and also leads to repressed expression of immune-regulatory genes (transrepression). ICS may also have post-transcriptional/translational effects such as degradation of mRNA and reduced inflammatory protein secretion. Effects of ICS on transcription of genes relevant to COPD are shown in table 1.2.
Increased transcription (transactivation)

- Anti-inflammatory cytokines: IL-10, IL-12, IL-1 receptor antagonist
- β2 adrenoceptors
- IκB
- SLPI
- Lipocortin 1
- MAP kinase phosphatase-1 (MKP-1)

Reduced transcription (transrepression)

- Inflammatory cytokines: IL-1β, IL-6, IL-13, IL-15, TNF-α, GM-CSF
- Chemokines: CCL5/RANTES, CXCL10/IP-10, CXCL8/IL-8, MCP-1
- Inflammatory enzymes: cyclo-oxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS)
- Adhesion molecules: ICAM-1, vascular cell adhesion molecule-1 (VCAM-1)
- Mediator receptors: neurokinin-1 receptors, bradykinin (B2) receptors

Table 1.2: Effects of ICS on transcription of genes relevant to COPD
(Adapted from Barnes (2011)[225])

1.4.2.3 Anti-inflammatory effects of inhaled corticosteroids in stable COPD

Although ICS are highly effective in reducing eosinophilic and Th2-mediated inflammation in asthma, COPD is believed to be associated with a resistance to the anti-inflammatory effects of ICS.[227] Histone deacetylase-2 (HDAC-2) is an enzyme that is recruited following activation of GRs and reverses histone acetylation leading to suppression of NFκB-activated inflammatory genes.[228] The oxidative stress present in COPD reduces the expression and activity of HDAC-2 in lung tissue and alveolar macrophages and thus leads to resistance to ICS effects.[229]

Despite the relative resistance to ICS therapy in COPD, several studies have reported suppressive effects on airway inflammation in stable state, although conflicting data exists. Studies have reported suppressed neutrophilic inflammation by ICS in BAL [230, 231] but conversely increased or no effect on neutrophil counts in bronchial biopsies.[232-234] Reduced numbers of CD4+ and CD8+ lymphocytes have also been observed with ICS treatment [233, 235, 236] but other studies report no
or minimal effects.[234, 237, 238] ICS treatment has been shown to increase macrophage numbers in BAL.[230, 231]

1.4.2.4 Beneficial clinical effects associated with inhaled corticosteroid use

Clinical studies have reported a number of potentially beneficial effects of ICS use in COPD. Reductions in exacerbation frequency have been reported in trials of ICS in patients with COPD with most studies reporting ~20-25% reduction.[217, 239, 240] The largest longitudinal study of ICS in COPD, the Towards a Revolution in COPD Health (TORCH) study reported a 25% reduction associated with combination fluticasone/salmeterol therapy. However, the group receiving salmeterol alone also had significantly reduced exacerbation rates compared to placebo and no difference was found between salmeterol alone and combined FP/salmeterol regarding severe exacerbation rates.[217] This has led some authors to speculate that the benefit on exacerbation reduction with combination therapy may be primarily related to the LABA component. Consistent with this hypothesis, some studies assessing budesonide or fluticasone therapy alone vs. placebo have reported no effect of ICS on exacerbation frequency.[241-243] Overall, the effect of ICS use on reducing exacerbation rates in COPD is relatively modest.

Other potential beneficial effects associated with ICS therapy include increases in post-bronchodilator FEV1 [217, 244] and improvements in health-related quality of life measures.[217, 239, 245] No convincing data exists to suggest that ICS therapy has any significant effects on rate of lung function decline or mortality in patients with COPD.

1.4.3 Effects of inhaled corticosteroids on respiratory virus infections

As mentioned, respiratory virus infections are common triggers for acute exacerbations of COPD. Since ICS have been shown to have relatively modest effects on exacerbation frequency, there has been some speculation that they may have a relative lack of benefit in the context of acute respiratory virus infections in particular.[57]

1.4.3.1 Effects of corticosteroids on innate anti-viral responses

Emerging evidence suggests that corticosteroids may suppress innate anti-viral immune responses. *In vitro* studies have shown suppression of RV-induction of IFN-α by budesonide in peripheral blood mononuclear cells [246] and suppression of RV-induction of ISG CXCL10/IP-10 by budesonide in bronchial epithelial cells.[247] Additionally, dexamethasone administration has been shown to
inhibit IFN–induced ISGs in bone-marrow derived macrophages.\textsuperscript{[248]} The specific components of type IFN signalling affected by steroids have not been fully characterised.

1.4.3.2 Effects of corticosteroids on virus control

The \textit{in vivo} consequences of this potential impairment of anti-viral immune responses by ICS on virus control have also not been extensively studied. Gustafson \textit{et al} reported that oral prednisolone administration prior to experimental RV challenge in healthy subjects led to increased viral titres in nasal lavage \textsuperscript{[249]} and Puhakka \textit{et al} showed prolonged virus shedding with intranasal FP administration during naturally occurring colds \textsuperscript{[250]}. In a mouse model, systemic administration of hydrocortisone in combination with pneumovirus infection increased viral replication.\textsuperscript{[251]} Conversely, Singam \textit{et al} showed in a mouse model of allergic asthma that nebulised FP administration reduced virus loads following RSV challenge \textsuperscript{[252]} and Bauer \textit{et al} showed no effect of oral dexamethasone administration on virus titres in cigarette-smoke exposed mice infected with influenza \textsuperscript{[253]}. None of these studies have performed detailed evaluations of the \textit{in vivo} effects of corticosteroids on anti-viral innate immune responses and/or correlated impairment with changes in virus loads.

1.4.3.3 Effects of corticosteroids on virus-induced airway inflammation

Studies that have assessed the effects of inhaled corticosteroids on virus-induced airway inflammation have reported conflicting results. Intranasal administration of FP in a long-term house dust mite (HDM)-induced mouse model of asthma with RV1B infection led to suppressed BAL eosinophil numbers but had no effect on numbers of neutrophils, lymphocytes or macrophages in BAL.\textsuperscript{[254]} Another study assessed oral prednisolone therapy administration in combination with influenza or RSV infection, also in a mouse model of HDM-induced asthma. Prednisolone had no effect on virus-induced BAL macrophage or neutrophil recruitment but reduced eosinophils in response to RSV infection and lymphocytes in response to influenza or RSV infection.\textsuperscript{[255]} A study that assessed inhaled budesonide in a human model of experimental RV-induced asthma exacerbations showed only minimal effects on cellular airways inflammation.\textsuperscript{[67]} These studies have therefore further reinforced the belief that ICS may be ineffective at specifically reducing virus-induced airway inflammation.

1.4.3.4 Effect of corticosteroids on adaptive immunity

Very few studies have assessed the effects of ICS on components of the adaptive immune response to respiratory virus infection. Grunberg \textit{et al} evaluated the effect of inhaled budesonide treatment in
asthmatic patients experimentally infected with RV16 and showed no significant effect on CD4+ or CD8+ T cell numbers in airway epithelium.\[67\] To date, there are no studies that have assessed the effect of corticosteroids on antibody production following respiratory virus infection.

1.4.4 ICS use and pneumonia

An increased risk of pneumonia associated with use of ICS in COPD has been reported by a number of recent studies and this signal has raised considerable concern about the potential safety of these commonly used therapies. Several large randomised controlled trials have reported a consistent and statistically significant increased incidence of pneumonia associated with use of FP in COPD, either as monotherapy or in combination with LABA.\[217, 256-259\] TORCH, the largest study to date, included 4788 patients over a 3 year follow-up and reported 19.6% incidence of pneumonia associated with FP/Salmeterol vs. 12.9% with placebo (p<0.001).\[217\] A recent study which assessed a novel FP derivative compound, fluticasone furoate, in combination with the LABA vilanterol, also reported increased frequency of pneumonia.\[260\] Evaluation of alternative ICS/LABA combinations containing budesonide have shown a less consistent association with pneumonia, with one study reporting increased risk \[245\] but others showing no effect \[261-263\]. Although an earlier industry funded meta-analysis concluded that budesonide use was not associated with increased pneumonia risk \[264\], a more recent Cochrane review that included 43 studies reported an increased risk of pneumonia hospitalisation associated with use of either FP or budesonide \[265\]. The association of ICS use with pneumonia risk has also been evaluated in population based cohort studies which have reported an increased incidence of pneumonia with an estimated relative risk between 26–70%.\[222, 266-270\] Some of these studies have additionally suggested that the risk may be dose dependent with increasing doses associated with greater risk.\[266, 267, 270\]

Although the risk of pneumonia with ICS has been reported by a number of studies, some have questioned this association due to a number of potential methodological limitations and confounding factors. Very few existing studies have been designed to specifically assess pneumonia as an endpoint with most trials relying on a physician diagnosis of pneumonia rather than the gold standard of radiographic imaging.\[271\] Diagnosis of pneumonia on clinical grounds alone has been shown to have poor sensitivity and specificity \[272, 273\] and this has led to suggestions that some episodes of non-pneumonic COPD exacerbation may have been misreported as pneumonia, thus leading to an over-estimation of risk. Results of observational studies may be further confounded by
over-representation of unmeasured factors such as smoking, immunisation history, functional status and co-morbidities that could influence the risk of pneumonia in ICS-users.

Intriguingly, although several clinical trials show an increased risk of development of pneumonia with ICS use in COPD, many have not reported a corresponding increase in pneumonia-related mortality.\[217, 257\] Observational studies of patients admitted with community-acquired pneumonia (CAP) provide further weight to this theory by demonstrating that ICS use causes either reduced mortality \[269, 274, 275\] or at least has no effect on outcome in patients with COPD.\[221\] A recent study also showed that prior treatment with ICS reduces the risk of development of parapneumonic effusions in patients with CAP.\[276\] In contrast, some observational studies have shown an increase in pneumonia hospitalisations and pneumonia-related mortality associated with ICS use.\[222, 267\]

**1.4.5 Effects of ICS on anti-bacterial host defence mechanisms and bacterial infection**

As discussed above, studies of ICS use in COPD report an increased risk of development of pneumonia and a variable effect on pneumonia-related mortality. Interestingly, ICS use in asthma has not been shown to correlate with a similar increased risk of pneumonia.\[277\] The reasons for this are unclear but may be theoretically related to a number of factors including higher drug doses used in COPD, greater frequency of colonising bacteria in the lower airways and/or differences in effects of ICS on immune responses in asthma and COPD.

**1.4.5.1 Innate anti-bacterial immune responses**

A number of studies have assessed the effect of corticosteroids on various components of anti-bacterial host-defence in the lungs and some of the reported impairments may contribute to the increased risk of pneumonia observed in clinical practice. MacRedmond et al reported that FP downregulates TLR4 mRNA and protein in an airway epithelial cell line in vitro.\[163\] As discussed, severe COPD is also associated with reduced TLR4 expression and therefore ICS and COPD may interact synergistically to lead to impaired expression of TLR, reduced bacterial recognition and suppressed initiation of anti-bacterial immune responses which may increase risk of pneumonia. Conversely, TLR2 has been shown to be enhanced by dexamethasone with an 8-fold increase in mRNA expression observed in an airway epithelial cell line.\[278\] A study which assessed the effect of dexamethasone on expression of NLRs, the other major family of PPRs for *S. pneumoniae*, showed an increase in NLRP3 mRNA and protein expression in macrophage cell lines.\[279\]
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The effect of corticosteroids on phagocytosis by macrophages is unclear with some studies showing an impairment of function [280-282] and others reporting no effect [170, 283]. Phagocytosis of *E. coli* by neutrophils *ex vivo* has been reported to be unaffected by hydrocortisone [284] and studies have also shown no effect of fluticasone on production of reactive oxygen species by neutrophils [285] and dexamethasone on formation of neutrophil extracellular traps [286]. A number of *in vitro* studies in various cell types and *in vivo* studies have shown that corticosteroids can suppress induction of number of inflammatory cytokines and chemokines that are known to have roles in anti-bacterial host defence including IL-6 [283, 287-289], TNF-α [288-291], CXCL8/IL-8 [287, 288] and type I IFNs (discussed previously in section 1.4.3.1).

Variable effects of corticosteroids on AMP expression have been reported. *In vitro*, dexamethasone had no effect on spontaneous release of lactoferrin or lysozyme in nasal explants in one study [292] but a 40% reduction in lactoferrin was reported in another study [293]. Thompson *et al* showed a reduction in BAL lactoferrin and lysozyme levels in patients with COPD treated with 6 weeks of beclomethasone compared to placebo control.[231] Conversely, Schoonbrood *et al* showed no effect on sputum lactoferrin levels following 4 weeks treatment with beclomethasone.[294] *In vitro*, SLPI mRNA expression in airway epithelial cells has been shown to be induced by administration of FP [295] but these effects have not been replicated *in vivo* with no effect of FP treatment on sputum levels of SLPI shown in patients with COPD [296] and no effect of FP treatment on BAL levels of SLPI reported in healthy controls [297]. Biphasic effects of corticosteroids have been shown on expression of *surfactant protein-A in vitro* with induction at low drug concentration but inhibition with prolonged exposure to high concentrations.[298] *In vivo*, ICS treatment has been associated with increased expression of surfactant proteins-A and -B in a mouse model [299] and increased surfactant protein-D in induced sputum from patients with COPD [300]. ϒ-defensin 2 has been shown to be inhibited by corticosteroids *in vitro* [301, 302] and *in vivo* [302] while cathelicidin-related antimicrobial peptide (CRAMP, the mouse homolog of human LL37/cathelicidin) has been shown to be suppressed by budesonide administration in mice [303].

The effects of corticosteroids on mucociliary clearance have not been extensively studied. High dose prednisolone administration has been shown to impair mucociliary clearance in rats [304] but nebulised budesonide had no effect on mucociliary clearance in human asthmatics [305]. Varying effects of corticosteroids on expression of mucins have been reported with some *in vitro* studies reporting suppression of baseline MUC5AC expression [306] and suppression of MUC5AC induced by *P. aeruginosa or H. influenzae* in airway epithelial cell lines [307, 308]. Conversely *in vivo* studies have shown no effect of intranasal corticosteroids on MUC5AC expression in nasal epithelium [309] and
no effect of ICS on MUC5AC and MUC5B expression in bronchial biopsies from asthmatic patients [310] while dexamethasone has been shown to increase IL-13 induced MUC5AC in vitro in human bronchial epithelial cell lines [311].

Some of the possible impairments in anti-bacterial host defence in COPD (as discussed in section 1.3.2) and in relation to ICS use are summarised in figure 1.1.

![Figure 1.1: Potential mechanisms of impaired anti-bacterial host defence associated with ICS use and COPD.](image)

Abbreviations: COPD chronic obstructive pulmonary disease; IL interleukin; TLR toll-like receptor; TNF tumour necrosis factor. Blue dashed arrow represents anti-bacterial impairment related to COPD. Red dashed arrow represents anti-bacterial impairment related to inhaled corticosteroid use.

1.4.5.2 Bacterial clearance

As discussed in the previous section, ICS may impair or enhance a number of anti-bacterial host defence factors in the lungs. Only a few studies have assessed the in vivo effects of ICS administration on bacterial clearance in mouse models and they report conflicting findings. Chu et al
showed that nebulised administration of FP during *M. pneumoniae* infection in mice reduced bacterial loads in lung tissue. [312] Barbier *et al* similarly showed that intranasal FP administration led to ~50% reduction in bacterial loads in mice infected with *S. pneumoniae* and additionally showed that FP administration reduced *in vitro* invasion of airway epithelial cells by *S. pneumoniae*. [313] Conversely, Patterson *et al* showed increased bacterial burden in lung tissue and blood associated with nebulised FP administration in a mouse model of *Klebsiella pneumoniae* infection [283] and Wang *et al* showed a similar effect with nebulised budesonide following *P. aeruginosa* infection in an ovalbumin-induced mouse model of asthma [303]. These studies suggest that ICS may either accelerate or impair bacterial clearance from the lungs but variation in a number of factors including dose and type of ICS administered, bacterial pathogen and strain of mouse used may all influence the observed effects. The relevance of these animal studies to ICS use in patients with COPD is unclear and there are no existing human models of experimental bacterial respiratory infection in COPD to translate these findings into.

### 1.4.5.3 Bacterial colonisation and the lower respiratory microbiome

In addition to studies that have assessed the effects of ICS in models of bacterial pneumonia, other studies have focussed on whether ICS have any effects on colonising bacteria in the lungs during clinical stability. A study that used culture based techniques showed that ICS use in children with asthma increased oropharyngeal colonisation by *S. pneumoniae*. [314] Other studies which have employed molecular techniques have shown that ICS use in COPD is associated with increased sputum bacterial loads measured by quantitative PCR in stable state. [315] Pragman *et al* showed segregation of bacterial communities associated with use of ICS by performing 16S pyrosequencing in BAL samples from patients with COPD. [120] Similarly, Huang *et al* observed a trend towards greater richness and diversity of the microbiota in samples from ICS-exposed vs. non-exposed patients with COPD. [316] These studies all provide intriguing evidence that ICS use may potentially alter the respiratory microbiota in COPD, leading to proliferation of existing PPMs and subsequent increased risk of bacterial pneumonia.

### 1.4.5.4 Virus-induced secondary bacterial infection

As discussed in section 1.3.3, there is evidence that a primary respiratory virus infection may directly precipitate secondary bacterial infection. Whether ICS use can influence virus-induced secondary bacterial infection and interact to favour development of pneumonia is unclear. In the Investigating New Standards for Prophylaxis in Reduction of Exacerbations (INSPIRE) study, pneumonia was shown to occur more frequently following an unresolved exacerbation in COPD patients taking ICS but the
aetiology of exacerbations could not be determined.[317] This finding raises speculation that ICS use in COPD may potentiate pneumonias that follow viral exacerbations. Jamieson et al showed that dexamethasone treatment led to increased bacterial loads in the liver during sequential infection with intranasal influenza and intravenous *Listeria monocytogenes* in mice.[318]

1.5 Animal models of COPD exacerbation

As discussed above, COPD is a complex, heterogeneous disorder and human studies have previously focused on characterising the underlying disease pathogenesis. There has also been interest in development of clinically relevant animal models of COPD to enable controlled investigation of pathogenic pathways involved in stable disease and during acute exacerbations. However, there remains an incomplete understanding of the different disease phenotypes in humans and therefore, developing a clinically relevant model in animals has been somewhat challenging.

1.5.1 Models of stable COPD

Most existing animal models of COPD have been reported in mice and broadly employ three main strategies: inhalation of noxious stimuli (most commonly cigarette smoke), instillation of tissue-degrading proteinases such as elastase or genetic manipulation.[319]

Models using genetic manipulation include gene knockout mouse strains such as *tissue inhibitor of metalloproteinases-3 (TIMP-3)* -/- [320] or *surfactant protein-D* -/- [321] or transgenic strains over-expressing mediators such as IL-13 and IFN-γ.[322, 323] Although these models have provided some insight into COPD pathogenesis and susceptibility, it is recognized that the disease in humans is caused by a combination of genetic susceptibility and environmental exposures and therefore, the relevance of these monogenetic approaches to clinical disease may be questionable.

Cigarette smoke based models have the advantage of using the primary disease-causing agent in humans. Smoke can be administered by whole-body exposure or nose-only dosing protocols.[324] Regardless of the method of exposure, studies have consistently shown that cigarette smoke induces a number of pathophysiological changes that are indicative of COPD including enhanced cellular airways inflammation, increased BAL inflammatory mediator production, increased oxidative stress and mucus hypersecretion.[325-330] Although these features may be representative of those seen in healthy human smokers or early stage COPD, the overt emphysematous tissue destruction seen in advanced disease cannot be recreated with use of cigarette smoke exposure in mice, even if
administration occurs for prolonged periods.[331] It has been estimated that cigarette smoke exposure induces pathology associated with mild COPD (GOLD stage 1).[331] Given that most cases of COPD in humans are not diagnosed until the disease has become more severe (usually GOLD stage 2 or above)[332, 333], cigarette exposure models may be considered irrelevant to the spectrum of patients commonly encountered in clinical practice. In particular, acute exacerbations of disease become more frequent as the disease progresses [25] and, therefore, other methods of modelling COPD may be more appropriate when studying pathophysiological mechanisms involved in exacerbations.

Instillation of elastase into the airways produces a rapid onset of emphysematous tissue destruction in the lungs and may be considered to be the best and most rapid method for modelling severe disease and therefore, the optimum method for producing a phenotype relevant to patients encountered in clinical practice.[319, 331] Additionally, elastase administration in mice also produces other disease-relevant features including increased airway inflammation, mucus hypersecretion and impaired lung function.[334-337] However, elastase does not model all the complicated events associated with exposure to cigarette smoke, which contains over 4000 chemicals and >10^{15} free radicals per inhalation [331] and therefore administration of a single substance is an over-simplified method of modeling COPD. Some studies have also reported models of chronic lipopolysaccharide (LPS) administration leading to features of airway inflammation, remodelling, emphysema and altered lung function in mice [338, 339] but these models are generally believed to be even less representative of human disease as they fail to accurately recreate a number of key features.

1.5.2 Models of viral exacerbation

Several studies have combined models of stable COPD with viral challenge to directly mimic acute exacerbation. Most studies have used cigarette smoke exposure prior to infection with either influenza or respiratory syncytial virus (RSV). Studies vary in terms of duration of smoke exposure and also with regards to the specific virus used. However, most studies report that cigarette smoke exposure leads to enhanced virus-induced airway inflammation.[253, 340, 341] Conversely, Han et al reported that 21 days of cigarette smoke exposure led to suppressed cytokine production and airway inflammation in response to challenge with pandemic H1N1 influenza [342] and Robbins et al reported suppressed inflammation with low dose influenza but enhanced inflammation when higher doses of virus were combined with cigarette smoke exposure [343]. Studies that have assessed the effect of cigarette smoke on virus loads have reported varying effects with one study showing
increased virus titres [341], another showing reduced titres [343] and two reporting no effect [253, 342]. Other endpoints that are relevant to COPD exacerbation such as mucus hyper-secretion and lung function impairments have not generally been reported in the existing cigarette smoke exacerbation models.

Despite being the most frequently identified viral cause for COPD exacerbation, no existing studies have assessed the effect of cigarette smoke exposure on RV infection in mice. The only published mouse model of RV-induced exacerbation in COPD reported that weekly intranasal administration of porcine pancreatic elastase combined with LPS for four weeks, followed by RV1B infection, led to increased lung tissue mRNA expression of TNF-α, IL-13 and MUC5AC, increased airway hyper-responsiveness (AHR) to methacholine (MCH) challenge, deficient type I IFN responses and delayed virus clearance from the airways.[344] These features are similar to those reported in human studies of naturally occurring exacerbations and those induced by the experimental RV infection human COPD model, as discussed previously.

1.5.3 Models of bacterial infection

1.5.3.1 Bacterial exacerbation

A number of studies have also combined cigarette smoke exposure or elastase (+/- LPS) administration with bacterial challenge to model exacerbation. Cigarette smoke models have reported enhanced inflammation and increased bacterial clearance in response to challenge with H. influenzae [345] or P. aeruginosa.[346] Models of elastase administration have reported suppressed inflammation in response to S. pneumoniae [347] with other studies showing enhanced inflammation to H. influenzae [348] or S. pneumoniae [349]. Ganesan et al reported that H. influenzae infection following combined four dose elastase/LPS administered led to delayed bacterial clearance, increased airway inflammation, enhanced expression of MUC5AC and exaggerated AHR.[350]

1.5.3.2 Colonisation

An alternative approach to modelling COPD adopted by some studies has been to try to recreate the lower respiratory bacterial colonisation component of the disease. A major problem in establishing models of chronic lung infection is that common human PPMs are rapidly cleared by the rodent immune system, typically within 24-48h of administration when low infecting doses are instilled.[351] Haste et al reported a model of low dose S. pneumoniae infection in CBA/Ca mice where recoverable numbers of pneumococci were present at 7d post-infection and showed that the
dose and strain of bacteria and also the strain of mouse used can impact upon whether chronic infection can be successfully established.[352] Other studies have attempted to encase bacteria in agar beads to restrict *in vivo* phagocytosis and have shown longer term persistence of bacteria in the airways.[353, 354]

**1.5.3.3 Lower respiratory microbiome in mice**

Studies have also started to characterise the lower respiratory microbiota in samples including BAL, lung tissue and nasal lavage taken from specific pathogen free mice and have shown the presence of similar phyla to those identified in humans including *Proteobacteria, Firmicutes* and *Bacteroidetes*. [355, 356] Krone *et al* used 16S pyrosequencing to show that the microbiota in nasal lavage differed between young and elderly mice and also showed that challenge with *S. pneumoniae* led to a decrease in microbial community diversity.[356] Goulding *et al* used clone library sequencing of BAL samples from mice and showed that influenza infection led to dysbiosis of the airway microbiota.[199]
1.6 Aim, Hypothesis and Thesis Outline

1.6.1 Aim and Hypothesis

The overall aim of this project was to investigate the effects of ICS therapy on rhinovirus-induced secondary bacterial infection in COPD. The central hypothesis was that ICS suppress anti-viral and anti-bacterial host defence responses and thus predispose to secondary bacterial infection following RV-induced exacerbation of COPD. Focussed aims and hypotheses are presented at the start of each of the results chapters.

1.6.2 Thesis Outline

In the Introduction section of this chapter, the background to the topics covered within this thesis is outlined including COPD, the role of RVs and bacteria in precipitating COPD exacerbations and secondary bacterial pneumonias and the effects of ICS on anti-viral and anti-bacterial host-defence. Chapter 2 details the materials and methods used. This is followed by the results chapters: Chapter 3 describes experiments to define a representative mouse model of RV-induced COPD exacerbation; Chapter 4 investigates the effects of inhaled corticosteroid administration on RV infection in mice; Chapter 5 investigates the effects of inhaled corticosteroid administration on respiratory bacterial infection in mice. Finally, in Chapter 6, the results presented in the thesis are discussed and plans for future work are outlined.
Chapter 2: Materials and methods

2.1 Materials

Details of commonly used buffers and cell/virus culture media are shown in table 2.1. Further miscellaneous reagents are also given in table 2.2.

<table>
<thead>
<tr>
<th>Medium / Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium-chloride- Potassium (ACK) lysis buffer</td>
<td>0.155M Ammonium chloride (Sigma-Aldrich, Shaftesbury, UK), 10mM potassium bicarbonate (Sigma-Aldrich) and 0.1mM disodium ethylenediaminetetraacetic acid (EDTA; Invitrogen, Paisley, UK) in dH2O. Filter-sterilised through a 0.22μm filter.</td>
</tr>
<tr>
<td>BAL fluid</td>
<td>12mM lidocaine hydrochloride monohydrate (Sigma-Aldrich) and 5.5 mM EDTA (Invitrogen) in phosphate buffered saline (PBS; PAA Laboratories, Yeovil, UK).</td>
</tr>
<tr>
<td>10%/2% Dulbecco’s Modified Eagles Medium (DMEM) medium</td>
<td>10%/2% (v/v) foetal calf serum (FCS, PAA) in DMEM (PAA) with 20µM HEPES buffer (Invitrogen) and 1% sodium bicarbonate (v/v) (Gibco, Paisley, UK).</td>
</tr>
<tr>
<td>ELISA wash buffer</td>
<td>0.05% (v/v) Polyethylene glycol sorbitan monolaurate (‘Tween20”; Sigma-Aldrich) in PBS.</td>
</tr>
<tr>
<td>ELISA reagent diluent</td>
<td>1% bovine serum albumin (w/v) (BSA; Sigma-Aldrich) in PBS.</td>
</tr>
<tr>
<td>Fluorescence activated cell sorting (FACS) buffer</td>
<td>1% (w/v) BSA (Sigma-Aldrich) and 0.01% (w/v) sodium azide in PBS.</td>
</tr>
<tr>
<td>Lung/BAL cell culture medium</td>
<td>100µg/ml streptomycin (Sigma-Aldrich), 100 units/ml penicillin (Sigma-Aldrich), 5% FCS (v/v) (PAA), 2mM L-glutamine, 20 µM HEPES buffer (Invitrogen) in Roswell Park Memorial Institute 1640 medium (RPMI; PAA).</td>
</tr>
<tr>
<td>Lung digestion buffer</td>
<td>Lung/BAL cell culture medium containing 1mg/ml collagenase type XI (Sigma-Aldrich) and 80 units/ml bovine pancreatic DNAse type IV (Sigma-Aldrich).</td>
</tr>
<tr>
<td>4% Paraformaldehyde (PFA)</td>
<td>4% solution (w/v) of paraformaldehyde (Sigma-Aldrich) in water. Stirred at 55°C for 30 minutes. pH 7.2-7.4. Filter sterilised through a 0.22μm filter.</td>
</tr>
<tr>
<td>10%/2% RPMI medium</td>
<td>10%/2% (v/v) heat inactivated FCS (PAA) in RPMI (PAA) supplemented with 20µM HEPES buffer (Invitrogen); 0.075% (v/v) sodium bicarbonate (Invitrogen), 100 units/ml penicillin (Invitrogen) and 100μg/ml streptomycin (Invitrogen)</td>
</tr>
<tr>
<td>Tris -EDTA (TE) buffer</td>
<td>1M Tris Base (Sigma-Aldrich) with 0.5M EDTA (Invitrogen) in dH2O 496ml.</td>
</tr>
</tbody>
</table>

Table 2.1 Buffers and Media
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Details</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA ladder</td>
<td>1kB plus DNA ladder</td>
<td>Invitrogen</td>
</tr>
<tr>
<td></td>
<td>10bp-12kB DNA standard</td>
<td></td>
</tr>
<tr>
<td>Elastase</td>
<td>Porcine pancreatic elastase. High purity, crystallised</td>
<td>Merck, Nottingham, UK</td>
</tr>
<tr>
<td>Fluticasone propionate</td>
<td>5mg powder</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>‘Isoflurane-Vet’ 100% (w/w)</td>
<td>Merial, Harlow, UK</td>
</tr>
<tr>
<td>LPS</td>
<td>LPS from <em>Escherichia coli</em> 026:B6. Lyophilised powder, purified by phenol extraction</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Pentobarbitone</td>
<td>Pentoject Pentobarbitone sodium 20% (w/v)</td>
<td>AnimalCare Ltd, York, UK</td>
</tr>
<tr>
<td>Polyinosinic-polycytidylic acid (poly(I:C))</td>
<td>Long synthetic analog of dsRNA</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Poly(I:C) (HMW)/LyoVec</td>
<td>MDAS agonist (transfected poly(I:C))</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>RNALater</td>
<td>RNA stabilisation buffer</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>Recombinant IFN-β</td>
<td>Mouse IFN-β, carrier free</td>
<td>R &amp; D Systems, Abingdon, UK</td>
</tr>
<tr>
<td>Streptavidin–Horse radish peroxidase (HRP)</td>
<td>ELISA grade Streptavidin-HRP conjugate (streptavidin from <em>Streptomyces avidinii</em>)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Tetra methyl benzidine (TMB) substrate</td>
<td>3,3’,3,5’ TMB single solution chromagen</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Table 2.2 Miscellaneous reagents
2.2 Methods

2.2.1 Virological techniques

2.2.1.1 RV1B propagation

The minor group (LDL-R binding) human *Enterovirus* rhinovirus A, serotype 1B was obtained from the American Type Tissue Culture Collection (ATCC, Teddington, UK) and passaged 7 times in Ohio Hela cells (European Collection of Cell Cultures, Health Protection Agency, Porton Down, UK) to create laboratory working stocks. Working stocks were periodically neutralised with HRV-specific antisera (ATCC) to confirm serotype.

25 x 175 cm² flasks of Ohio HeLa cells were grown to approximately 90% confluence in 10% DMEM medium (v/v) at 37°C with 5% CO₂. Cells were washed twice with PBS and infected with 10ml of 2 x 10⁷ 50% tissue culture infectious dose (TCID₅₀) working stock RV1B in 2% DMEM medium (v/v). Flasks were then incubated at room temperature for 1h with gentle shaking before addition of a further 10ml 2% DMEM medium (v/v) with subsequent incubation at 37°C with 5% CO₂ for a further 24h or until close to 100%. Cytopathic effect (CPE) was observed. The cells and medium were then harvested, concentrated to 36ml in PBS by repeated centrifugation at 2,700 x g at 4°C and stored at -80°C.

2.2.1.2 Virus purification

The cell pellet/PBS mixture was freeze-thawed twice to lyse cells and then centrifuged at 2,700 x g for 15 minutes at 4°C to remove cellular debris. Virus was precipitated from the supernatant by adding 2.8g Polyethylene Glycol-6000 (Sigma-Aldrich) and 4ml of 5M (final concentration) NaCl, followed by incubation on ice for 1h. The virus was then pelleted by centrifugation at 2,700 x g for 15 minutes and was then re-dissolved in 15ml of PBS and further centrifuged to remove insoluble matter. The supernatant was filtered through a 0.2µm syringe filter and then concentrated to ~2.5ml by repeated centrifugation at 2,500 x g, at 4°C using a 100,000MW 15ml Amicon centrifugal filtration device (Milipore, Billerica, USA). Purified RV1B stocks were stored at -80°C.

2.2.1.3 Virus titration

As separate virus batches were used for each study, these were assessed for infectivity by *in vitro* culture before use *in vivo*. Ohio Hela cells were seeded in 96 well plates at 1.5 x 10⁴ cells per well in 150µl 10% DMEM medium (v/v). RV1B stocks were serially diluted to give concentrations from 10⁻¹ to 10⁻⁸ and 50µl of each dilution was added to 8 replicate wells of HeLa cells. Plates were incubated...
Chapter 2: Materials & Methods

at 37°C in 5% CO2 for 96h and then assessed for CPE by light microscopy. The Spearman-Karber formula was used to calculate the TCID₅₀.[357]

2.2.1.4 Virus inactivation
Purified virus stocks were inactivated for use as a control inoculum by irradiation with 1200µJ/cm² ultraviolet (UV) light using a UV crosslinker (CX-2000; UVP, Cambridge, UK).

2.2.2 Animal models and methods

2.2.2.1 Mice
8-10 week old female C57BL/6 mice were purchased from Charles River (Margate, UK) and housed in individually ventilated cages in specific pathogen-free conditions within the CBS facility at St. Mary’s campus, Imperial College London. All work was completed in accordance with UK Home office guidelines (UK project licence PPL 70/7234). During all experiments, animal welfare was monitored at least twice daily and any mice that displayed signs of significantly impaired health status were euthanised.

2.2.2.2 Anaesthesia
For all intranasal challenges, mice were lightly anaesthetised in an exposure box with inhaled isofluorane and oxygen at 2 litres per minute (Merial). Terminal anaesthesia was achieved by intraperitoneal injection of 0.2ml 20% (w/v) pentobarbitone solution (Pentoject).

2.2.2.3 RV1B infection
Mice were challenged intranasally with 50µl inoculum containing 2x10⁶ TCID₅₀ RV1B or UV-inactivated RV1B (UV-RV1B), or mock infected with PBS. Mice were culled by terminal anaesthesia performed at various time-points post challenge.

2.2.2.4 RV-induced exacerbation of COPD model
Mice were challenged intranasally with 1.2 units of porcine pancreatic elastase (Merck) on d1 and with 70 endotoxin units of LPS from Escherichia coli 026:B6 (Sigma-Aldrich) on d4 of the week for up to 4 consecutive weeks. In an alternate model, mice were treated with a single dose of 1.2 units of elastase alone. Mice treated with intranasal PBS instead of elastase or LPS were used as controls. For exacerbation, mice were infected intranasally with 50µl inoculum containing 2 x 10⁶ TCID₅₀ RV1B or UV-inactivated RV control, either 7d after final LPS challenge in the case of combined elastase and LPS models or 10d after elastase challenge in the single dose elastase model. The four dose elastase/LPS RV-induced exacerbation of COPD mouse model is outlined schematically in figure 2.1 and the nomenclature used for each treatment group is explained in table 2.3.
Figure 2.1 Four dose combined elastase/LPS RV-induced exacerbation of COPD model

Table 2.3 Four dose combined elastase/LPS COPD RV exacerbation model, treatment group nomenclature
The single dose elastase RV-induced exacerbation of COPD model is outlined in figure 2.2 and the nomenclature used for each treatment group is explained in table 2.4.

![Figure 2.2 Single dose elastase RV-induced exacerbation of COPD model](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Elastase</th>
<th>RV1B Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase + RV</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Elastase + UV</td>
<td>Yes</td>
<td>No (UV-RV1B)</td>
</tr>
<tr>
<td>PBS + RV</td>
<td>No (PBS)</td>
<td>Yes</td>
</tr>
<tr>
<td>PBS + UV</td>
<td>No (PBS)</td>
<td>No (UV-RV1B)</td>
</tr>
</tbody>
</table>

Table 2.4 Single dose elastase RV-induced exacerbation of COPD model, treatment group nomenclature

2.2.2.5 FP and RV1B infection model

FP powder (Sigma-Aldrich) was resuspended at a concentration of 357µg/ml in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and then diluted 1:1000 in PBS. Mice were treated intranasally with 50µl of
this solution (equating to approximately 1mg/kg FP dose) or vehicle DMSO diluted 1:1000 in PBS as control. One hour after FP or vehicle administration, mice were infected intranasally with 50µl RV1B (5 x 10^6 TCID<sub>50</sub>) or UV-inactivated RV1B control. The FP and RV1B model is outlined in figure 2.3 and the nomenclature used for each treatment group is explained in table 2.5.

![Figure 2.3 FP and RV1B infection model](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Fluticasone propionate</th>
<th>RV1B Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP + RV</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FP + UV</td>
<td>Yes</td>
<td>No (UV-RV1B)</td>
</tr>
<tr>
<td>Vehicle + RV</td>
<td>No (DMSO)</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle + UV</td>
<td>No (DMSO)</td>
<td>No (UV-RV1B)</td>
</tr>
</tbody>
</table>

Table 2.5 FP and RV1B infection model, treatment group nomenclature
2.2.2.6 Recombinant IFN-β administration; FP and RV1B infection model

Mice were intranasally dosed with 1mg/kg FP or vehicle DMSO, 1h prior to infection with 50µl RV1B (5 x 10^6 TCID_{50}) or UV-inactivated RV1B control, as described for the FP and RV1B infection model (section 2.2.2.5). One hour following RV1B or UV-RV1B challenge, mice were then additionally treated intranasally with 50µl of PBS containing 10^4 units of recombinant IFN-β (R & D systems). This model is outlined in figure 2.4 and the nomenclature used for each treatment group is explained in table 2.6.

Figure 2.4 Recombinant IFN-β administration in FP and RV1B infection model


<table>
<thead>
<tr>
<th>Group</th>
<th>Fluticasone propionate</th>
<th>RV1B Infection</th>
<th>Recombinant IFN-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP + RV</td>
<td>Yes</td>
<td>Yes</td>
<td>No (PBS)</td>
</tr>
<tr>
<td>FP + UV</td>
<td>Yes</td>
<td>No (UV-RV1B)</td>
<td>No (PBS)</td>
</tr>
<tr>
<td>vehicle + RV</td>
<td>No (DMSO)</td>
<td>Yes</td>
<td>No (PBS)</td>
</tr>
<tr>
<td>vehicle + UV</td>
<td>No (DMSO)</td>
<td>No (UV-RV1B)</td>
<td>No (PBS)</td>
</tr>
<tr>
<td>FP + RV + IFN-β</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FP + UV + IFN-β</td>
<td>Yes</td>
<td>No (UV-RV1B)</td>
<td>Yes</td>
</tr>
<tr>
<td>vehicle + RV + IFN-β</td>
<td>No (DMSO)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>vehicle + UV + IFN-β</td>
<td>No (DMSO)</td>
<td>No (UV-RV1B)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2.6 Recombinant IFN-β in FP and RV1B infection model, treatment group nomenclature

2.2.2.7 FP and *Streptococcus pneumoniae* infection model

Mice were dosed intranasally with FP (1mg/kg) or vehicle DMSO, as described for the FP and RV1B infection model (section 2.2.2.5), 1h prior to infection with 50µl of *S.pneumoniae* (2.5 x 10^5 colony forming units (CFU)) capsular serotype 2 strain (D39). This strain had been cultured previously at 37°C in Todd-Hewitt broth (Oxoid, Basingstoke, UK) supplemented with 0.5% yeast extract (w/v) (Sigma-Aldrich) and aliquots of known concentration had been stored at -80°C in 10% glycerol (v/v) (Sigma-Aldrich). This model is outlined in figure 2.5 and the nomenclature used for each treatment group is explained in table 2.7.
Figure 2.5 Fluticasone propionate and *S. pneumoniae* model

<table>
<thead>
<tr>
<th>Group</th>
<th>Fluticasone propionate</th>
<th><em>S. pneumoniae</em> Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FP + S. pneumoniae</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Vehicle + S. pneumoniae</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>(DMSO)</td>
<td></td>
</tr>
<tr>
<td><strong>Vehicle + PBS</strong></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>(DMSO)</td>
<td>(PBS)</td>
</tr>
</tbody>
</table>

Table 2.7 FP and *S. pneumoniae* infection model, treatment group nomenclature

### 2.2.2.8 Airway hyper-responsiveness assessment

AHR was assessed using an unrestrained whole body plethysmography system (Electromedical Measurement Systems (EMMS; Alton, UK). Mice were challenged for 1 minute with increasing doses of acetyl-β-methyl-choline chloride (methacholine) (Sigma-Aldrich) in H₂O up to a maximum of 100mg/ml and enhanced pause (Penh) was assessed over the following 5 minute period. Data were
acquired and analysed using eDaq v1.8 software (EMMS) and are presented as Penh average for 5 minute log period post-challenge. The equation for calculation of Penh is as follows:

\[
\text{Penh} = \left( \frac{\text{PEF}}{\text{PIF}} \right) \times \left( \frac{\text{Te}}{\text{Rt}} - 1 \right)
\]

PEF = Peak Expiratory flow (ml/s)
PIF = Peak Inspiratory flow (ml/s)
Te = Expiratory Time (Total)
Rt = Relaxation Time (time take for expiration of 65% total expiratory volume)

2.2.3 Sample recovery and processing

2.2.3.1 Bronchoalveolar lavage

Mice were cannulated via the trachea and lavaged with 1.5ml of BAL fluid. For 16S sequencing work, a modified protocol was adopted with use of autoclaved polytetrafluoroethylene (PTFE) tubing (VWR chemicals, Lutterworth, UK) and autoclaved instruments. Cells and supernatants from recovered fluid were separated by centrifugation at 12,000 x g for 1 minute. Cells were treated with 1ml ACK buffer for 30 seconds to lyse red blood cells and then resuspended in 1ml of 10% RPMI medium (v/v). BAL supernatants were stored at -80°C until use. Total live BAL cell counts were assessed by trypan blue (Sigma-Aldrich) exclusion.

2.2.3.2 Nasal lavage

Nasal lavage was performed by cannulation via the trachea upward to the nasopharynx. The nasal cavity was gently perfused back to front with 1.0 ml of PBS via the catheter, and the lavage fluid was collected at the nares. Autoclaved tubing and instruments were used, as described for BAL.

2.2.3.3 Differential cell counts

100µl of cells from the 1ml total BAL suspension (from section 2.2.3.1) was spun down (110 x g, 5 minutes) onto Shandon Cytoslides using a cytocentrifuge (Shandon Cytospin 3; Thermo Scientific, Basingstoke, UK). Slides were air-dried and stained with Quick Diff kit (Reagena, Toivala, Finland) and, using light microscopy, approximately 300 cells per slide were differentially counted blind to experimental conditions.
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2.2.3.4 Isolation of lung cells for flow cytometry
The left lung was excised post BAL and crudely dissociated in gentleMACS C-tubes (Miltenyi Biotech, Bisley, UK) containing 5ml of lung digestion buffer using the gentleMACS Dissociator system (Miltenyi Biotech) followed by incubation for 45 minutes at 37°C (5% CO₂). A second step of mechanical dissociation was then carried out to generate a single cell suspension and cells were pelleted by centrifugation (445 x g for 10 minutes). Erythrocytes were lysed in 5ml ACK buffer for 5 minutes and then neutralised using 10ml PBS. Finally, cells were filtered through a 100μm cell strainer, washed in PBS and resuspended in 10% RPMI medium (v/v). Total live lung cell counts were determined by light microscopy using trypan blue exclusion.

2.2.3.5 Lung tissue processing for RNA extraction
The right apical lung lobe was excised post BAL, rinsed in sterile PBS and stored at -80°C in RNAlater (Qiagen).

2.2.3.6 Lung tissue processing for measurement of bacterial loads
For quantification of bacterial loads in the S.pneumoniae mouse model, the left lung was excised post BAL using autoclaved instruments, rinsed in sterile PBS and placed into 1ml sterile PBS. Samples were processed immediately, as described in section 2.2.9.1. For 16S quantitative PCR, the right upper lobe, the left lung or both lungs were excised post BAL, immediately placed into an autoclaved 1.7ml tube and snap frozen in liquid nitrogen followed by storage at -80°C.

2.2.3.7 Nuclear protein extraction
The left lung was excised post BAL, immediately placed into a 1.7ml tube and snap frozen in liquid nitrogen followed by storage at -80°C. Lung tissue was manually homogenised whilst immersed in liquid nitrogen using a mortar and pestle. Nuclear Protein was then extracted using a nuclear extraction kit (Active Motif, La Hulpe, Belgium) according to manufacturer’s instructions and samples stored at -80°C.

2.2.3.8 Histological evaluation of lungs.
For histological evaluation, following BAL, the lungs were perfused with PBS via the heart and inflated with 4% paraformaldehyde (w/v) (PFA) and then immersion fixed in 4% PFA for 24h. Further processing was carried out by the St Mary’s Hospital Cellular Pathology Service (Imperial College NHS Trust, London). Lungs were embedded in 4% paraffin and 5μm thick sagittal sections were stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) reagent. Alveolar chord length was determined by measuring the diameter of air spaces in 10 random fields per slide using Zeiss Axiovision software (version 4.8.3.0; Carl Zeiss, Oberkochen, Germany). PAS stained lung sections

70
were scored using a modified system, as previously described [358] and 10-20 airways were counted per section. All counting was performed blind to experimental conditions.

2.2.3.9 Blood sampling
For measurement of RV-specific antibodies in the RV1B mouse model, peripheral blood was collected from the carotid arteries into microtainer serum separation tubes (BD Biosciences, Oxford, UK) and whole blood was allowed to coagulate for 1h at room temperature. Serum was then separated by centrifugation at 13,000 x g for 2 minutes and stored at -80°C.

For measurement of bacterial loads in blood in the *S.pneumoniae* model, samples were obtained by cardiac puncture and immediately transferred into lithium heparin microtainer blood collection tubes (BD Biosciences). Samples were processed immediately, as described in section 2.2.9.2.

2.2.4 Flow cytometry

2.2.4.1 Surface staining of BAL and lung leukocytes.
1 x 10^6 lung cells or 1 x 10^5 BAL cells were plated into a 96 v-well plate and incubated in 50µl of 5µg /ml of Fc Block in FACS buffer (anti-mouse CD16/CD32, BD Biosciences) for 15 minutes at room temperature. Fluorochrome-conjugated antibodies specific for cell surface markers diluted in 50µl FACS buffer per well were directly added to cells with further incubation for 30 minutes at 4°C in the dark. The antibodies used and their working concentrations are displayed in table 2.8. Cells were then washed in PBS and stained with live/dead cell marker (Invitrogen) at 1:1000 dilution in PBS for 20 minutes at 4°C in the dark. Cells were then washed with PBS and fixed by addition of 100µl per well of 2% formaldehyde (v/v) (Sigma-Aldrich) in PBS for 20 minutes at room temperature in the dark. Cells were again washed with PBS, resuspended in FACS buffer and stored at 4°C in the dark, prior to analysis within 24h.
Table 2.8 Antibodies used for flow cytometry and working concentrations

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Conjugate</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster anti-mouse CDε3</td>
<td>500A2</td>
<td>Pacific Blue</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse CD4</td>
<td>RM4-5</td>
<td>APC</td>
<td>0.25µg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse CD8a</td>
<td>53-6.7</td>
<td>Alexa Fluor 488</td>
<td>0.5µg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse C19</td>
<td>1D3</td>
<td>PerCP-Cy5.5</td>
<td>0.25µg/ml</td>
</tr>
<tr>
<td>Hamster anti-mouse CD69</td>
<td>H1.2F3</td>
<td>FITC</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Mouse anti-mouse NK1.1</td>
<td>PK136</td>
<td>PE</td>
<td>1µg/ml</td>
</tr>
</tbody>
</table>

2.2.4.2 Flow cytometry data acquisition and analysis

Stained cells were acquired on an LSR II flow cytometer (BD Biosciences) and analysed using FlowJo software (version 10.0.6; Tree Star, Ashland, USA). Representative gating strategies used for analysis of surface staining of lung and BAL cells are shown in figure 2.6.
Figure 2.6 Representative flow cytometry gating; Lung and BAL lymphocytes in RV infection model

Abbreviations: FSC-A Forward scatter area; SSC-A Side scatter area; SSC-H Side scatter height
2.2.5 Enzyme-linked immunosorbent assay (ELISA)

2.2.5.1 BAL soluble mediators

Protein levels of chemokines and cytokines were assessed in BAL fluid, diluted as required. All ELISAs were carried out using reagents from Duoset kits (R&D Systems), according to manufacturers’ instructions. All steps were performed at room temperature and plates were washed 3 times with ELISA wash buffer between steps. Briefly, the primary antibody was incubated in a 96-well plate (Nunc MaxiSorp, ThermoScientific) overnight at room temperature and blocked with 1% BSA in PBS (w/v) (reagent diluent) for 2h. One hundred µl of sample or standard (recombinant protein serially diluted 1:2 in reagent diluent) was added and incubated for 2h. Biotin conjugated detection antibody in reagent diluent was then added with further incubation for 2h. HRP conjugated to streptavidin (Invitrogen) was added at 0.2µg/ml in reagent diluent and incubated for 15 minutes. Seventy-five µl of TMB substrate (Invitrogen) was then added and development of colour monitored before addition of equal volume of 1M H₂SO₄ to stop the reaction.

IFN-α and -β protein levels in BAL were assessed using specific kits (R&D Systems/PBL Interferon Source, Piscataway, USA) with plates pre-coated with capture antibody. For the IFN-β ELISA, sample/standard, secondary antibody and HRP were simultaneously incubated for 1h. For the IFN-α ELISA, sample/standard was simultaneously incubated with detection antibody for 1h at room temperature with shaking at 450rpm, followed by overnight incubation at 4°C. Subsequently, plates were washed and incubated with HRP conjugate for 2h, prior to reaction development.

2.2.5.2 Mucin proteins

MUC5AC and MUC5B proteins in BAL were measured after adhesion to a 96 well plate by allowing samples to evaporate at 37°C overnight. Plates were washed 3 times with ELISA wash buffer between steps. The following day, plates were blocked for 2h at room temperature with 2% BSA/PBS (w/v). For measurement of MUC5AC, the detection antibody used was biotinylated anti-MUC5AC (ThermoScientific) at 400 ng/ml in 0.2% BSA/PBS (w/v) and subsequent steps were as described in section 2.2.5.1. For the MUC5B assay, detection antibody was mouse anti-MUC5B clone EH-MUC5Ba, as previously described.[359] Bound anti-MUC5B antibody was detected with peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) diluted 1:1000 in 0.2% BSA/PBS. Standard curves for mucin ELISAs were generated by serial 1:2 dilutions of BAL supernatants previously taken from ovalbumin-induced hyper-allergic mice.
2.2.5.3 RV-specific IgG
96 well plates were coated with 50µl per well of concentrated RV1B or HeLa cell lysate, diluted 1:100 in PBS and incubated at 4°C overnight. Plates were washed 3 times with ELISA wash buffer between all steps. Plates were blocked with 100µl 5% (w/v) skimmed milk powder (Marvel, Knighton, UK) dissolved in PBS at room temperature for 2h. 50µl serum diluted 1:50 in 5% skimmed milk/PBS was added with further incubation at room temperature for 2h. Biotinylated rat anti-mouse IgG1 or IgG2a detection antibodies (BD Biosciences) were then added and incubated at room temperature for 2h. Subsequent stages were as described in section 2.2.5.1. For analyses, values generated for HeLa cell lysate coated wells were subtracted from that of RV coated wells for each sample.

2.2.5.4 Nuclear DNA-binding ELISAs
Activation of transcription factors GR, NFκB p65 subunit and IRF-3 were assessed in lung tissue using commercially available DNA binding assays (Active Motif). 20 µg per well of nuclear protein (extracted as described in section 2.2.3.7) was added per well and the assay was carried out according to manufacturers’ instructions.

2.2.5.5 ELISA analyses
Plates were analysed by a Spectramax Plus plate reader (MDS Analytical Technologies, Wokingham, UK) at 450nm with corrections made for plate and background absorbance at 540nm and concentrations calculated from the standard curve. Data were analysed using Softmax Pro software (version 5.2; Molecular Devices, Sunnyvale, USA). The lower limits of detection for ELISA assays are shown in table 2.9.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Lower limit of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRAMP</td>
<td>0.32 ng/ml</td>
</tr>
<tr>
<td>CCL5/RANTES</td>
<td>31.2 pg/ml</td>
</tr>
<tr>
<td>CCL17/TARC</td>
<td>31.2 pg/mL</td>
</tr>
<tr>
<td>CCL22/MDC</td>
<td>7.81 pg/ml</td>
</tr>
<tr>
<td>CXCL1/KC</td>
<td>15.6 pg/ml</td>
</tr>
<tr>
<td>CXCL2/MIP-2</td>
<td>15.6 pg/ml</td>
</tr>
<tr>
<td>CXCL9/MIG</td>
<td>15.6 pg/ml</td>
</tr>
<tr>
<td>CXCL10/IP-10</td>
<td>62.5 pg/ml</td>
</tr>
<tr>
<td>IFN-α</td>
<td>15.6 pg/ml</td>
</tr>
<tr>
<td>IFN-β</td>
<td>15.6 pg/ml</td>
</tr>
<tr>
<td>IFN-λ</td>
<td>31.2 pg/ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>15.6 pg/ml</td>
</tr>
<tr>
<td>Pentraxin-3</td>
<td>21.8 pg/ml</td>
</tr>
<tr>
<td>TNF-α</td>
<td>31.2 pg/ml</td>
</tr>
</tbody>
</table>

Table 2.9: Lower limit of detection for ELISA assays

2.2.6 Neutralisation assay
Neutralisation of RV1B was measured in Ohio HeLa cells on 96 well plates. HeLa cells were grown until ~90% confluent. Pooled sera for each given treatment group (sampled as described in section 2.2.3.9) at d14 post-challenge were incubated with purified RV1B or medium control at room temperature with shaking for 1h and then added to HeLa cells with further incubation at 37°C for 48-72h. Protection from CPE was measured by crystal violet cell viability assay. Cells were stained with 0.1% (w/v) crystal violet (Sigma-Aldrich) at room temperature for 10 minutes. Cells were then
washed with water, air-dried and crystal violet was solubilised with 1% sodium dodecyl sulphate (SDS) solution (w/v). Absorbance was measured by a Spectramax Plus plate reader at 560 nm.

2.2.7 Quantitative PCR

2.2.7.1 RNA extraction and cDNA preparation
Lung lobes which had been stored in RNAlater were removed and lysed in RLT buffer by bead milling using a TissueLyser LT (Qiagen). RNA was extracted using an RNeasy kit (Qiagen) according to manufacturers’ instructions. Briefly, cellular debris was removed by centrifugation at 13,000 x g for 3 minutes. RNA was precipitated with 70% ethanol and then bound to an RNeasy spin column (Qiagen), washed and contaminating DNA removed using DNase I (Qiagen). RNA was eluted in 35µl RNase-free water.

For conversion to cDNA, 10µl RNA was added to a reaction mix containing 10µM random hexamer primers (Promega, Southampton, UK), 0.5mM deoxynucleotide triphosphates (dNTPs) and 0.2units/µl reverse transcriptase (both from Omniscript kit, Qiagen) and incubated at 37°C for 1h.

2.2.7.2 Bacterial DNA extraction
To extract bacterial DNA present in lavage or lung tissue samples, the FastDNA Spin Kit for Soil (MPBiomedicals, Cambridge, UK), was used according to manufacturers’ instructions. BAL or nasal lavage samples were centrifuged at 20,000 x g for 30 minutes to pellet cell debris and bacteria. The pellet was then resuspended in 998µl sodium phosphate buffer and added to a lysing matrix E tube with 122µl MT buffer (all MPBiomedicals) and homogenised using a Precellys24 high-throughput tissue homogeniser (Precellys, Ann Arbor, USA) at a speed of 6,800rpm with two cycles of 30 seconds. For lung tissue, the sample was added directly to 998µl sodium phosphate buffer and 122µl MT buffer in a lysing matrix E tube and homogenised as described above. Protein was then removed from the supernatant by addition of protein precipitation solution (MPBiomedicals) followed by centrifugation at 14,000 x g for 5 minutes. From the resultant supernatant, DNA was bound by addition of matrix beads. The beads/DNA were then washed with ethanol after which DNA was eluted into 100µl DNase/Pyrogen Free water (MPBiomedicals). The extracted DNA was stored at -80°C until further use.

2.2.7.3 Taqman quantitative PCR
Taqman quantitative PCR was carried out using 1µl cDNA or appropriately diluted plasmid standard, added to a reaction mix of 6.25µl Quantitect Probe PCR Mastermix (Qiagen) with primers and FAM/TAMRA-labelled probes specific for the 5’-untranslated region of RV, 18S ribosomal RNA or the
the gene of interest (table 2.10) in a total volume of 12.5µl per well of a qPCR plate (MicroAmp fast 96-well reaction plate; Applied Biosystems, Paisley, UK). The reaction was run on a 7500 Real-Time PCR System (Applied Biosystems) for 45 cycles with the following conditions: 1 cycle of 50°C (2 minutes) and 95°C (10 minutes); 45 cycles at 95°C (15 seconds) and 60°C (1 minute). The cycle at which fluorescence of free FAM in the sample passed a threshold value (Ct) appropriate to the data, as set by the user, was determined. cDNA for 18s ribosomal RNA was diluted 1:100 in dH2O prior to analysis. All samples were analysed in duplicate.

Gene/virus copy number was quantified by comparison to a plasmid DNA standard and normalised to 18s ribosomal RNA levels. The lower limit of detection for all Taqman assays was 10 copies/µl cDNA. When no standard was available (as it was not possible to clone some qPCR products) the relative quantity was calculated first by normalisation to an endogenous control (to 18s rRNA copies using the $2^{\Delta\Delta Ct}$ method) and then subsequently normalised to the mean of the control group giving fold change of gene expression over control, as previously described.[360]
<table>
<thead>
<tr>
<th>Assay</th>
<th>Sequence (5’ – 3’)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>β</em> Defensin-2</td>
<td>CACTCCAGCTGTTGGAAAGTTTAAA GCTCTGACACAGTGACTCCATT ATACGAGCAAGAACTTGAACACTGCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>900nM 900nM 100nM</td>
</tr>
<tr>
<td>IFN-β</td>
<td>CCATCTAGAAGACGTTGAGAT GAGAGGCTGTTGGAGAAATCG TCCACGACCTCTTCAATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300nM 300nM 100nM</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TCAAGTGCAATAGATGTTGAAAGAT TGGCTCTGAGAAAATTCATG TCACCATCTTTTGCCAGATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>900nM 900nM 100nM</td>
</tr>
<tr>
<td>IFN-λ</td>
<td>AAAGAGATGCAACATTGCT TCAAGACGCCCTTCTCGAT TCCCAAAAGAGCTGAGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>900nM 900nM 100nM</td>
</tr>
<tr>
<td>IL-13</td>
<td>GATATTGCACTGTTGAAATCC GGTACTCTGGATTTGCTCATC ATCCGCTACTTCTGTCCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300nM 300nM 100nM</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>AGAGGACGGAGAGACACTCTGT CTCCATCTCTCTTCAGGATGTCTT CAGAGGCCATCAGGCTGATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300nM 300nM 100nM</td>
</tr>
<tr>
<td>MUC5B</td>
<td>GAGCGTGGCTATGTGAAATGAG CAGGGGCTGTTGCTTTCAT ATCCGCTACTTCTGTCCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300nM 300nM 100nM</td>
</tr>
<tr>
<td>RV</td>
<td>GTGAAGAGCCACTTGCTT TGGCTACAGGGTTAGCTCC TGCAGCTCTCAGTCTG</td>
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<td></td>
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<tr>
<td>Surfactant protein- A</td>
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<td></td>
<td>GCCAGAACCTCACTAAGGAA</td>
<td>300nM 300nM 100nM</td>
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<tr>
<td></td>
<td>TGTCACTAGGCTCTTGGCCTCCACCC</td>
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<tr>
<td>SLPI</td>
<td>CACTCCAGCTGTGGAAAGTTTAAA GCTCTGACACAGTGACTCCATT AGTCAGCTCTG</td>
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<tr>
<td>TLR2</td>
<td>CAAAGGCCTAAATCTCAGAGGAT ACACCCAGAGCATCAGTACATG CAGTCTCTCTCGCAAGCCTGTTGC</td>
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<td></td>
<td></td>
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<td>TNF-α</td>
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<tr>
<td>18S</td>
<td>CGCCGCTAGAGGTGAATAACT CATCTTGGGAAATGCTTTTC ACCGGGCGAAGCACGGAAG GA</td>
<td></td>
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<td></td>
<td></td>
<td>300nM 300nM 100nM</td>
</tr>
</tbody>
</table>

Table 2.10 Taqman quantitative PCR primers and probes
2.2.7.4 SYBRGreen quantitative PCR
SYBRGreen quantitative PCR for bacterial DNA content of all extracted samples was carried out using the 7500 Real-Time PCR System (Applied Biosystems). Triplicate reactions were performed for each sample using 1µl template per reaction. For creation of the standard curve a 1:10 dilution series (1 x 10^8 to 1 x 10^2 copies/µl) of Pseudomonas aeruginosa PAO1 full length 16S rRNA gene cloned into PCR II vector (Invitrogen) was used. In addition to template, each PCR reaction mix contained 0.2µl of forward primer 520F (10µm; 5'-AYTGGYDTAAAGNG-3'), 0.2 µl reverse primer 802R (10µm; 5'-TACNVGGGTATCTAATCC-3'), 5µl SYBR Fast Universal master mix (Kapa Biosystems, Woburn, USA) and 3.6µl H_2O. PCR cycling conditions were: 1 cycle of 90°C for 3 minutes, followed by 40 cycles of 95°C (20 seconds), 50°C (30 seconds), 72°C (30 seconds) and melt conditions of 1 cycle of 95°C (15 seconds) and 1 cycle of 60°C (1 minute) followed by dissociation at 95°C (15 seconds). The lower limit of detection for SYBRGreen quantitative PCR was 1000 copies/µl cDNA.

2.2.8 16S rRNA sequencing methods

2.2.8.1 Amplicon preparation
The 16S rRNA gene was amplified using the reverse primer 5’CCATCTCATCCCTGGCTGTCTCCGACTCAGNNNNNNNNNCCGTCAATTTTTRAGT-3’ and the forward primer 5’-CTATCCCCGTGGCCTGCAATTTTTRAGT-3’. The reverse primers contain the A adaptor sequence for 454 pyrosequencing (underlined) followed by a 12 nucleotide multiplex identifier barcode (represented as NNNNNNNNNNNN) used to tag each PCR product and then the bacterial primer 926R (italicised). The forward primer lacks the barcode but contains an adaptor sequence (underlined) followed by the bacterial primer 357F (italicised). Twenty five µl PCR reactions were set up with 1µl of template (extracted DNA), 1µl Forward Primer (10µM), 1µl Reverse primer (10µM), 0.5µl dNTP mix (10mM), 1µl BSA (20 mg/ml), 6.5µl Glycine Betaine (1.3M), 2.5µl Faststart 10 x Buffer, 0.2µl FastStart HiFidelity Polymerase (5units/µl)(all Sigma-Aldrich) and 11.25µl H_2O. Cycling conditions were: 1 cycle of 95°C for 2 minutes, 30 cycles of 94°C (20 seconds), 50°C for (30 seconds) and 72 °C (5 minutes).

2.2.8.2 Gel electrophoresis
To confirm amplification and that no PCR contamination had occurred, amplicons were visualised on 1% agarose gels (w/v). These were prepared by addition of 1g agarose (Sigma-Aldrich) to 100ml of TE Buffer with 5µl of Gel Red (Biotium, Cambridge, UK). The mixture was heated until the agarose dissolved and left to cool until a temperature of approximately 60°C was reached. The mixture was then poured onto an agarose gel slab, a well comb inserted and the gel left to set at room
temperature for 1h. When loading the gel, 10µl of 100 bp DNA ladder (Invitrogen) was loaded into the well of one lane of the gel to allow the length of DNA fragments to be determined post electrophoresis. 5µl of each amplicon was mixed with 1 µl of loading dye (Invitrogen) before loading onto gel. Bands were visualised using the EpiChemi3 Darkroom UV transilluminator system (Ultra-Violet Products Inc., Upland, USA).

2.2.8.3 Amplicon purification
Quadruplicate PCR products were pooled together to ensure sufficient DNA for sequencing. The AMPure XP kit (Agencourt, Takeley, UK) was used to purify the pooled amplicon products with a ratio of 0.7 magnetic beads to PCR product, giving a size selection that removes oligonucleotides smaller than ~250 base pairs. The purified PCR amplicons were then washed with ethanol, eluted from the magnetic beads and stored at -80°C until further use.

2.2.8.4 Amplicon quantification, dilution and pooling
Purified amplicons were quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen) according to manufacturers’ instructions. A standard curve was created from phage λ DNA standard (Invitrogen) in TE buffer to allow DNA concentration of each amplicon to be determined. DNA samples of sufficient quantity for subsequent sequencing (>2.5 ng/µl) were pooled together in equimolar concentrations. The Amplicon pool was then purified (as described in section 2.2.8.3) and quantified using the Quant-iT PicoGreen dsDNA assay. The amplicon pool was then diluted to a concentration of 10⁵ molecules/µl prior to setting up the emulsion PCR.

2.2.8.5 Emulsion PCR
Emulsion PCR was carried out according to the manufacturers’ instruction. Briefly, a live amp mixture was made consisting of 382.5 µl H₂O, 515 µl additive, 297.5 µl amp mix, 80 µl amp primer, 70 µl enzyme mix, 2 µl PPiase (all Roche). The live amp mixture was then added to a Turrex stirring tube containing 4 ml emulsion oil (both Roche), 30 µl of the amplicon pool diluted to 10⁵ molecules/µl and DNA capture beads (Roche). The contents were mixed using an Ultra Turrax Tube Drive (Roche) for 5 minutes at 2,000 rpm. The emulsion mix was then transferred to a 96 well plate and amplification performed with the following cycling conditions: 1 cycle of 94°C (4 minutes); 50 cycles of 94°C (30 seconds) and 60°C (10 minutes).

2.2.8.6 16S pyrosequencing
Single direction pyrosequencing was carried out using the 454 Life Sciences GS Junior (Roche), according to manufacturers’ instructions. The barcoded sequence reads were processed using Quantitative Insights into Microbial ecology (QIIME) software package (version 1.7.0), as previously...
described.[361] Sequence reads containing ambiguous bases, primer sequence mismatches, homopolymer runs or mean quality score of <25 were removed. Remaining sequences were clustered into Operational Taxonomic Units (OTUs) at 97% identity, aligned to full length 16S rRNA sequences and then assigned a taxonomic identity using the Ribosomal Database Project Classifier [362] via the Silva reference database.[363]

2.2.9 Bacterial culture methods

2.2.9.1 Measurement of *S. pneumoniae* colony forming units in lung tissue

Lung tissue was homogenised by bead milling using a TissueLyser LT (Qiagen) and then serial tenfold dilutions of the homogenate were made in sterile PBS up to a maximum dilution of 1:10,000. One hundred µl of each dilution was plated onto a separate Columbia horse blood agar plate (Oxoid, Basingstoke, UK) using a sterile spreader (Thermo Scientific). Plates were incubated for 18h at 37°C and colonies were counted on plates where 50-300 distinct colonies were present. The total number of CFUs was determined as the number of colonies x dilution factor x the original cell suspension volume.

2.2.9.2 Measurement of *S. pneumoniae* colony forming units in blood

Heparinised blood samples (taken as described in section 2.2.3.9) were tenfold serially diluted in PBS up to a maximum dilution of 1:10,000. One hundred µl of each dilution was then plated onto Columbia horse blood agar plates and processed as described in section 2.2.9.1.

2.2.10 Measurement of bacterial phagocytosis by BAL macrophages

2.2.10.1 Fluorescein isothiocyanate labelling of *S. pneumoniae*

*S. pneumoniae* D39 (1 x 10^6 CFU) was heat killed by incubation at 60°C for 1h. Bacteria were centrifuged at 12,000 x g for 3 minutes and washed with 1ml of ice-cold 1% BSA/Hank’s balanced salt solution (w/v) (HBSS; Sigma-Aldrich). Bacteria were again centrifuged at 12,000 x g for 3 minutes and the resulting pellet resuspended in 100µl fluorescein isothiocyanate (FITC) isomer I solution at 0.1 mg/ml in PBS (Sigma-Aldrich). The solution was mixed on a rotator at 4°C for 30 minutes. Nine hundred µl of ice-cold 1% BSA/HBSS was then added with further centrifugation at 12,000 x g for 3 minutes. The bacterial pellet was resuspended in 1ml ice-cold 1% BSA/HBSS and again centrifuged at 12,000 x g for 3 minutes. Resuspension in 1%BSA/HBSS followed by
centrifugation was repeated 3 further times until supernatant was clear of residual FITC. Labelled bacteria were stored at -20°C until use.

2.2.10.2 Ex vivo bacterial phagocytosis assay

BAL samples from 5 mice taken 4h after treatment with 50µl FP 1mg/kg or vehicle DMSO control (as described in section 2.2.2.5) were pooled and cells were pelleted by centrifugation at 12,000 x g for 1 minute. Cells were treated with 1ml ACK buffer for 30 seconds to lyse red blood cells and then resuspended in 1ml of 10% RPMI medium (v/v). Total live BAL cell counts were assessed by trypan blue exclusion and 1 x 10⁵ cells per group were removed into a separate 1.7ml tube for use in the assay. FITC-labelled S. pneumoniae (1 x 10⁶ CFU) was added to each tube and a control tube of BAL cells with unlabelled heat-killed S. pneumoniae was also prepared. 50µl ice-cold mouse serum (taken from untreated C57/BL6 mice, as described in section 2.2.3.9) was added to each tube. Tubes were rotated end-over-end on a shaker for 25 minutes at ~8 rpm followed by centrifugation at 250 x g for 8 minutes. The pellet was then resuspended in 1ml ice cold HBSS. Centrifugation and resuspension then occurred a further five times to remove extracellular bacteria. Finally, the pellet was resuspended in 200µl ice-cold 5% FCS/PBS (v/v).

2.2.10.3 Visual assessment of phagocytosis by fluorescence microscopy

A 100µl aliquot of final macrophage/bacteria suspension from section 2.2.10.2 was removed and mixed with ethidium bromide to a final concentration of 50µg/ml. Ten µl of this suspension was then placed immediately onto a microscope slide and overlayed with a coverslip. Slides were kept in the dark and viewed within 2h (under oil immersion for 1000x magnification) using a fluorescence microscope (LSM 5 Pascal Laser Scanning microscope, Carl Zeiss) with a long-pass FITC filter.

2.2.10.4 Measurement of phagocytosis by flow cytometry

1x10⁵ cells of the final macrophage/bacteria suspension from section 2.2.10.2 was plated into a 96 v-well plate and incubated with live/dead cell marker (Invitrogen) at 1:1000 dilution in PBS for 20 minutes. Cells were then washed with PBS and fixed by addition of 100µl per well of 2% formaldehyde (v/v) (Sigma-Aldrich) in PBS for 20 minutes at room temperature in the dark. Cells were again washed with PBS, resuspended in FACS buffer and stored at 4°C in the dark, prior to analysis within 4h. Data were acquired and analysed as described in section 2.2.4.2.
2.2.11 In vitro methods

2.2.11.1 BEAS2B cell culture
The epithelial cell line BEAS2B was cultured in 175cm² tissue culture flasks in 10% RPMI medium (v/v) at 37°C with 5% CO₂. Cells were passaged when approximately 90% confluent.

2.2.11.2 Transfection of BEAS2B cells with reporter constructs
Transfections were performed on 12 well culture plates (Nunc). BEAS2B cells were grown and transfected when 80-90% confluent. A reaction mix of reporter plasmid (IFNβ-luciferase) at 0.8µg/well was diluted in serum-free RPMI containing internal control vector (Renilla, 0.2µg/well) and incubated for 3 minutes at room temperature. Superfect (Qiagen) at 3µl/well was added to the mix with further incubation for 15 minutes at room temperature. The mix was then diluted 1:5 with serum-free RPMI. Cells were washed twice with PBS and 475ml of the mix was added to each well. Cultures were then incubated at 37°C for 3h with 5% CO₂. The transfection mixture was then removed, cells washed twice with PBS and 1ml 2% RPMI medium (v/v) added to each well.

2.2.11.3 Treatment of BEAS2B cells with FP and receptor agonists
FP (Sigma-Aldrich) was dissolved in DMSO at a stock concentration of 0.1 M, and stored at -20 °C. Twenty four hours following transfections, cells were treated with FP at 1nM and 10nM concentrations in 1ml of 2% RPMI medium (v/v). Control wells were treated with 2% RPMI medium alone. One hour following FP treatment, 5µg/ml TLR3 agonist (Poly(I:C); Invitrogen) or 250ng/ml MDA5 agonist (transfected Poly(I:C); Invitrogen) in 1ml 2% RPMI medium was added to cells. 2% RPMI medium alone was used as negative control for either agonist.

2.2.11.4 Dual-luciferase reporter assay system
At 24h following treatment with agonist, cells were washed with PBS and then lysed in 100µl of 1 x passive lysis buffer (Promega). Cells were then collected, subjected to two freeze-thaw cycles and centrifuged at 16,200 x g for 1 minute to pellet cell debris. Twenty µl of each sample was assessed for relative light units (RLU) using a Dual Luciferase kit (Promega) according to the manufacturers’ instructions. Relative promoter activity was calculated by dividing the RLU of the reporter plasmid (IFN-β-Luc) over the RLU of the internal control vector (Renilla).
2.2.12 Statistical analysis

For animal experiments, group sizes of at least 4 mice per experimental condition were used. All data shown is representative of at least 2 independent experiments with individual figures depicting data from a single experiment (unless otherwise indicated). Data were analysed using Prism (version 4, Graphpad Software, San Diego, USA) and expressed as group mean, displaying error bars as standard error of the mean (SEM). Data were analysed by Kruskal-Wallis test with differences between groups assessed by Dunn’s post-test when three or more groups were compared at a single time-point. Two-way ANOVA was used for comparison between multiple groups at more than one timepoint with significant differences between groups assessed by Bonferroni’s multiple comparison test. For in vitro data, mean +/- SEM of 3 independent experiments is shown. Data were analysed by Kruskal-Wallis test with differences between groups assessed by Dunn’s post-test. Differences were considered significant when $p<0.05$. Specific p values are indicated in figure legends.
Chapter 3: Results - Characterisation of a mouse model of RV-induced COPD exacerbation

3.1 Introduction

Rhinovirus (RV) infections are common triggers for acute exacerbations of chronic obstructive pulmonary disease (COPD).[27, 364] Inflammatory responses during RV-induced exacerbations of COPD are poorly characterized. Some insight has been gained from naturally occurring COPD exacerbation studies, but these studies are limited by potential confounding factors such as varying time between virus infection and presentation, and treatments being initiated prior to sampling. A recently described human experimental RV infection model in COPD has allowed controlled measurement of a range of inflammatory parameters and provided a clearer understanding of the relationship between virus infection, anti-viral innate responses and airways inflammation.[35] In comparison to stable state, exacerbations are associated with increased neutrophilic [31, 35, 87, 88, 91] and lymphocytic [35, 84, 87, 88, 96] cellular airways inflammation, exaggerated production of cytokines such as TNF-α [35, 89], CXCL10/IP-10 [91, 95] and CCL5/RANTES [87, 91] in the airways, deficient type I IFN responses, impaired virus clearance [35] and increased airway mucus production [35]. Additionally, the human experimental model has allowed comparison of inflammatory responses in RV-induced exacerbations of COPD compared to RV infection in healthy controls and showed increased airway neutrophil and lymphocyte recruitment, increased neutrophil chemokine CXCL8/IL-8 expression and increased levels of neutrophil elastase in sputum.[35] Positive correlations were also observed between virus loads and sputum levels of CXCL8/IL-8, IL-6 and TNF-α in patients with COPD but not in healthy controls.[35]

Modelling COPD in mice can be achieved by a number of approaches including cigarette smoke administration, instillation of tissue-degrading proteinases such as elastase, or genetic manipulation.[331] Models that employ elastase administration produce a rapid onset of emphysematous destruction of the lungs and may be considered the best short-term method for modelling severe disease. A number of studies have described elastase-induced models of stable COPD [365, 366] or exacerbation triggered by bacterial infection.[347, 348, 350] The only published mouse model of RV-induced exacerbation in COPD reported that weekly intranasal administration of porcine pancreatic elastase to induce histological emphysema combined with LPS for four weeks to mimic chronic bacterial colonisation, followed by RV1B infection, led to increased lung tissue mRNA expression of TNF-α, IL-13 and MUC5AC, increased AHR to MCH challenge, deficient type I IFN responses and delayed virus clearance from the airways.[344] Given that this model appeared to display many of the key features reported in human disease, we attempted to recreate the model...
using the same dosing protocol so that it could subsequently be used to test the effects of ICS administration in exacerbation of COPD induced by RV.

3.2 Aim and hypotheses

3.2.1 Aim
To develop a clinically relevant mouse model of RV-induced exacerbation of COPD that mimics what is known of human disease including: histological emphysematous lung changes, deficient IFN responses and impaired virus control, increased cellular airways inflammation and inflammatory mediator production and mucus hypersecretion.

3.2.2 Hypotheses
1) Once weekly intranasal administration of elastase and LPS for four weeks to mice causes histological abnormalities consistent with emphysema and models other features of stable COPD including increased airway inflammation, mucus hypersecretion and increased AHR.

2) Once weekly intranasal administration of elastase and LPS for four weeks to mice impairs RV induction of type I and III IFNs leading to increased virus loads in vivo.

3) Once weekly intranasal administration of elastase and LPS for four weeks to mice leads to enhanced RV induction of inflammatory chemokines and cytokines, as previously reported and also increases other features of RV-induced inflammation reported in human studies of COPD such as cellular airways inflammation.

4) RV infection further increases AHR induced by intranasal administration of elastase and LPS.
3.3 Results

3.3.1 RV1B infection in C57BL/6 mice

The mouse model of RV1B infection was initially described in BALB/c mice [367] and relatively few studies from our group have been conducted in the C57BL/6 strain. Given that the RV-induced exacerbation of COPD mouse model that we were aiming to reproduce was reported in C57BL/6 mice [344], we therefore initially carried out a time-course experiment assessing primary RV1B infection in C57BL/6 mice and measured a range of inflammatory parameters. Consistent with previous observations in BALB/c mice, C57BL/6 mice challenged with RV1B had increased lung tissue viral RNA copies at d1, 2 and 4 post-infection compared to mice challenged with UV-RV1B (fig 3.1a). Additionally, IFN-λ BAL protein at d1 post-infection, CXCL1/KC BAL protein at d1 and 2 post-infection and CXCL10/IP-10 BAL protein at d1, 2 and 4 post-infection were increased in mice challenged with RV1B vs. UV-RV1B (fig 3.1b–d). Assessment of cellular airways inflammation showed increased BAL neutrophils at d1 and 2 and increased BAL lymphocytes at d2, 4 and 7 post-infection in mice challenged with RV1B vs. UV-RV1B (fig 3.1e&f).
Figure 3.1 Virus loads and inflammatory responses to RV1B infection in C57BL/6 mice.
C57BL/6 mice were challenged intranasally with RV1B or UV inactivated RV1B (UV-RV1B). (a) Lung tissue was harvested at the indicated timepoints post-infection, RNA was extracted from tissue and cDNA generated as described. RV RNA copies were assessed by Taqman quantitative PCR. (b-d) BAL was performed at the indicated timepoints post-infection and (b) IFN-λ (c) CXCL1/KC and (d) CXCL10/IP10 proteins in lavage supernatants were measured by ELISA. BAL cell cytospin slides were prepared as described. (e) neutrophils and (f) lymphocytes were differentially counted blind to experimental conditions. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, ***p<0.001).
Chapter 3: Results

3.3.2 Administration of four doses of elastase and LPS in combination with RV1B infection to model COPD exacerbation

The only previously published model of COPD RV exacerbation reported that weekly administration of porcine pancreatic elastase (1.2 units) and LPS (7 endotoxin units) for four weeks followed 7d later by infection with RV1B in C57BL/6 mice led to inflammatory features consistent with human COPD RV exacerbation.[344] We therefore attempted to recreate this model using the same dosing protocol and evaluated similar parameters to those reported, along with additional features that we deemed to be indicative of human COPD exacerbation based on what has been previously reported in experimental and naturally occurring infection studies.

3.3.2.1 Lung histology

Emphysematous lung changes are characteristic of COPD in humans and in the reported four dose elastase/LPS mouse model, histological changes consistent with severe COPD were observed.[344] Using this protocol, exactly as reported [344], we treated mice with four weekly doses of intranasal elastase and LPS and harvested lung tissue at 7d post final LPS or PBS challenge (fig 3.2a). Emphysematous changes were apparent in lung sections from mice treated with elastase regardless of LPS treatment (elastase + LPS and elastase + PBS groups) and to a lesser extent with PBS + LPS treatment compared to PBS + PBS treatment, both visually (fig 3.2c-f) in H&E stained sections and when quantified by measuring mean linear intercept (fig 3.2b). The highest alveolar chord lengths were observed in lung sections from mice treated with combined elastase and LPS with significant increases compared to elastase + PBS, PBS + LPS or PBS + PBS treatment (fig 3.2b). Mice treated with elastase + PBS or PBS + LPS had increased mean linear intercept values compared to control mice dosed with PBS alone (fig 3.2b). Despite the histological changes induced by intranasal elastase and LPS administration, none of the animals studied showed any outward signs of illness or respiratory compromise.
Figure 3.2 Histological changes in four dose elastase/LPS model.
(a) C57BL/6 mice were challenged intranasally with elastase on d1 and LPS on d4 of each week, or with PBS as control, for four weeks. At d7 following final LPS or PBS challenge, lungs were harvested, formalin fixed, paraffin embedded and stained with haematoxylin and eosin (H&E). (b) The diameter of air spaces were measured in at least 10 random fields per slide and averaged to determine alveolar chord length. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001). (c-f) Representative images are shown from mice treated with four doses of (c) elastase + LPS (d) elastase + PBS (e) PBS + LPS and (f) PBS + PBS.
3.3.2.2 Anti-viral immune responses and virus loads

Having established that combined elastase and LPS administration led to histological airway changes consistent with COPD, we next added RV1B infection to this baseline model (fig 3.3a), exactly as reported.[344] Experimental infection studies in patients with COPD have reported deficient IFN responses to RV infection [35] and this was also shown in the previously reported four dose elastase/LPS COPD exacerbation model where elastase/LPS + RV treated mice had suppressed type I IFN mRNA expression and delayed RV clearance [344]. We found that IFN-β and IFN-λ mRNAs were increased at 8h post-infection in mice treated with PBS and infected with RV (PBS+RV treatment; modelling RV infected healthy subjects) vs. treatment with PBS and UV-RV1B (PBS + UV; modelling uninfected healthy subjects). Induction of IFNs-β and -λ mRNAs in lung tissue by RV was reduced in mice treated with four doses of elastase/LPS (elastase/LPS + RV treatment; modelling RV infected patients with COPD) compared to treatment with PBS + RV (fig 3.3b&c) at 8h post-infection. However, elastase/LPS + RV treatment led to reduced rather than increased lung virus loads compared to PBS+RV treatment at d1 post-infection (fig 3.d).
Figure 3.3 Virus loads and interferon responses in four dose elastase/LPS + RV1B model.
(a) C57BL/6 mice were challenged intranasally with elastase on d1 and LPS on d4 of each week, or with PBS control, for four weeks. At d7 following final LPS or PBS challenge, mice were additionally challenged with RV1B or UV-inactivated RV1B. Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (b) IFN-β, (c) IFN-λ mRNA and (d) RV RNA copies were assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.001).

3.3.2.3 BAL cellular inflammation
In the previously reported model, the effect of four dose elastase/LPS administration as a baseline model of the inflammatory changes of stable COPD was assessed and shown to increase numbers of macrophages, neutrophils and lymphocytes in BAL compared to PBS dosed controls [344], which are all features characteristic of stable COPD in humans. In contrast to the reported model, we observed no increases in BAL neutrophil and a non-significant trend towards increased lymphocyte numbers associated with four dose elastase/LPS administration (comparison between elastase/LPS + UV and PBS + UV groups) (fig 3.4a&b). However, in keeping with the reported model, we observed increased macrophages at d1 and d4 post administration in elastase/LPS + UV vs. PBS + UV treated mice (fig 3.4c). Therefore, in our hands, the four-dose elastase/LPS model was only partially representative of the inflammatory changes of stable COPD reported in human studies.
Acute exacerbation of COPD is associated with a further enhancement of airway inflammation with increases in airway neutrophil and lymphocyte recruitment above stable state, as reported by several human naturally occurring exacerbation studies [31, 87, 96] and the human experimental RV infection model in COPD also reported increased inflammatory responses in patients with COPD compared to healthy controls [35]. This feature was not reported in the previously published four dose elastase/LPS model [344] but would be considered to be an important characteristic of a clinically relevant mouse model and we therefore considered these features in our assessment of the model. As previously observed, mice treated with PBS + RV had increased BAL neutrophils at d1 and increased lymphocytes at d4 post-infection vs. PBS + UV treatment (fig 3.4a&b). Elastase/LPS administration suppressed RV induced neutrophilia (~60% reduction) at d1 post-infection (fig 3.4a) but neutrophil number was greater in elastase/LPS + RV vs. elastase/LPS + UV treated mice (modelling exacerbated COPD vs. stable COPD) at d1 post-infection (fig 3.4a). Elastase/LPS + RV treated mice had increased BAL lymphocyte numbers (~6 fold) vs. PBS + RV treatment at d1 post-infection but there were no significant differences in BAL lymphocyte numbers between elastase/LPS + RV and elastase/LPS + UV treated mice (fig 3.4b). Macrophage numbers were no different in elastase/LPS + RV treated mice vs. PBS + RV or elastase/LPS + UV treated mice at either d1 or d4 post-infection (fig 3.4c).

Therefore, as a model of enhanced inflammation associated with RV infection in COPD vs. RV infection in healthy controls, 4 dose elastase/LPS mimicked the increased lymphocyte but not neutrophil component. When considering this dosing strategy as a model for exacerbated vs. stable COPD, the opposite was observed with enhanced neutrophilic but not lymphocytic inflammation observed. Therefore, four dose elastase/LPS was only partially representative of airway inflammatory changes in RV-induced exacerbation of COPD.
Figure 3.4 BAL cellular inflammation in four dose elastase/LPS + RV1B model.
C57BL/6 mice were challenged intranasally with elastase on d1 and LPS on d4 of each week or PBS as control for four weeks. At d7 following final LPS or PBS challenge, mice were additionally challenged with RV1B or UV-inactivated RV1B. BAL was performed at the indicated timepoints. BAL cell cytospin slides were prepared as described. (a) neutrophils, (b) lymphocytes and (c) macrophages were differentially counted blind to experimental conditions. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).

3.3.2.4 Chemokine and cytokine expression
In addition to cellular inflammation, we also evaluated chemokine and cytokine production in the four dose elastase/LPS model. The previous report of this model showed increased CXCL2/MIP-2, IL-6, TNF-α and IL-1β in the baseline model of elastase/LPS vs. PBS administration [344], consistent with observations in human studies. We therefore assessed these features and, in contrast to that reported, found no significant differences in BAL protein levels of CXCL2/MIP-2 or IL-6 with elastase/LPS + UV vs. PBS + UV treatment (fig 3.5a&b). We were unable to detect TNF-α or IL-1β protein in BAL in the model but availability of a Taqman qPCR assay for TNF-α allowed measurement of lung tissue mRNA expression of this cytokine with no difference also observed between elastase/LPS + UV and PBS + UV treated mice (fig 3.5e). Additionally, BAL protein levels of CXCL10/IP-10 and CCL5/RANTES were not affected by elastase/LPS + UV treatment (fig 3.5c&d). Therefore, in
In contrast to data reported by Sajjan et al [344], we failed to observe increased chemokine and cytokine expression representative of stable COPD in the baseline four dose/elastase LPS model.

In the previously reported model, four dose elastase/LPS administration also led to increases in RV induction of TNF-α and IL-13 mRNAs in lung tissue.[344] We therefore measured these parameters and also extended our analyses to include other chemokines and cytokines that have previously been shown to be upregulated in human COPD exacerbations. RV infection increased BAL protein levels of CXCL2/MIP-2, IL-6 and CCL5/RANTES (all at d1 post-infection) and CXCL10/IP-10 (at d1 and d4 post-infection) compared to PBS + UV treatment (fig 3.5a-d). RV infection also increased lung tissue mRNA expression of TNF-α at d1 post-infection (fig 3.5e). However, lung tissue IL-13 mRNA was not increased by RV infection at either d1 or d4 post-infection (fig 3.5f).

In contrast to the previous report of the four dose elastase/LPS model, we observed suppressed (~90% inhibition) rather than increased RV induction of TNF-α mRNA in lung tissue of elastase/LPS treated mice at d1 post-infection with no difference observed at d4 (fig 3.5e). There was, however, increased TNF-α mRNA in elastase/LPS + RV vs. elastase/LPS + UV treated mice (~7 fold) at d1 post-infection (fig 3.5e). Similar to the reported model, increased IL-13 mRNA expression was observed in elastase/LPS + RV vs. PBS+ RV treatment groups at d1 (~3 fold) and d4 (~4 fold) post-infection and vs. elastase/LPS + UV (~2 fold) at d1 post-infection (fig 3.5f).

Assessment of a number of additional disease-relevant chemokines/cytokines in the four dose model showed that elastase/LPS suppressed RV induction of CXCL2/MIP-2 (~25% inhibition) and IL-6 (~75% inhibition) proteins in BAL at d1 post-infection and completely inhibited induction of CXCL10/IP-10 protein in BAL at d4 post-infection (fig 3.5a-c). There was no difference in CCL5/RANTES BAL protein levels between elastase/LPS + RV and PBS + RV treated mice at d1 or 4 post-infection (fig 3.5d). However increases were observed in protein levels of CXCL2/MIP-2, IL-6, CXCL10/IP-10 and CCL5/RANTES in BAL of elastase/LPS + RV vs. elastase/LPS + UV treated mice (fig 3.5a-d). Therefore, four doses of elastase/LPS was a poor model of enhanced inflammatory cytokines and chemokine expression associated with RV infection in COPD vs. healthy controls but appeared to be a better model of exacerbated vs. stable COPD.
Figure 3.5 Chemokine and cytokine induction in four dose elastase/LPS + RV1B model.

CS7BL/6 mice were challenged intranasally with elastase on d1 and LPS on d4 of each week or PBS as control for four weeks. At d7 following final LPS or PBS challenge, mice were additionally challenged with RV1B or UV-inactivated RV1B. (a-d) BAL was performed at the indicated time-points post-infection and (a) CXCL2/MIP-2, (b) IL-6, (c) CXCL10/IP-10 and (d) CCL5/RANTES proteins in lavage supernatants were measured by ELISA. (e-f) Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (e) TNF-α and (f) IL-13 mRNA copies were assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).

3.3.2.5 Airway mucins

Mucus hypersecretion is a prominent feature of stable COPD and has been shown to be increased by RV infection in vitro and in vivo [367-369]. The published mouse model reported increased MUC5AC
mRNA expression in lung tissue with four dose elastase/LPS administration, thus demonstrating the mucus hypersecretion indicative of stable COPD.[344] In contrast, we observed no difference in lung tissue mRNA expression of MUC5AC or the other major airway mucin MUC5B between elastase/LPS + UV and PBS + UV treated mice and, therefore, in our hands the four dose elastase/LPS model did not appear to be representative of mucus hypersecretion, as reported in human studies of stable COPD.

In the previous report of this model, addition of RV infection to four doses of elastase/LPS led to increases in MUC5AC mRNA expression over elastase/LPS or RV challenge alone.[344] We therefore assessed MUC5AC and additionally MUC5B mRNA in the four dose model. RV infection increased lung tissue MUC5AC mRNA expression at d4 post-infection but had no effect on MUC5B mRNA at d1 or 4 post-infection (PBS + RV vs. PBS + UV treatment) (fig 3.6a&b). There were no significant differences in MUC5AC or MUC5B expression between elastase/LPS + RV and PBS + RV treated mice or between elastase/LPS + RV and elastase/LPS + UV treated mice (fig 3.6a&b). Therefore, four dose elastase/LPS administration did not appear to accurately model the mucus hyper-secretion that is indicative of RV-induced COPD exacerbation.

**Figure 3.6 Airway mucins in four dose elastase/LPS + RV1B model.**

C57BL/6 mice were challenged intranasally with elastase on d1 and LPS on d4 of each week or PBS as control for four weeks. At d7 following final LPS or PBS challenge, mice were additionally challenged with RV1B or UV-inactivated RV1B. Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (a) MUC5AC and (b) MUC5B mRNA copies were assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.01).
3.3.2.6 Airway hyper-responsiveness

In the published four dose elastase/LPS model, invasive techniques were used to measure lung function in mice. Since these techniques were not available to us, we used whole body plethysmography to assess AHR to nebulised MCH at d1 and d7 in the model. As reported in BALB/c mice [367], RV infection alone did not lead to increased AHR measured by whole body plethysmography at d1, or at d7 post-infection (fig 3.7 a&b). We observed that elastase/LPS + UV treatment led to increased PenH average values at d1 (30mg/mL and 100mg/mL doses of MCH) and d7 post-infection (100mg/mL MCH dose) and lower PC20 values at d1 post-infection compared to treatment with PBS + UV. This contrasts with data reported by Sajjan et al using invasive measurement techniques where elastase/LPS treatment had no effect on baseline airway resistance or AHR in response to MCH.[344]

Increased AHR to MCH challenge following RV infection was reported in the published four dose elastase/LPS model at d7 and d14 post-infection when measured in anesthetized mice by invasive techniques with increases seen in comparison to elastase or RV challenge alone.[344] We observed that elastase/LPS + RV treated mice had increased AHR compared to PBS + RV treatment at d1 (30 and 100 mg/mL MCH doses) and d7 (100mg/mL) and a trend towards lower PC20 values at d1 post-infection (figure 3.7 a-c). However, AHR was reduced in mice treated with elastase/LPS + RV compared to elastase/LPS + UV treatment at d1 with no difference at d7 post-infection and no differences in PC20 values at either timepoint (fig 3.7a-d).
Chapter 3: Results

3.3.3 Comparison of single vs. multiple dose elastase and LPS in combination with RV1B infection to model COPD exacerbation

As shown above, in our hands, the four dose elastase/LPS plus RV model failed to produce an exacerbation of a number of features of disease that the existing literature suggests are indicative of human COPD exacerbation and we also failed to produce many of the reported features from the previously published study [344]. Some authors have previously speculated that inducing very severe lung damage with high doses of elastase may lead to impaired inflammatory responses to infectious...
agents [347] perhaps explaining our observations with the published four dose elastase/LPS protocol. We therefore determined whether reducing the number of doses of elastase/LPS, to reduce lung damage, would more accurately model human COPD exacerbation.

### 3.3.3.1 Lung histology

Comparison of one, two, three and four weekly doses of elastase and LPS indicated a dose dependent increase in emphysematous lung changes apparent both visually in H&E stained lung sections (fig 3.8a–e) and when quantified by measuring mean linear intercept (fig 3.8f). A single dose of elastase and LPS was, however, sufficient to induce emphysematous lung changes with significantly increased mean linear intercept compared to control PBS treated mice (fig 3.8f).

![Figure 3.8](image.png)

**Figure 3.8 Effect of differing elastase and LPS dosing regimes on histological lung changes.** C57BL/6 mice were challenged intranasally with elastase on d1 and LPS on d4 of each week for 1, 2, 3 or 4 weeks or PBS control. At d7 following final LPS or PBS challenge, lungs were harvested, formalin fixed, paraffin embedded and stained with haematoxylin and eosin (H&E). Representative images of mice treated with (a) PBS or (b) one dose (c) two doses (d) three doses or (e) four doses of elastase and LPS. (f) The diameter of air spaces were measured in at least 10 random fields per slide and averaged to determine alveolar chord length. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, ***p<0.001).
3.3.3.2 Virus loads and airway inflammation

Having observed that only a single administration of elastase and LPS is required to induce emphysematous changes, we next assessed inflammatory and virological parameters following single or multiple doses of elastase/LPS plus RV infection. Regardless of the number of doses administered, elastase/LPS + RV treated mice had reduced RV RNA levels in lung tissue at d1 (fig 3.9a), reduced or no difference in BAL neutrophilia (fig 3.9b) at d1 post-infection and no difference or increased BAL lymphocytosis at d4 post-infection (fig 3.9c) vs. PBS + RV treated mice. We also observed reduced or similar BAL protein levels of inflammatory chemokines CXCL10/IP10, CCL5/RANTES and cytokine IL-6 in BAL at d1 vs. PBS + RV treated mice (fig 3.9d-f). The number of doses of elastase and LPS therefore had little effect on the efficacy of this approach for modelling RV enhanced airway inflammation in COPD when comparing elastase/LPS + RV treatment to RV infection alone. However, a number of inflammatory endpoints including BAL neutrophilia (1 and 4 dose protocols), BAL lymphocytosis, BAL protein levels of CCL5/RANTES and IL-6 (1 and 2 dose protocols) and BAL protein levels of CXCL10/IP-10 (1,2, and 3 dose protocols) were increased in elastase/LPS + RV treated mice compared to elastase/LPS + UV treated mice (fig 3.9b-f). Therefore, although combined elastase/LPS appeared to be a poor model of RV infection in COPD vs. infection in healthy controls (elastase/LPS + RV vs. PBS + RV), it was a more representative model of exacerbated vs. stable disease.
Figure 3.9 Effect of differing elastase and LPS dosing regimes on virus loads and virus-induced airway inflammation.

C57BL/6 mice were challenged intranasally with elastase on d1 and LPS on d4 of each week for 1, 2, 3 or 4 weeks or PBS control. At d7 following final LPS or PBS challenge, mice were additionally challenged with RV1B or UV-inactivated RV1B. (a) Lung tissue was harvested at d1 post-infection, RNA was extracted from tissue, cDNA generated as described and RV RNA copies were measured in lung tissue by Taqman quantitative PCR. (b-f) BAL was performed at d1 and d4 post-infection. BAL cell cytospin slides were prepared as described and (b) neutrophils at d1 and (c) lymphocytes at d4 were differentially counted blind to experimental condition. (d) CXCL10/IP-10, (e) CCL5/RANTES and (f) IL-6 proteins in lavage supernatants at d1 were measured by ELISA. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
3.3.4 Evaluation of single dose elastase without LPS administration in combination with RV1B infection to model COPD exacerbation

Since the combination of elastase and LPS with RV did not produce a phenotype that was consistent with human COPD exacerbation, regardless of the number of doses administered, we next determined if the removal of LPS from the model would provide any benefit. Models of COPD exacerbation induced by bacterial challenge in mice have successfully used single dose elastase administration protocols with enhanced inflammatory responses reported following infection with *H. influenzae* or *S. pneumoniae* challenge.[348, 349]

3.3.4.1 Anti-viral immune responses and virus clearance

We assessed innate anti-viral immune responses and virus loads in the single dose elastase model (fig 3.11a). As previously observed with the four dose elastase/LPS model, lung tissue *IFN-λ* mRNA levels were reduced (~70% inhibition) in elastase + RV compared to PBS + RV treated mice on d1 post-infection (fig 3.11b). However, there was no significant difference between these treatments in lung *IFN-β* mRNA levels (fig 3.11c) and no effect of elastase treatment on lung tissue RV RNA levels on either d1 or d4 post-infection (fig 3.11d). Therefore, similar to single or multiple dose elastase/LPS administration, single dose elastase alone did not accurately model the impaired virus clearance that is believed to be indicative of human disease.
Figure 3.10 Effect of single dose elastase administration on virus loads and anti-viral immune responses. (a) C57BL/6 mice were challenged intranasally with elastase and additionally with RV1B or UV-inactivated RV1B, 10d later. Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (b) IFN-λ (c) IFN-β mRNA and (d) RV RNA copies were measured in lung tissue by Taqman quantitative PCR. Data represent mean (+/-SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (***p<0.001).

3.3.4.2 BAL cellular inflammation

We also assessed cellular airways inflammation in the single dose elastase model. In contrast to four dose elastase/LPS administration, single dose elastase led to increases in BAL lymphocytes at d1, macrophages at d1 and d4 and a trend towards increased BAL neutrophils at d1 compared to PBS + UV treatment (fig 3.11a-c). Therefore, single dose elastase was a more representative model of the increased cellular airway inflammation of stable COPD than four doses of elastase + LPS.

Also in contrast to combined elastase/LPS models, single dose elastase plus RV administration led to significant increases in BAL neutrophilia vs. PBS + RV treatment (~2 fold at d1 and ~5 fold at d4) and also vs. elastase + UV treatment (~5 fold at d1 and ~12 fold at d4) (fig 3.11a). BAL lymphocytes were also increased with elastase + RV treatment vs. PBS + RV treatment at d1 (~4 fold) and vs. elastase + UV treatment at d4 (~6 fold) post-challenge (fig 3.11b). Therefore, single dose elastase was also a
more accurate model of RV-induced airways inflammation in COPD vs. healthy controls and in exacerbated vs. stable disease than combined elastase/LPS models.

Figure 3.11 Effect of single dose elastase administration on RV1B-induced cellular airways inflammation
C57BL/6 mice were challenged intranasally with elastase and additionally with RV1B or UV-inactivated RV1B, 10d later. BAL was performed at the indicated timepoints post-infection. BAL cell cytospin slides were prepared as described and (a) neutrophils, (b) lymphocytes and (c) macrophages were differentially counted blind to experimental conditions. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).

3.3.4.3 Chemokine and cytokine expression
We also measured inflammatory chemokine and cytokines in the single dose elastase + RV model. As with the four dose elastase/LPS model, no increases in inflammatory chemokines or cytokines were observed with elastase + UV vs. PBS + UV treatment and therefore the single dose elastase model also failed to mimic the enhanced airway cytokine levels associated with stable COPD.

Elastase administration increased RV induction of CXCL10/IP-10 (~80% increase), CCL5/RANTES protein (~80% increase) in BAL at d1 (fig 3.12a&b) but had no effect on CXCL2/MIP-2 BAL protein (fig 3.12c). Neither IL-13 or TNF-α proteins were detectable in BAL in the model but, as with the four
dose elastase/LPS model, we were able to measure the mRNA expression of these cytokines in lung tissue by Taqman qPCR. Elastase administration suppressed RV induction of *IL-13* mRNA (~50% reduction) at d1 post-infection (fig 3.12e). Additionally, *TNF-α* mRNA in lung tissue was significantly increased in elastase + RV treated mice vs. PBS + RV at d1 (~5 fold) and d4 (~2 fold) post-infection (fig 3.12d). Increases in *CXCL10/IP-10*, *CCL5/RANTES* and *CXCL2/MIP-2* BAL protein at d1 and *TNF-α* mRNA in lung tissue at d4 were also observed in elastase + RV vs. elastase + UV treated mice (fig 3.12a-d). Therefore, in addition to being a more accurate model of enhanced cellular airways inflammation in COPD RV exacerbation, single dose elastase also more accurately modelled the increased inflammatory cytokine levels of RV infection in COPD vs. infection in healthy controls.
Figure 3.12 Effect of single dose elastase administration on RV1B-induced chemokine and cytokine production.

C57BL/6 mice were challenged intranasally with elastase and additionally with RV1B or UV-inactivated RV1B, 10d later. (a–c) BAL was performed at the indicated timepoints post-infection. (a) CXCL10/IP-10, (b) CCL5/RANTES and (c) CXCL2/MIP-2 proteins in lavage supernatants were measured by ELISA. (d–e) Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (d) IL-13 and (e) TNF-α mRNA copies were measured in lung tissue by Taqman quantitative PCR. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
3.3.4.4 Airway mucins

We next assessed airway mucins in the single dose elastase model to determine whether single dose elastase +/- RV1B would model the mucus hypersecretion that is characteristically observed in stable and exacerbated COPD. In contrast to four dose elastase/LPS, single dose elastase administration increased MUC5AC mRNA expression in lung tissue at d1 post administration (comparison of elastase + UV and PBS + UV groups) (fig 3.13a). Similar expression levels of MUC5B mRNA in lung tissue were observed between elastase + UV and PBS + UV treated mice (fig3.13b). We also assessed mucin proteins in BAL and observed increases in MUC5AC at d4 and MUC5B at d1 and 4 post-administration with elastase + UV vs. PBS + UV treatment (fig 3.13c&d). Therefore, in addition to more accurately modelling enhanced airway inflammation of stable COPD, single dose elastase administration was also more representative of mucus hypersecretion associated with stable disease than the four dose elastase/LPS model.

Increases in MUC5AC mRNA in lung tissue and MUC5AC protein in BAL at d1 and d4 were observed in mice treated with elastase + RV vs. PBS + RV (fig 3.13a&c). MUC5AC mRNA at d4 and BAL protein at d1 were also increased in elastase + RV vs. elastase + UV treated mice (fig 3.13a&c). Increased MUC5B mRNA in lung tissue and protein in BAL were observed in mice treated with elastase + RV vs. PBS + RV at d4 post-infection but no significant differences were observed between elastase + RV and elastase + UV treatments (fig 3.13b&d). Therefore, single dose elastase also more accurately modelled increased mucin expression following RV infection in COPD.
C57BL/6 mice were challenged intranasally with elastase and additionally with RV1B or UV-inactivated RV1B, 10d later. (a-b) Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (a) MUC5AC and (b) MUC5B mRNA copies were measured in lung tissue by Taqman quantitative PCR. (c-d) BAL was performed at the indicated timepoints post-infection. (c) MUC5AC and (d) MUC5B protein in lavage supernatants were measured by ELISA. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).

### 3.3.4.5 Lung periodic acid schiff staining

Having observed that the single dose elastase model more accurately modelled mucus hypersecretion, we proceeded to further assess this characteristic of the model by staining lung sections with PAS to identify mucin-producing goblet cells. Abundant PAS-positive mucus producing cells were present in the airways of elastase + RV treated mice at d4 following RV challenge and, to a lesser extent, in the airways of elastase + UV treated mice (fig 3.14a&b). No PAS positive cells were visible in the airways of mice receiving PBS + RV or PBS+ UV treatments (fig 3.14c&d). PAS staining scores were increased in elastase + UV vs. PBS + UV treated mice and also in elastase + RV treated mice compared to elastase + UV or PBS + RV treated mice (fig 3.14e).
Figure 3.14 Effect of single dose elastase administration and RV1B infection on PAS staining.
C57BL/6 mice were challenged intranasally with elastase and additionally with RV1B or UV-inactivated RV1B, 10d later. At d4 post RV challenge, lungs were formalin fixed, paraffin embedded and stained with periodic acid Schiff (PAS). Representative images of mice treated with (a) elastase + RV1B (b) elastase + UV-RV1B (c) PBS + RV1B (d) PBS + UV-RV1B. (e) Scoring for PAS positive mucus producing cells, using a system described previously.[358] Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
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3.3.4.6 Airway hyper-responsiveness

Whole body plethysmography was used to assess AHR to nebulised MCH at d1 post-infection in the single dose elastase + RV model. Neither RV infection (PBS + RV) nor elastase treatment (elastase + UV) in isolation caused increased AHR compared to PBS + UV treated double negative controls. However, mice exposed to single dose elastase followed by RV infection had significantly increased PenH average values at MCH dose of 100mg/mL compared to PBS + RV and elastase + UV treated mice (fig 3.15a). Mice treated with elastase + RV or elastase + UV showed a trend towards lower PC_{20} values than mice treated with PBS + RV or PBS + UV (figure 3.15b). Therefore single dose elastase administration did not demonstrate any lung function abnormalities consistent with stable COPD when assessed by whole body plethysmography. However, it modelled the increased AHR associated with RV infection in COPD vs. RV infection in healthy controls or vs. stable disease.

Figure 3.15 Airway hyper-responsiveness in single dose elastase and RV1B infection model.

C57BL/6 mice were challenged intranasally with elastase and additionally with RV1B or UV-inactivated RV1B, 10d later. Airway hyper-responsiveness expressed as (a) average PenH over a 5 minute period following MCH challenge and (b) expressed as PC_{20}, the provocative concentration of MCH that produces a 20% increase in PenH was measured by whole body plethysmography at d1 post-infection. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (***) denotes statistical comparison between elastase + RV vs. PBS + RV groups (p<0.001), ¥¥ denotes comparison between elastase + RV vs. elastase + UV groups (p<0.01)).
3.4 Summary

In contrast to the previously published description of the model, we found that administration of four doses of elastase and LPS in combination with RV1B led to unchanged or impaired rather than enhanced inflammatory responses compared to PBS + RV treatment. Therefore, in our hands, this model was not a representative model of RV infection compared to infection in healthy controls. However, we did observe exacerbation of some inflammatory parameters compared to elastase + UV treatment, modelling some aspects of exacerbated vs. stable disease. We also failed to demonstrate the previously reported impaired virus control associated with four dose elastase/LPS administration [344] and therefore this model also did not recreate this key feature that has been reported in the human experimental model.[35] Reducing the number of elastase/LPS doses led to less severe histological emphysematous changes but also did not improve the accuracy of the model for mimicking the key features of exacerbation.

In contrast to combined elastase/LPS models, we found that single dose elastase without LPS administration in combination with RV infection modelled many of the key pathological features reported in human experimental and naturally occurring disease, including enhanced neutrophilic and lymphocytic airways inflammation, exaggerated inflammatory cytokine production and increased airways mucin production compared to PBS + RV and elastase + UV treatments. Therefore, this model was a more representative model of the enhanced inflammation and exaggerated mucus secretion following RV infection in COPD vs. either RV infection in healthy controls or stable COPD. Despite this, single dose elastase also failed to recreate the impaired virus control that has been reported in human experimental infection disease models. Since impaired virus control was a key component of our hypothesis that COPD and ICS administration would synergistically suppress IFN responses to RV and increase virus replication, none of the models we evaluated were deemed to be suitable for further testing of this hypothesis.
Chapter 4: Results – Effects of inhaled corticosteroids on rhinovirus infection

4.1 Introduction

ICS are prescribed as maintenance therapy in up to 70% of patients with COPD, either alone or in combination with long acting β2-adrenoreceptor agonists.[370] Their use is supported by clinical guidelines which recommend prescription in patients with forced expiratory volume in the first second (FEV1)<50% predicted and history of frequent disease exacerbations.[9] Randomised controlled trials have reported that inhaled corticosteroid use in COPD reduces exacerbation frequency by only a modest 20-25%.[217, 239] There has been speculation that ICS may have a relative lack of benefit in the context of acute respiratory virus infections in particular.[57]

Emerging evidence suggests that this lack of efficacy of ICS in respiratory virus-induced exacerbations could be due to suppression of innate anti-viral immune responses. In vitro studies have reported suppression of RV-induced IFN-α by budesonide in peripheral blood mononuclear cells (PBMCs) and suppression of IFN-stimulated genes by dexamethasone in fibroblasts and by budesonide in bronchial epithelial cells.[246-248] In vivo, Gustafson et al reported that oral prednisolone administration prior to experimental RV challenge in healthy subjects led to increased viral titres in nasal lavage [249] and Puhakka et al showed prolonged virus shedding with intranasal FP administration during naturally occurring colds.[250] Other studies assessing ICS in experimental RV-induced asthma exacerbations have shown only minimal effects on cellular airways inflammation, further reinforcing the belief that these drugs may be ineffective at reducing virus-induced airway inflammation.[67] As discussed in chapter 3, COPD is believed to be associated with an inherent deficiency in IFN responses to RV infection [35] and given that ICS are frequently used in patients with COPD, they may interact with the disease to synergistically inhibit IFN responses and thus potentially lead to impaired virus clearance and increased exacerbation severity.

To date, however, no study has provided a thorough analysis of the in vivo effects of ICS on anti-viral immunity and virus clearance. The mouse model of RV1B infection allows us to investigate effects of ICS administration on innate and adaptive immune responses to RV in vivo and to assess disease-relevant features including airway inflammation and mucin production.
4.2 Aims and Hypotheses

4.2.1 Aims
To characterise the effects of FP on anti-viral immune responses to minor group RV infection:

i) To determine the effects of inhaled FP on innate immune responses to RV infection in a mouse model including type I and III IFNs, ISG induction and virus replication.

ii) To evaluate the effects of inhaled FP on adaptive immunity, specifically pulmonary CD4+ and CD8+ T cell responses and RV-specific serum IgG and neutralising antibody levels.

iii) To evaluate the effects of inhaled FP on human disease associated endpoints including airway inflammatory cytokine production, cellular airways inflammation and airway mucin levels.

4.2.2 Hypotheses

1) FP suppresses RV induction of type I and III IFNs and ISGs, leading to increased virus loads in vivo.

2) FP suppresses adaptive immunity to RV infection by inhibiting CD4+ and CD8+ T cell responses and impairs production of RV specific serum IgG and neutralising antibody.

3) Increased virus loads associated with FP administration leads to increased airway inflammation and increased mucin production following RV infection.
Chapter 4: Results

4.3 Results

In the initial data presented within this chapter, mice were treated as described in section 2.2.2.5. For clarity, the nomenclature used for the treatment groups in the FP plus RV model is summarised in table 4.1 below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fluticasone propionate</th>
<th>RV1B infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP + RV</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FP + UV</td>
<td>Yes</td>
<td>No (UV-RV1B)</td>
</tr>
<tr>
<td>Vehicle + RV</td>
<td>No (DMSO)</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle + UV</td>
<td>No (DMSO)</td>
<td>No (UV-RV1B)</td>
</tr>
</tbody>
</table>

Table 4.1: Treatment group nomenclature in FP and RV1B infection model

4.3.1 Determination of FP dosing strategy

The intranasal dosing route was chosen for administration of FP in our experimental model due to familiarity with this method and because it was reasoned that this would be a more efficient strategy for consistent dosing compared to nebulised drug administration. At the time of commencement of these experiments there were few published studies using intranasal administration of FP in mice and so advice was sought from an industrial collaborator who had expertise in mouse models of steroid administration in asthma. The dose of 1mg/kg FP used was chosen as a high dose that was expected to have effects on immune responses. Lower doses of FP were also assessed and are presented in section 4.3.8. In preliminary experiments, vehicle DMSO and PBS administration were compared for a range of inflammatory parameters including BAL differential cell counts and BAL chemokine and cytokine levels and no differences between these treatments were observed (data not shown). Therefore vehicle was used as the negative control for FP in all subsequent experiments shown.
4.3.2 Effect of FP administration on nuclear transcription factor activation

The pharmacological actions of ICS are mediated by binding of the drug to the GR in the cell cytoplasm, forming a complex that subsequently translocates to the nucleus to interact with GREs located in the promoter region of target genes.[223] To initially determine the efficacy and duration of action of intranasally administered FP, we assessed GR activation in lung tissue by measuring nuclear GR-DNA binding by ELISA. We found increased activated GR in the lung tissue of mice treated with FP compared to mice treated with vehicle DMSO at 8h post administration which returned to basal levels of vehicle treated mice by 24h post administration (fig 4.1a). For subsequent experiments, we therefore administered FP 1h prior to infection with RV1B since gene expression of a number of inflammatory mediators also peaks at 8h post-infection in the mouse model [367] and this dosing strategy would allow a direct assessment of the anti-inflammatory effects of FP at the most appropriate timepoint.

The immune response to RV infection involves an innate anti-viral response mediated by type I and III IFNs and regulation of IFN synthesis requires participation of several transcription factors including IRF-1, -3 and -7, AP-1 and NFkB, which are activated in response to virus—specific signals.[56, 57] Glucocorticoids are known to interact directly or indirectly with these transcription factors and thus alter the expression of pro-inflammatory genes.[371] We therefore used DNA binding ELISAs to study transcription factor activation in response to RV infection +/- FP administration. As previously reported [56], RV infection (vehicle + RV treatment) induced an increase in NFkB p65 activation at 8 and 24h post-infection which was suppressed at 8h post-infection in mice dosed with FP prior to infection with RV1B (FP + RV treatment) (fig 4.1b).

Activation of IRF-3 was initially assessed at the same time-points as assessed for GR and NFkB activation but we found no effect of RV infection on activation at 8 or 24h post-infection. We reasoned that IRF-3 activation may occur earlier and therefore subsequently evaluated activation at 2h post-infection. RV infection increased IRF-3 activation vs. UV-RV administration at 2h post-infection and FP administration suppressed this effect (fig 4.1 c).
Figure 4.1 Effect of FP administration on RV1B-induced nuclear transcription factor activation.
(a) C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO control intranasally and glucocorticoid receptor nuclear DNA binding in lung tissue was measured by ELISA. (b&c) 1h after FP or vehicle administration, mice were additionally challenged with RV1B or UV-inactivated RV1B and (b) NFκB p65 and (c) IRF-3 activation in lung tissue was assessed by measuring nuclear DNA binding by ELISA. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.01, ***p<0.001).

4.3.3 Effect of FP administration on RV1B-induced inflammation

4.3.3.1 BAL cellular inflammation
A key effect of ICS is their ability to reduce the number of inflammatory cells in the airways [372] but effects on virus-induced inflammation have not been extensively studied. We therefore evaluated the effect of FP on RV-induced cellular airways inflammation in BAL by assessment of cell counts on cytospins. RV infection led to increases in neutrophils at 8 and 24h, lymphocytes at d2 and 7 and macrophages at d2 post-challenge compared to UV-inactivated RV challenge. Administration of FP prior to RV infection led to suppressed numbers of BAL neutrophils (complete inhibition at 8h and ~60% reduction at 24h post-infection), lymphocytes (~80% reduction at d2 and ~65% reduction at d7 post-infection) and macrophages (~80% reduction at d2 post-infection) in comparison to mice treated with vehicle + RV (fig 4.2a-c).
Figure 4.2 Effect of FP administration on cellular airways inflammation.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. BAL was performed at the indicated timepoints. BAL cell cytospin slides were prepared as described and (a) neutrophils, (b) lymphocytes and (c) macrophages were differentially counted blind to experimental conditions. Data represent mean (+/− SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.01, ***p<0.001).
4.3.3.2 BAL chemokines

To investigate whether the effects of FP on cellular airways inflammation were associated with suppression of RV-induced chemokine expression, we measured BAL protein levels of neutrophil and lymphocyte chemokines by ELISA. RV1B infection led to increases in neutrophil chemokines CXCL2/MIP-2 at 8h and CXCL1/KC at 8 and 24h (fig 4.3a&b). RV infection also increased Th1 lymphocyte chemokines CXCL9/MIG (24h post-infection) and CCL5/RANTES (8 and 24h post-infection) and Th2 lymphocyte chemokines CCL22/MDC and CCL17/TARC (8 and 24h post-infection).

Mice treated with FP + RV infection had suppressed levels of neutrophil chemokines CXCL2/MIP2 (~70% reduction) and CXCL1/KC proteins (~60% reduction) in BAL at 8h post-infection (fig 4.3a&b) and suppressed levels of Th1 lymphocyte chemokines CXCL9/MIG (~65% reduction) and RANTES (~55% reduction) proteins in BAL at 24h post-infection (fig 4.3c&d) compared to treatment with vehicle + RV. Conversely, BAL protein levels of Th2 lymphocyte chemokine CCL17/TARC were no different at 8h and significantly increased in FP + RV vs. vehicle + RV treated mice at 24h post-infection with similar trends observed for CCL22/MDC (fig 4.3e&f).
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Figure 4.3 Effect of FP administration on RV1B-induced BAL chemokine production.
C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. BAL was performed at the indicated timepoints post-infection. Chemokines (a) CXCL2/MIP-2 (b) CXCL1/KC (c) CXCL9/MIG (d) CCL5/RANTES (e) CCL22/MDC and (f) CCL17/TARC in lavage supernatants were measured by ELISA. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, ***p<0.001).
4.3.3.3 BAL proinflammatory cytokines

We also assessed the effect of FP administration on RV induction of proinflammatory cytokines by ELISA. RV infection led to increased BAL protein levels of IL-6 at 8 and 24h post-infection and TNF-α at 8h post-infection (fig 4.4a&b). FP suppressed RV induction of IL-6 (~70% inhibition) and TNF-α (~85%) at 8h post-infection (fig 4.4a&b).

Figure 4.4 Effect of FP administration on RV1B-induced BAL proinflammatory cytokine production.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. BAL was performed at the indicated timepoints post-infection. Cytokines (a) IL-6 and (b) TNF-α in lavage supernatants were measured by ELISA. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (***p<0.001).

4.3.4 Effect of FP administration on innate anti-viral immune responses to RV1B infection

4.3.4.1 Type I and III interferons

*In vitro* studies have recently reported that ICS pre-treatment of fibroblasts and PBMCs can suppress the induction of types I and III IFNs by RV [246, 248] but these effects have not been demonstrated in vivo. We therefore assessed the effect of FP on innate anti-viral immune responses to RV infection in the mouse model. RV infection induced IFN-β and -λ mRNAs in lung tissue (8h post-infection), IFN-α and β protein in BAL (24h post-infection) and IFN-λ protein in BAL (8 and 24h post-infection) (fig 4.5a-e). FP suppressed RV induction of *IFN-β* (complete inhibition) and *−λ* (~40% reduction) mRNA expression in lung tissue at 8h post-infection (fig 4.5a&b). FP also almost completely inhibited RV-induced IFN-α and −β proteins in BAL at 24h post-infection and suppressed IFN-λ protein in BAL at 8 and 24h post-infection (~65% and ~40% inhibition respectively) (fig 4.5c-e).
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Figure 4.5 Effect of FP administration on type I and III IFN induction by RV1B infection.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. (a&b) Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue, cDNA generated as described and (a) IFN-β and (b) IFN-λ mRNA copies were assessed by Taqman quantitative PCR. (c-d) BAL was performed at the indicated timepoints post-infection and (c) IFN-α, (d) IFN-β and (e) IFN-λ protein in lavage supernatants were measured by ELISA. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.01, ***p<0.001).
4.3.4.2 Interferon-stimulated genes

Interferons act by inducing expression of ISGs which encode a range of proteins that selectively interfere with virus replication, protein synthesis or protein trafficking.[57, 373] RV infection increased lung tissue mRNA levels of the ISGs oligoadenylate synthetase (OAS), viperin, IL-15 and CXCL10/IP-10 protein in BAL at 8 and 24h post-infection (fig 4.6a-d). Consistent with the suppression of type I and III IFNs, FP also attenuated RV-induced lung tissue mRNA expression of OAS at 8h (near complete inhibition), viperin at 8h and 24h post-infection (~60 % and ~50% inhibition respectively) and IL-15 at 8h post-infection (~75% inhibition) (fig 4.6 a-c). FP also suppressed RV induction of CXCL10/IP10 protein in BAL at 8h post-infection (~90% inhibition) (fig 4.6d).

Figure 4.6 Effect of FP administration on RV1B induction of interferon stimulated genes.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. (a-c) Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue, cDNA generated as described and (a) OAS, (b) viperin and (c) IL-15 mRNA copies were assessed by Taqman quantitative PCR. (d) BAL was performed at the indicated timepoints post-infection. CXCL10/IP-10 protein in lavage supernatants was measured by ELISA. Data represent mean (+/− SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
4.3.4.3 Natural killer cells in lung tissue and BAL

NK cells also play an important role in the innate immune response against respiratory virus infections by directly eliminating virally infected cells.\[61, 69\] Type I IFN signalling has previously been shown to be required for NK cell responses to RV.\[56\] We therefore assessed NK cell populations in the lung and BAL by flow cytometry staining for the cell surface markers NK.1.1 and CD69. RV infection had no effect on total numbers of NK cells in lung but increased numbers of activated NK cells in lung (d2 post-infection) and numbers of total and activated NK cells in BAL (d2 and d7 post-infection)(fig 4.7a–d). There was no difference in total NK cell numbers in lung tissue between mice treated with FP + RV vs. vehicle + RV (fig 4.7a). Mice treated with FP + RV had suppressed numbers of activated NK cells in lung tissue (~60% reduction) and suppressed numbers of total and activated NK cells in BAL (~60% reduction) at d2 post-infection compared to mice treated with vehicle + RV (fig 4.7b–d).

Figure 4.7 Effect of FP on RV1B-induced natural killer cell response.
C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Lung tissue was harvested and BAL was performed at the indicated timepoints post-infection. Lung and BAL cells were stained for CD3 and the NK cell marker NK1.1 and the early activation marker CD69 (as indicated) and analysed by flow cytometry. (a) Lung CD3- NK1.1+ cell number, (b) Lung activated (CD69+) CD3- NK1.1+ cell number (c) BAL CD3- NK1.1+ cell number and (d) BAL activated (CD69+) CD3- NK1.1+ cell number. Data represent mean (+/− SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
4.3.5 Effect of FP administration on adaptive immune responses to RV1B infection

T cell and antibody responses may be important for both viral clearance and prevention of reinfection.[64, 71, 72] In addition to evaluating the effect of FP administration on innate immune responses to RV infection, we therefore also determined if there were any effects on adaptive immunity including T cell responses, virus-specific serum IgG and serum neutralising antibodies.

4.3.5.1 BAL and lung CD4+ T cells

Harvested BAL and lung cells were stained for T cell markers CD3 and CD4 and the activation marker CD69 and analysed by flow cytometry. RV infection had no effect on lung CD3+CD4+ cell numbers but increased total CD3+CD4+ cells in BAL and activated CD3+CD4+ cell numbers in lung and BAL at d2 and d7 post-infection (fig 4.8a-d). Total CD3+CD4+ cell numbers in lung tissue were no different between FP+RV treated and vehicle+RV treated mice (fig 4.8a). Decreased numbers of CD3+CD4+ cells in BAL at d2 and d7 post-infection (~45% and ~75% reduction) and decreased numbers of activated CD3+CD4+ cells in lung tissue (~70% reduction at d2 and d7 post-infection) and BAL (~50% reduction at d2 and ~75% reduction at d7 post-infection) were observed in mice treated with FP + RV compared to mice treated with vehicle + RV (fig 4.8b-d).
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Figure 4.8 Effect of FP on lung and BAL CD4+ T cell responses to RV1B infection.
C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Lung tissue was harvested and BAL was performed at the indicated timepoints post-infection. Lung and BAL cells were stained with antibodies specific for CD3, CD4 and CD69. (a) Lung CD3+ CD4+ T cell number and (b) BAL CD3+ CD4+ T cell number. (c) Lung activated (CD69+)CD3+ CD4+ T cell number and (d) BAL activated (CD69+)CD3+ CD4+ T cell number. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).

4.3.5.2 BAL and lung CD8+ T cells

We also measured the effect of FP administration on RV-induced CD8+ T cell responses. Harvested BAL or lung cells were stained for T cell markers CD3, CD8 and the activation marker CD69 and analysed by flow cytometry. RV infection had no effect on lung CD3+CD8+ cell numbers but increased activated CD3+CD8+ cell numbers in lung (d2 post-infection) and total and activated CD3+CD8+ cells in BAL (d2 and d7 post-infection) (fig 4.9 a-d).

As with CD4+ T cells, total numbers of CD8+ cells in lung tissue were no different between FP + RV treated and vehicle + RV treated mice (fig 4.9a). Decreased numbers of total CD8+ cells in BAL (~60% reduction) and decreased numbers of activated CD8+ cells in lung tissue and BAL (~70 and ~60% reduction respectively) were observed in mice treated with FP + RV compared to mice treated with vehicle + RV at d2 but not d7 post-infection (fig 4.9b-d).
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Figure 4.9 Effect of FP on lung and BAL CD8+ T cell responses to RV1B infection. C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Lung tissue was harvested and BAL was performed at the indicated timepoints post-infection. Lung and BAL cells were stained with antibodies specific for CD3, CD8 and CD69. (a) Lung CD3+CD8+ T cell number and (b) BAL CD3+CD8+ T cell number. (c) Lung activated (CD69+) CD3+CD8+ T cell number and (d) BAL activated (CD69+) CD3+CD8+ T cell number. Data represent mean (+/-SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).

4.3.5.3 RV1B-specific serum IgG and neutralising antibodies

Peripheral blood was collected on d14 post-infection and RV1B-specific IgG1 and IgG2a in serum were measured by ELISA. RV infection led to increased levels of RV1B-specific IgG1 and IgG2a compared to treatment with UV-RV1B. Sera from mice treated with FP + RV had reduced levels of RV1B-specific IgG1 and a non-significant trend towards reduced IgG2a compared to vehicle + RV treatment (fig 4.10a&b). We also assessed the effect of FP treatment on the ability of sera to neutralise RV infection of HeLa cells in vitro. Sera taken at d14 from mice infected with RV prevented any CPE to a dilution of at least 1 in 320 with no protection observed in sera from mice treated with UV-RV1B. Neutralisation of RV1B CPE was reduced in sera from mice treated with FP + RV with loss of protection observed at 1 in 40 dilution (fig 4.10c).
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Figure 4.10 Effect of FP on RV1B-specific serum IgG and neutralising antibody production.
C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. On d14 post-infection, peripheral blood was harvested and (a) RV-specific IgG1 and (b) RV-specific IgG2a in serum were quantified by ELISA. Data represent mean (+/SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.01, ***p<0.001; ns = non significant). (c) Sera were assayed for their ability to prevent cytopathic effect caused by the same RV serotype used for in vivo challenge. Cytopathic effect was quantified by crystal violet staining. Top dotted lines; serum only (uninfected) controls. Bottom dotted lines; virus infected (no serum) control. Data points represent sera pooled from 4 mice per treatment group from one representative experiment with findings reproduced in at least 2 independent experiments.

4.3.6 Effect of FP administration on virus loads

In our model, FP therefore suppressed IFN and ISG induction and adaptive cellular and humoral immune responses. Suppressed IFN has previously been shown to correlate with increased RV replication in asthma and COPD [35, 374] but the influence of ICS therapy has not been previously assessed in vivo. We therefore evaluated whether the effects of FP on suppression of anti-viral immune responses in the mouse model would have any impact on virus loads in vivo.
Measurement of RV RNA in lung tissue by Taqman quantitative PCR showed increased copy numbers in mice treated with FP + RV vs. vehicle + RV at 8h (~3 fold), 24h (~2 fold) and 48h (~3 fold) post-infection. There was no difference between RV RNA copies at 1h post-infection between FP + RV and vehicle + RV groups, suggesting that initial binding of RV to lung epithelial cells in the mouse model is not affected by FP (fig 4.11a). To further interrogate this finding, we also quantified rhinovirus load in lung tissue by titration of lung homogenate supernatants in HeLa cells and found increased titres in FP + RV compared to vehicle + RV treated mice (~ 0.5 log) at 24h post-infection (fig 4.11b).

Figure 4.11 Effect of FP on lung tissue virus loads.
C57BL/6 mice were treated with intranasal FP (1mg/kg) or vehicle DMSO and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Lung tissue was harvested at the indicated timepoints post-infection. (a) RNA was extracted from tissue, cDNA generated as described and RV RNA copies were assessed by Taqman quantitative PCR. (b) Lungs were homogenised and infectious virus in lung tissue homogenate supernatant was measured by titration in HeLa cells. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments. Statistical significance is indicated in (a) for FP + RV vs. vehicle + RV treatments only (*p<0.05, ***p<0.001, n.s not significant).

4.3.7 Effect of FP administration on RV1B-induced airway mucins
Mucus hypersecretion is a prominent feature of COPD and may contribute to airways obstruction. [18] Rhinoviruses have been shown to induce mucin expression in the mouse model of disease [367] and in vitro in epithelial cells in a replication dependent manner. [369] Studies evaluating the effect of glucocorticoids on mucin expression in vitro have reported conflicting results with some studies showing suppression of MUC5AC [306, 307], others showing no effect [309] and one study reporting increased IL-13 induced MUC5AC in response to dexamethasone administration in bronchial epithelial cells [311]. The effect of glucocorticoids on mucin expression in response to
RV infection has not been investigated previously. We therefore assessed lung tissue mRNA expression and BAL protein levels of the major airway mucins MUC5AC and MUC5B in the mouse model.

RV infection had no effect on MUC5AC mRNA but significantly increased MUC5B mRNA in lung tissue at d4 post-infection (fig 4.12a&b). BAL protein levels of MUC5AC at d7 post-infection and MUC5B protein at d1, 2, 7 and 14 post-infection were increased by RV infection (fig 4.12c&d). Lung tissue mRNA and BAL protein levels of MUC5AC were increased (~3 fold and 1.5 fold respectively) in mice dosed with FP + RV compared to vehicle + RV administration at d7 post-infection (fig 4.12a&c). There was no difference in lung tissue MUC5B mRNA expression between FP + RV vs. vehicle + RV administration (fig 4.12b). MUC5B protein in BAL was reduced (~75% suppression) at d1 but increased (~1.5 fold) at d14 post-infection in FP + RV treated mice compared to vehicle + RV treatment (fig 4.12d).

Figure 4.12 Effect of FP on RV1B-induced airway mucins.
C57BL/6 mice were treated with intranasal FP (1mg/kg) or vehicle DMSO and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. (a&b) RNA was extracted from harvested lung tissue, cDNA was generated as described and (a) MUC5AC and (b) MUC5B mRNA copies were assessed by Taqman quantitative PCR. (c&d) BAL was performed and (c) MUC5AC and (d) MUC5B proteins in lavage supernatants were measured by ELISA. Data represent mean (±/ SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments. *denotes statistical comparison for FP + RV vs. vehicle + RV treatments and †denotes comparison for vehicle + RV vs. vehicle + UV treatments (*p<0.05, ***p<0.001; †p<0.05, ††p<0.01, †††p<0.001).
4.3.8 Dose responsive effects of FP on innate anti-viral immune responses, virus loads and airway inflammation

The dose of FP (1mg/kg) used in experiments described above was based on that defined as optimal in asthma models by an industry collaborator. Although it is difficult to compare intranasal dosing in mice with inhaled dosing in patients with COPD, this dose is likely to be considerably higher than FP doses used in clinical practice which are typically around 1000μg per day (~0.014mg/kg for a 70kg human). We therefore evaluated the effects of lower 0.5mg/kg and 0.1mg/kg doses of FP on anti-viral immunity and airway inflammation to determine if similar effects are observed at these lower, perhaps more realistic, doses. Endpoint analysis was limited to 8h and d1 post-infection and therefore dose responsive effects of FP on parameters expressed later in the infection timecourse such as BAL lymphocytes and mucins were not assessed.

Mice dosed with 1mg/kg FP + RV and 0.5mg/kg FP + RV had significantly reduced BAL neutrophils (~50% suppression) at 24h post-infection compared to vehicle + RV treatment (fig 4.13a). There was no difference in BAL neutrophil numbers between mice dosed with 0.1mg/kg FP + RV and vehicle + RV (fig 4.13a). RV RNA copy numbers in lung tissue measured by Taqman quantitative PCR were increased with FP 1mg/kg + RV (~5 fold) and 0.5mg/kg FP + RV (~3 fold) administration at d1 post-infection in comparison with vehicle + RV administration but no difference was observed between 0.1mg/kg FP + RV and vehicle + RV administration (fig 4.13b). IFN-λ mRNA in lung tissue was suppressed in mice administered 1mg/kg FP + RV (~75% inhibition) compared to vehicle + RV at 8h post-infection with non-significant trends towards suppression observed in mice receiving 0.5mg/kg or 0.1mg/kg FP + RV (fig 4.13c). We also observed trends towards reduced IL-6 and CXCL10/IP-10 protein in mice treated with 1mg/kg or 0.5mg/kg FP + RV vs. vehicle + RV at 8h post-infection. These differences at 1mg/kg failed to reach statistical significance which contrasts the significant reduction observed with 1mg/kg FP + RV vs. vehicle + RV treatment shown in sections 4.3.3.2 and 4.3.4.2. This is likely to be due to the increased number of groups required for these experiments thus leading to multiple post-test comparisons, preventing significant differences from being observed.

In summary, effects of FP on virus loads and virus-induced airway inflammation appeared to be dose responsive with a 50% dose reduction (0.5mg/kg) showing similar effects to the highest dose (1mg/kg) of FP assessed. At 90% dose reduction (0.1mg/kg), impairment of anti-viral responses and anti-inflammatory effects of FP are lost. We were unable to identify a dose of FP where anti-inflammatory effects were retained but detrimental effects on anti-viral immune responses were absent.
Figure 4.13 Effect of FP dose on airway inflammation, virus loads and innate anti-viral immunity.

C57BL/6 mice were treated with FP at 1mg/kg, 0.5mg/kg, 0.1mg/kg or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Lung tissue was harvested and BAL was performed at 8 and 24h post-infection. RNA was extracted from tissue and cDNA generated as described. Cell cytospin slides were prepared as described and (a) neutrophil numbers in BAL at 24h post-infection were differentially counted blind to experimental conditions. (b) RV RNA at 24h and (c) IFN-λ mRNA at 8h post-infection were measured in lung tissue by Taqman quantitative PCR. (d) IL-6 and (e) CXCL10/IP10 protein were measured in lavage supernatants at 8h post-infection. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
4.3.9 Evaluation of the effects of recombinant IFN-β administration in the FP and RV1B infection model

The effects on virus loads by FP could be explained at least in part by suppression of type I and III IFN. To evaluate whether the effects of FP in suppressing innate immune responses and enhancing virus loads were causally related to suppression of IFN, we therefore administered recombinant IFN-β to FP treated mice. A dose of $10^4$ units of recombinant IFN-β was administered 1h after RV1B infection as previous experiments had shown that this dosing strategy caused upregulation of ISGs comparable to RV1B infection at 8h post-challenge (N.Glanville, unpublished data). This model is summarised in table 4.2.

<table>
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<th>Group</th>
<th>Fluticasone propionate</th>
<th>RV infection</th>
<th>IFN-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP + RV</td>
<td>Yes</td>
<td>Yes</td>
<td>No (PBS)</td>
</tr>
<tr>
<td>FP + UV</td>
<td>Yes</td>
<td>No (UV-RV1B)</td>
<td>No (PBS)</td>
</tr>
<tr>
<td>Vehicle + RV</td>
<td>No (DMSO)</td>
<td>Yes</td>
<td>No (PBS)</td>
</tr>
<tr>
<td>Vehicle + UV</td>
<td>No (DMSO)</td>
<td>No (UV-RV1B)</td>
<td>No (PBS)</td>
</tr>
<tr>
<td>FP + RV + IFN-β</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FP + UV + IFN-β</td>
<td>Yes</td>
<td>No (UV-RV1B)</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle + RV + IFN-β</td>
<td>No (DMSO)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Vehicle + UV + IFN-β</td>
<td>No (DMSO)</td>
<td>No (UV-RV1B)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 4.2: Treatment group nomenclature in FP and RV1B infection with recombinant IFN-β model
4.3.9.1 Interferons and interferon-stimulated genes

We initially evaluated whether administration of recombinant IFN-β led to reconstitution of FP suppressed ISG expression. Administration of recombinant IFN-β alone (vehicle + UV + IFN-β treatment) significantly induced lung tissue OAS mRNA and IP-10 BAL protein vs. vehicle + UV treatment at 8h post-administration (fig 4.14a&b) and led to a non-significant trend towards increased viperin mRNA in lung tissue at 8h post-administration (fig 4.14c). There was no effect of IFN-β administration on lung tissue mRNA expression or BAL protein levels of IFN-λ (fig 4.14d&e). Lung tissue mRNA expression of OAS was increased in mice receiving FP + RV in combination with IFN-β (FP + RV + IFN-β treatment) vs. FP + RV treatment, back to levels similar to those observed with vehicle + RV treatment (fig 4.14a) at 8h post-infection. BAL protein levels of CXCL10/IP-10 and IFN-λ were also increased in mice dosed with FP + RV + IFN-β compared to treatment with FP + RV at 8h post-infection with CXCL10/IP-10 levels restored to similar levels as those observed with vehicle + RV treatment but only partial restoration of IFN-λ was observed (fig 4.14b&e). Conversely, there were no significant differences in IFN-λ or viperin mRNA levels in lung tissue between FP + RV + IFN-β and FP + RV treated mice (fig 4.14c&d).
Figure 4.14 Effect of recombinant IFN-β administration on FP suppressed innate immune responses to RV1B infection.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Mice were additionally dosed intranasally with recombinant IFN-β 10^4 units 1h after RV infection. Lung tissue was harvested and BAL was performed at 8h post-infection. RNA was extracted from lung tissue and cDNA generated as described. (a) OAS mRNA copies were assessed by Taqman quantitative PCR. (b) CXCL10/IP-10 protein in lavage supernatants were measured by ELISA. (c) viperin and (d) IFN-λ mRNA copies were assessed by Taqman quantitative PCR. (e) IFN-λ protein in lavage supernatants was measured by ELISA. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
4.3.9.2 Airway inflammation

We also assessed whether administration of recombinant IFN-β had any effect on the (perhaps desirable) suppression of RV-induced airway inflammation by FP. There was no difference between BAL neutrophil (fig 4.15a) and lymphocyte numbers (fig 4.15b) and BAL IL-6 protein levels (fig 4.15c) between mice treated with FP + RV + IFN-β vs. treatment with FP + RV. BAL CXCL2/MIP2 protein levels were also no different between FP + RV and FP + RV + IFN-β treated mice but CXCL1/KC protein in BAL was suppressed (~60% reduction) in FP + RV + IFN-β vs. FP + RV treated mice at 8h post-infection. Therefore, recombinant IFNβ had either no effect or enhanced (in the case of CXCL1/KC) FP suppression of RV-induced inflammation.
Figure 4.15 Effect of recombinant IFN-β administration in combination with FP on RV1B-induced airway inflammation

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Mice were additionally dosed intranasally with recombinant IFN-β 10^4 units 1h after RV infection. BAL was performed and BAL cell cytospin slides were prepared as described and (a) neutrophils at 24h and (b) lymphocytes at 48h post-infection were differentially counted blind to experimental conditions. (c) IL-6 (d) CXCL2/MIP-2 and (e) CXCL1/KC protein in lavage supernatants were measured by ELISA at 8h post-infection. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (***p<0.01, **p<0.001).
4.3.9.3 Adaptive immune responses

We also carried out an assessment of whether administration of recombinant IFN-β would have any effect on FP suppression of adaptive immunity including total numbers of CD4+ and CD8+ T cells in BAL and neutralising antibody production in serum. Numbers of CD8+ T cells at d2 post-infection and CD4+ T cells at d7 post-infection were no different between FP + RV + IFN-β treated mice compared to FP + RV treated mice (fig 4.16a&b). Neutralising antibodies were suppressed to the same extent in FP + RV and FP + RV + IFN-β treated mice compared to mice receiving vehicle + RV at d14 post-infection (fig 4.16c). This suggests that suppression of IFN does not explain the observed effects of FP on impaired adaptive immune responses.

Figure 4.16 Effect of recombinant IFN-β administration in combination with FP on adaptive immune responses to RV.

C57BL/6 mice were treated with intranasal FP (1mg/kg) or vehicle DMSO and infected with RV1B or UV-inactivated RV1B, 1h following FP administration. Mice were additionally dosed intranasally with recombinant IFN-β 10^5 units 1h after RV infection. (a & b) BAL was performed at d2 and d7 post-infection. BAL cells were stained with antibodies specific for (a) CD3 and CD8 at d2 or (b) CD3 and CD4 at d7 post-infection. Data represent mean (+/- SEM) of 4 mice per group. (* p<0.05, **p<0.01, n.s non significant). (c) Peripheral blood was harvested at d14 post-infection. Sera were assayed for their ability to prevent cytopathic effect caused by the same RV serotype used for in vivo challenge. Cytopathic effect was quantified by crystal violet staining. Top dotted lines; serum only (uninfected) controls. Bottom dotted lines; virus infected (no serum) control. Data points represent sera pooled from 4 mice per treatment group from one representative experiment with findings reproduced in at least 2 independent experiments.
4.3.9.4 Virus loads

We next assessed whether reconstitution of FP suppressed innate responses by administration of recombinant IFN-β would reduce the increased virus loads previously observed with administration of FP + RV (as shown in section 4.3.6). Increased virus loads were again seen in mice treated with FP + RV compared to vehicle + RV at 24h post-infection. Administration of recombinant IFN-β in combination with FP + RV (FP + RV + IFN-β) led to significantly reduced virus loads compared to FP + RV administration reducing loads down to similar levels as those observed with vehicle + RV treatment (fig 4.17).

Figure 4.17 Effect of recombinant IFN-β administration in combination with FP on virus loads.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Mice were additionally dosed intranasally with recombinant IFN-β 10^4 units 1h after RV infection. Lung tissue was harvested at 24h post-infection. RNA was extracted from tissue and cDNA generated as described. RV RNA copies were assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.01, ***p<0.001).
4.3.9.5 Airway mucins

We next assessed whether recombinant IFN-β administration had any effect on the increase in RV induced BAL mucin proteins observed with FP administration at the time-points that increases had previously been observed. There was no difference between levels of BAL MUC5AC at d7 (fig 4.18a) and MUC5B at d14 post-infection (fig 4.18b) in mice treated with FP + RV + IFN-β compared to treatment with FP + RV. Therefore, administration of IFN-β did not reverse FP induced enhancement of mucin production.

Figure 4.18 Effect of recombinant IFN-β administration in combination with FP on airway mucins.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. Mice were additionally dosed intranasally with recombinant IFN-β 10⁴ units 1h after RV infection. BAL was performed at d7 and d14 post-infection. (a) MUC5AC and (b) MUC5B protein at d7 and d14 respectively were measured in lavage supernatants by ELISA. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, ns = non-significant).

4.3.10 Investigation into components of IFN signalling pathways affected by FP

It is known that the type 1 IFN response is bimodal with an initial release of IFN in response to virus detection and a secondary response where type I IFN acts in an autocrine fashion via IFNAR to stimulate further production of IFN and induction of ISGs.[57] We therefore sought to assess which components of virus recognition and/or IFNAR signalling pathways were disrupted by ICS.
4.3.10.1 Evaluation of effect of FP on virus sensing pathway mediated expression of IFN

In order to determine whether ICS acted upon the intracellular cytosolic MDA5 virus sensing pathway or cell surface/endosomal TLR3 sensing pathway, we assessed IFN-β promoter activity using a luciferase (reporter) gene expression assay. Since primary airway epithelial cells were unavailable for these experiments, BEAS2B cells were chosen as a representative airway epithelial cell line that has been previously used in similar published studies from our group involving IFN pathway agonists [52]. Cells were transfected with IFN-β promoter reporter constructs and then treated with either 5μg/ml TLR3 agonist (Poly(I:C)) or 250ng/ml MDA-5 agonist (transfected Poly(I:C)) in the presence or absence of FP. FP led to suppressed TLR-3 induced IFN-β promoter activity (fig 4.19a) but had no effect on MDA-5 induced IFN-β promoter activity (fig 4.19b).

Figure 4.19 Effect of FP on virus sensing pathway mediated IFN expression in vitro.
BEAS-2B cells were transfected with interferon-β promoter reporter constructs and then treated with FP 1nM or 10nM or medium control 24h later. Cells were then stimulated with (a) 5μg/ml TLR3 agonist (Poly(I:C)) or medium control, (b) 250 ng/ml MDA-5 agonist (transfected Poly(I:C)) or medium control, harvested at 24h and relative light units (RLU) were determined. Data represent mean (+/- SEM) comprising 3 independent experiments. (**p<0.01, ***p<0.001, n.s non significant).

4.3.10.2 Evaluation of effect of FP on IFNAR signalling

We also planned to use in vitro systems to assess whether FP acts on the secondary component of IFN signalling via IFNAR but time constraints prevented these experiments from being carried out.
However, evaluation of the relevant control groups from the *in vivo* recombinant IFN-β experiment allowed an analysis of effects of FP administration on exogenous IFN-β induced ISG expression.

Recombinant IFN-β administration led to increased lung tissue mRNA expression of ISGs *OAS*, *viperin* and *IFN-λ* and CXCL10/IP-10 protein in BAL at 8h post administration (fig4.20a-d). Administration of FP suppressed IFN-β induced *OAS* (~25% reduction) and *IFN-λ* mRNAs (near complete inhibition) in lung tissue at 8h but had no effect on *viperin* mRNA expression (fig 4.20a–c). FP administration also reduced IFN-β induced CXCL10/IP-10 BAL protein (~50% reduction) at 8h post-infection (fig 4.20d). These results therefore suggest that ICS also impair the secondary component of IFN signalling via IFNAR.

Figure 4.20 Effect of FP on recombinant IFN-β induced ISGs in mice.
C57BL/6 mice were treated with intranasal FP (1mg/kg) or vehicle DMSO and additionally with intranasal recombinant IFN-β 10^4 units, 2h following FP administration. (a-c) Lung tissue was harvested at the timepoints indicated post IFN-β administration. RNA was extracted from lung tissue and cDNA generated, as described. (a) *OAS*, (b) *IFN-λ* and (c) *viperin* mRNA copies were assessed by Taqman quantitative PCR. (d) BAL was performed at the indicated timepoints post-infection. CXCL10/IP-10 protein was measured in lavage supernatants by ELISA. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
4.4 Summary

Consistent with previous observations from *in vitro* studies, administration of FP to the airways prior to infection with RV1B in mice led to suppressed induction of innate anti-viral responses including type I and III IFNs and ISGs. This was associated with increased virus loads *in vivo*. Despite these potentially adverse effects on innate anti-viral immunity, virus-induced airway inflammation including BAL neutrophil and lymphocyte numbers were suppressed by FP. However, the expression of airway mucins was conversely increased at later timepoints in mice treated with FP + RV compared to treatment with vehicle + RV. Further adverse immune effects of FP were demonstrated by an impairment of adaptive immunity including reduced CD4+ and CD8+ T lymphocyte recruitment to the airways and reduced RV-specific IgG1, IgG2a and neutralising antibody production in serum.

Administration of recombinant IFN-β in combination with FP led to reconstitution of suppressed innate responses, upregulation of ISGs and reduced virus loads without having any effect on FP suppression of inflammation, thereby confirming a direct causal role for suppression of IFN-β by FP for the observed effect on virus loads. Mechanistic studies in airway epithelial cells and *in vivo* show that suppression of IFN by FP occurs through inhibition of both the initial virus sensing release of IFN via TLR-3 mediated pathways and the secondary component of IFN signalling via IFNAR.
Chapter 5: Results

5.1 Introduction

Patients with COPD are known to be at increased risk of developing pneumonia [125, 375] and a large body of clinical evidence now demonstrates that this risk may be further increased by the use of ICS. Randomised controlled trials have reported an increased incidence of pneumonia in patients treated with FP, either alone or in combination with LABAs.[217, 256-258] This association has been further demonstrated in case-control studies that have additionally suggested that the risk of pneumonia may be dose dependent.[222, 266, 267]

Culture based studies have indicated that patients with COPD are frequently colonised with potentially pathogenic microorganisms (PPM) including Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis.[376-380] Acquisition of new bacterial strains increases risk of exacerbation [40] but the pathobiology underlying development of bacterial pneumonia in COPD is unclear. New culture independent molecular techniques have recently revealed the presence of complex bacterial communities in the lower airways of healthy individuals, with evidence of alterations in COPD.[114-116, 381] Studies have also begun to characterise the upper and lower respiratory microbiome in specific pathogen free mice and have shown similar communities to those present in humans.[199, 355, 356]

COPD has been shown to be associated with a number of specific impairments of innate anti-bacterial host defence in the lungs including reduced expression of PRRs [161-163], impaired macrophage function [169, 170], impaired mucociliary clearance [180, 181] and deficiencies in some AMPs [183, 185, 186, 188]. Additionally, there is a large body of evidence demonstrating that respiratory virus infection can impair anti-bacterial immune responses via a number of mechanisms, thus predisposing to secondary bacterial infection.[191, 192, 208] In a human experimental model of COPD exacerbation, RV infection was shown to directly precipitate secondary bacterial respiratory infection when assessed by either microbiological culture or culture-independent techniques.[122, 190]

The precise molecular mechanisms underlying the increased risk of pneumonia associated with ICS use in COPD are unclear. Some in vitro and in vivo studies have reported that corticosteroids can alter a number of components of anti-bacterial host defence including downregulation of TLR4 [163], impairment of macrophage phagocytosis [280-282], suppression of anti-bacterial cytokines including IL-6[283, 287-289], TNF-α [288-291] and CXCL8/IL-8 [287, 288] and suppression of AMPs
such as LL37/cathelicidin [303], β-defensins [301, 302] and lactoferrin.[231, 293] Whether all or any of these effects contribute to the increased risk of pneumonia associated with ICS use is unclear. Studies that have previously evaluated the effects of ICS in mouse models of pneumonia have yielded conflicting results with some studies showing improved bacterial clearance [312, 313] and others showing impairment.[283, 303] Studies have also focussed on whether ICS have any effects on colonising bacteria in the lungs during clinical stability. Increased sputum bacterial loads have also been shown to correlate with ICS use in COPD during stable state.[315] Pragman et al observed segregation of communities according to use or non-use of ICS, thereby suggesting that ICS may lead to alterations in the airway microbiome.[120] Similarly, Huang et al reported a trend towards increased richness and diversity of microbial communities associated with ICS use in COPD.[316]

These studies all provide intriguing evidence that ICS use and respiratory virus infection could potentially combine to synergistically impair anti-bacterial host defence. This may lead to alteration of the respiratory microbiome in COPD, promoting the proliferation of existing PPMs and subsequent increased risk of bacterial pneumonia.

5.2 Aims and Hypotheses

5.2.1 Aims

i) To evaluate anti-bacterial innate responses in the mouse model of RV-induced COPD exacerbation.

ii) To characterise the lower respiratory microbiota in naïve mice.

iii) To evaluate the effects of RV1B infection on the lower respiratory microbiota in mice in order to determine whether RV1B infection leads to proliferation of existing PPMs and/or acquisition of new bacterial species.

iv) To evaluate the effects of inhaled FP on anti-bacterial host defence responses following RV infection and to assess the effect of FP on the lower respiratory microbiota, when administered alone and in combination with RV1B infection.
5.2.2 Hypotheses

1) Administration of a single dose of elastase to model COPD in mice suppresses RV induction of innate anti-bacterial host responses, including PRR expression and production of AMPs.

2) The lower respiratory microbiome in naïve mice consists of PPMs that are relevant to human COPD disease including Streptococcus pneumoniae and Haemophilus influenzae.

3) RV1B infection in mice results in disturbance of the respiratory microbiome leading to proliferation of colonising PPMs in the airways.

4) FP administration prior to RV1B infection suppresses production of anti-bacterial host defence mediators. This further exaggerates disturbances of the respiratory microbiome leading to increased risk of bacterial pneumonia.
5.3 Results

5.3.1 Evaluation of innate anti-bacterial immune responses in the RV-induced COPD exacerbation model

Although we were unable to define a model of COPD RV exacerbation that accurately represented the impaired virus control reported in human disease, we showed in chapter 3 that single dose elastase administration was the most representative model of enhanced inflammation tested. We therefore evaluated whether this model displayed any of the features of impaired anti-bacterial host-defence that have been reported in human studies. We have already shown that mucins were increased in the model (chapter 3, section 3.3.4.4) and this would represent one feature that could be associated with impaired anti-bacterial function. We also evaluated a number of other features in the model including expression of PRRs and AMPs.

Elastase administration alone (elastase + UV vs. PBS + UV groups) was not associated with any changes in TLR2 or TLR4 mRNA expression in lung tissue (fig 5.1a-b) and therefore did not mimic findings from human studies which have reported reduced expression in patients with stable COPD.[161, 163] RV infection alone increased TLR2 but not TLR4 mRNA expression in lung tissue at d1 post challenge (fig 5.1a&amp;b). The combination of elastase and RV infection suppressed RV-induced TLR2 mRNA (~75% inhibition) but increased lung tissue TLR4 mRNA at d1 post challenge compared to either treatment alone. Elastase + RV treated mice had increased TLR4 mRNA vs. elastase + UV treated mice (~60%) but there was no difference in TLR2 mRNA between these groups (fig 5.1a-b).

A commercially available ELISA duoset for pentraxin-3 enabled measurement of protein levels of this AMP in BAL. Although similar assays were not available for surfactant protein-A, SLPI or β-defensin 2, we were able to measure mRNA expression of these AMPs in lung tissue by Taqman quantitative PCR. Elastase administration did not lead to any changes in pentraxin-3 protein in BAL or lung tissue mRNA expression of surfactant protein-A, SLPI or β-defensin-2 (comparison of elastase + UV vs. PBS + UV groups) (fig 5.1c-f) and therefore the baseline model of COPD did not show any deficiencies in expression of any of the AMPs measured. RV infection increased pentraxin-3 BAL protein at d1 and 4 post-challenge but had no effect on surfactant protein-A, SLPI or β-defensin 2 mRNAs in lung tissue. Elastase administration led to increased (~65%) RV induction of pentraxin-3 BAL and lung tissue mRNA expression of surfactant protein-A (~2.5 fold increase) and β-defensin 2 (~3 fold increase) at d1 post challenge but had no effect on RV induction of SLPI mRNA (fig 5.1c-f). Pentraxin-3 BAL protein and surfactant protein-A lung tissue mRNA were also increased in elastase + RV vs. elastase + UV treated mice at d1 post challenge (fig 5.1c&amp;e). Therefore, with the exception of RV induced TLR2...
expression, the single dose elastase was a model of enhanced or unchanged rather than impaired anti-bacterial host defence.

Figure 5.1 Effect of single dose elastase administration on RV1B induction of anti-bacterial host-defence responses.
C57BL/6 mice were challenged with elastase and additionally with RV1B or UV-inactivated RV1B, 10d later. (a-b & d-f) Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated, as described. (c) BAL was performed at the indicated timepoints post-infection. (a) TLR2 and (b) TLR4 mRNA expression was measured in lung tissue by Taqman quantitative PCR. (c) pentraxin-3 protein in lavage supernatants were measured by ELISA. (d) SLPI (e) surfactant protein-A and (f) β-defensin 2 mRNA copies were measured in lung tissue by Taqman quantitative PCR. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
5.3.2 Evaluation of effect of FP administration on RV induction of anti-bacterial immune responses

Previously, in chapter 4, we showed that FP suppresses RV1B induction of IL-6, TNF-α, type I IFNs and neutrophil recruitment to the airways, which are all components of anti-bacterial host defence that may theoretically alter the course of secondary bacterial infection following initial virus exacerbation.

We also measured similar additional components of anti-bacterial host defence in the FP and RV1B infection model as those measured in the single dose elastase model of COPD. RV infection induced mRNA expression of TLR2 and SLPI in lung tissue and increased pentraxin-3 protein in BAL (fig 5.2a,c&d). Treatment with FP completely inhibited RV-induced TLR2 mRNA expression at d1 post-infection but had no effect on TLR4 expression in lung tissue (fig 5.2a-b). FP administration also suppressed RV induction of pentraxin 3 protein at d1 (~70% inhibition) and d4 (~80% inhibition) post-infection but increased surfactant protein-A mRNA expression in lung tissue (~2.5 fold) at d4 post-infection (fig5.2c&e). FP administration had no significant effect on RV induction of SLPI, or β-defensin 2 mRNAs in lung tissue (fig 5.2d&f).
Figure 5.2 Effect of FP administration on RV1B induction of anti-bacterial host-defence responses. C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO intranasally and challenged with RV1B or UV-inactivated RV1B, 1h following FP administration. (a-b & d-f) Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (c) BAL was performed at the indicated timepoints post-infection. (a) TLR2 and (b) TLR4 mRNA expression was measured in lung tissue by Taqman quantitative PCR. (c) Pentraxin-3 protein in lavage supernatants were measured by ELISA. (d) SLPI (e) surfactant protein-A and (f) β-defensin 2 mRNA copies were measured in lung tissue by Taqman quantitative PCR. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).
5.3.3 Evaluation of the naïve mouse airway microbiota by 16S pyrosequencing

Previous studies have assessed the airway microbiome in upper and lower respiratory tract samples taken from human subjects by 16S rRNA pyrosequencing using the Roche 454 platform.[115, 116, 122] We therefore initially used this technique to evaluate whether the microbiome of the mouse airway could be measured in BAL from naïve wild-type mice.

Total DNA was extracted and the V3-V5 portion (550 base pairs (bp)) of the bacterial 16S rRNA gene was amplified, as previously described.[122] PCR products were separated on a 1% agarose gel in order to confirm successful amplification and presence of a measurable microbiome in these samples (fig 5.3). Although not quantitative, the presence of bands of reasonable intensity, and based on prior experience of the group, suggested that the bacterial DNA content would be sufficient to allow progression to amplicon sequencing for some of the samples.

Figure 5.3 Gel electrophoresis of 16S PCR products from naïve mouse BAL samples using standard sampling technique.
BAL was performed in naïve, wild type C57BL/6 mice. Total DNA was extracted from BAL samples, as described. Bacterial 16S rRNA gene was amplified by PCR using primers specific to the V3–V5 region. PCR products of DNA from individual BAL samples were separated on a 1% agarose gel. Arrows 1 and 2 denote representative experimental samples. Arrow 3 denotes positive control. Arrow 4 denotes negative no template control.

Quadruplicate 25µL PCRs were then set up, amplified and combined, purified and prepared for sequencing (as described in chapter 2, section 2.2.8). Pyrosequencing was carried out and 16S rRNA sequences were assigned a taxonomic identity with the ribosomal database project classifier (as described in chapter 2, section 2.2.8.6). A phylogenetic tree of the OTU sequences was constructed and a representative heat map of bacterial 16S rRNA sequences derived from mouse BAL samples is shown in fig 5.4. At the genus level, the microbiota in all samples was dominated by Herbaspirillum. This bacterial genus is a nitrogen-fixing Proteobacteria that is typically found in plants [382] and has not been described in previous studies that have evaluated the airway microbiome in either human
or mouse airway samples.[116, 122, 355, 356] It is therefore extremely unlikely to be a normal dominant component of the resident mammalian airway microbiome. We reasoned that presence of this bacterial genus represented an environmental contaminant that had been introduced most likely during the BAL sampling process or possibly at a later stage during DNA extraction or amplification.

**Figure 5.4 Heat map of 16S bacterial rRNA sequences from naïve mouse BAL samples.**

BAL was performed in untreated C57BL/6 mice. DNA was extracted and V3-V5 component of bacterial 16S gene was amplified by PCR. Quadruplicate 25µL PCR products were combined, purified and sequenced using the Roche 454 Junior pyrosequencer. A representative group of individual samples taken from naïve mice is shown, organized by taxonomy with abundance indicated by colour (see figure key). Columns represent individual samples with numerical sequence reads for each particular genus shown.

Given that BAL taken by standard sampling techniques was likely to have been the source of the environmental contaminant, we next proceeded to perform BAL by a more sterile technique. To minimize the risk of contamination, autoclaved BAL tubing and sterile instruments were used (as described in chapter 2, section 2.2.3.1). A similar technique was described in a recent study that reported successful characterization of the mouse airway microbiota in BAL samples.[355] Total DNA was extracted from these more optimally collected samples and PCR of the 16S rRNA gene was again carried out. As before, the obtained PCR product was separated on a 1% agarose gel, but only faint bands at ~550bp size were visible, suggesting a very low yield of 16S rRNA (data not shown). Formal quantification of PCR product using a Quant-iT PicoGreen dsDNA assay kit (as described in chapter 2, section 2.2.8.4) further confirmed a very low bacterial DNA yield with all samples containing levels below the threshold of 2.5ng/µL that is the minimal requirement for successful 16S pyrosequencing.
5.3.4 Quantitative PCR to evaluate 16S bacterial load in the lower respiratory tract of naïve mice

5.3.4.1 Bronchoalveolar lavage
Subsequent establishment of a 16S quantitative PCR assay (as described in chapter 2, section 2.2.7.4) enabled evaluation and comparison of overall bacterial loads in BAL samples taken from naïve mice via the modified sterile BAL sampling and normal BAL sampling methods. The mean 16S copy numbers in samples from sterile BAL technique was ~2 logs lower than samples that had been obtained with standard BAL sampling (mean 6.36 x 10^3 copies vs. 3.49 x 10^5 copies) and ~3 logs lower than a positive control of human sputum samples that had been previously successfully sequenced using the Roche 454 pyrosequencing platform (mean 5.02 x 10^6 copies) (fig 5.5). Although the minimum threshold of qPCR copy numbers that is sufficient to allow sequencing is currently unclear, these data further suggested that BAL samples obtained by sterile sampling techniques would not have a high enough 16S bacterial DNA content to enable sequencing.

Previous studies have reported that pooling BAL samples from multiple mice may increase the bacterial yield and allow 16S rRNA sequencing.[383] We therefore evaluated whether pooled BAL samples taken from four separate naïve mice would give higher 16S copy numbers but found similarly low levels of 16S rRNA copies in a single BAL sample compared to four pooled BAL samples (mean 6.36 x 10^3 copies for single BAL sample vs. 4.90 x 10^3 copies for four pooled BAL samples) (fig 5.5).

5.3.4.2 Nasal lavage
A recent study has reported successful characterisation of the microbiome in nasal lavage samples taken from mice.[356] We therefore assessed bacterial 16S copy numbers in nasal lavage taken from untreated mice using a sterile sampling technique. Once more, we found low yields of a similar level to BAL performed by sterile technique (mean 3.34 x 10^3 copies)(fig 5.5).
Figure 5.5 Evaluation of 16S rRNA gene copy number in mouse BAL and nasal lavage.

BAL or nasal lavage was performed in untreated C57BL/6 mice. DNA was extracted, as described and 16S rRNA gene copy numbers in 1µL of extracted DNA were measured by SYBR green quantitative PCR. 16S rRNA copy numbers were evaluated in BAL taken by normal sampling method, sterile sampling method (single sample or four pooled samples assessed) and nasal lavage by a sterile sampling method. Positive control of human sputum sample with known high bacterial DNA content and negative control of extracted DNA from fluid which had been passed through sampling tube but not been used to lavage lungs are also shown. Individual data points for single samples, or pooled samples where indicated and mean for group shown.

5.3.4.3 Lung tissue

Given the relatively low 16S qPCR copy numbers observed in BAL and nasal lavage samples, we next proceeded to measure bacterial loads in lung tissue taken from untreated mice, again using a sterile sampling technique to minimize contamination risk. We evaluated 16S rRNA copy numbers in different quantities of tissue including single lobe (right upper), single lung (left) and both lungs combined. 16S rRNA copy numbers were similar whether DNA was extracted from a single lobe, single whole lung or two whole lungs (mean $2.31 \times 10^5$, $1.64 \times 10^5$ and $1.20 \times 10^5$ copies for single lobe, single whole lung and two whole lungs respectively) (fig 5.6). This suggested that a single lung lobe provided sufficient DNA to reach the maximum amount that could be extracted via the FastPrep lysing matrix that is used to extract bacterial DNA. 16S rRNA copy numbers in lung tissue
were around 2 logs higher than copy numbers in BAL samples obtained by sterile sampling techniques. This suggested that use of lung tissue samples to characterize the respiratory microbiome by 16S pyrosequencing may be more feasible than use of BAL samples.

Figure 5.6: Evaluation of 16S rRNA gene copy number in mouse lung tissue samples.
16S rRNA copy numbers in lung tissue harvested from untreated wild-type C57BL/6 mice were measured in 1µL of extracted DNA by SYBR green quantitative PCR in varying quantities of lung tissue including single apical lung lobe, single whole lung and two whole lungs. Positive control of human sputum sample with known presence of high bacterial DNA content and negative control of extracted DNA from fluid which had been passed through sampling tube but not been used to lavage lungs are also shown. Individual data point for each sample and mean for group shown.

5.3.5 Evaluation of quantitative 16S rRNA copy numbers in mice treated with FP and/or RV1B
Given that quantitative PCR of extracted DNA samples from lung tissue of naïve mice showed relatively high copy numbers of 16S rRNA gene, we next used this technique to evaluate whether FP administration and/or RV1B infection in mice had any effects on total bacterial loads in the lungs. Time course analysis showed no effect of RV infection and/or FP administration on 16S rRNA copies in lung tissue at d1, 4 or 7 post-infection when compared to vehicle + UV treatment (fig 5.7). However, at 8h post-infection increased 16S rRNA copies (~4 fold) were observed in mice treated with FP + RV or FP + UV vs. vehicle + RV and increased 16S rRNA copies (~2 fold) were also observed with FP + RV vs. vehicle + UV treatment, suggesting that FP administration may transiently increase
bacterial loads in the lung, regardless of RV infection. Furthermore, 16S rRNA copy numbers were ~2 fold lower in mice treated with vehicle + RV compared to treatment with vehicle + UV (fig 5.7) suggesting that RV infection may transiently reduce bacterial loads in the lung.

Figure 5.7 Effect of FP and RV1B infection on 16S rRNA bacterial copy number in lung tissue. C57BL/6 mice were treated with FP 1mg/kg or vehicle DMSO control intranasally and challenged with RV1B or UV-inactivated RV1B, 1h after FP administration. Lung tissue was harvested at the indicated timepoints post-infection and DNA was extracted, as described. 16S rRNA copy numbers were assessed by SYBR green quantitative PCR. Data represent mean (+/- SEM) of 4 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01, ***p<0.001).

5.3.6 Exogenous administration of S.pneumoniae in mice to model bacterial colonisation in COPD

Since difficulties were experienced with attempts to undertake 16S pyrosequencing in lower respiratory tract samples from mice and following further delays due to a requirement for a change to use of the Illumina MiSeq sequencer at the Brompton Molecular Genetics and Genomics laboratory because of an unexpected discontinuation of the the Roche Junior 454 sequencing platform, the decision was made to establish a mouse model of low dose bacterial infection in order to directly model lower respiratory tract colonisation in COPD. *Streptococcus pneumoniae*, a PPM that is frequently cultured from sputum of patients with COPD in stable state [376, 378] and the most commonly implicated pathogen causing pneumonia in patients with COPD was the bacteria chosen for the model.[132] We aimed to use this model to assess the effects of FP on anti-bacterial
host defence and thus test the hypothesis that ICS use promotes proliferation of potentially pathogenic colonising bacteria and increases pneumonia risk in COPD.

*Streptococcus pneumoniae* capsular serotype 2 strain (D39) was used for all studies as frozen stocks of known concentration were available from previous *in vitro* work carried out the St Mary’s Airway Disease Infection group. This strain has previously been used in published mouse models of pneumococcal pneumonia.[384, 385]

5.3.6.1 Evaluation of varying infecting doses of *S.pneumoniae* on bacterial loads in lung tissue, systemic dissemination and survival

Colonisation is defined as presence of a pathogen in the airways without acute symptoms of infection.[98] We therefore sought to define a model of bacterial infection in which bacteria could be cultured from harvested lung tissue samples but only a mild inflammatory response was elicited *in vivo*.

We initially conducted a series of optimization experiments with the aim of defining a suitable infecting dose of *S.pneumoniae* to be used. Previous mouse models of acute pneumonia induced by *S.pneumoniae* D39 administration have used doses up to $2 \times 10^8$ CFU.[386] We therefore assessed a range of reducing doses from $1 \times 10^6$ CFU to define the optimum lowest dose for modelling bacterial colonisation. A dose dependent increase in bacterial CFU recovered from lung tissue harvested at 24h post-infection was observed up to $5 \times 10^5$ CFU infecting dose with no increase in recovery observed above this dose (fig 5.8). Very low bacteria loads were cultured from tissue of mice infected with the lowest dose evaluated $1 \times 10^5$ CFU (mean 176.7 CFU /mL recovered), thereby suggesting that this infecting dose was too low for reliable recovery of bacteria in lung tissue (fig 5.8).
Figure 5.8 Assessment of lung tissue bacterial loads following infection with varying doses of *S. pneumoniae*. C57BL/6 mice were challenged with *S. pneumoniae* D39 at doses of $1 \times 10^6$, $7.5 \times 10^5$, $5 \times 10^5$, $2.5 \times 10^5$, $1 \times 10^5$ CFU or PBS control. Lung tissue was harvested at 24h post-infection, homogenized, serially diluted, plated on columbia horse blood agar plates and incubated at 37$^\circ$C for 18h. Counts were performed on plates containing 50-300 colonies and CFU/mL calculated. Individual data points for each animal within group and mean for group shown. n= 5-6 mice per treatment group. Single experiment only.

Previous studies using *S. pneumoniae* D39 in mouse models of pneumonia have shown varying survival with one study reporting that administration of $2 \times 10^5$ CFU led to 50% mortality at d4 post-infection [384] and another which used the same dose reporting 0% mortality at the same timepoint [386]. In the mouse model of *S. pneumoniae*, mortality is caused by systemic dissemination of bacteria into the bloodstream leading to septicaemia.[352] We therefore assessed bacterial loads in the bloodstream and survival for a range of *S. pneumoniae* infecting doses. Systemic bacterial dissemination, defined by the presence of culturable *S. pneumoniae* from blood samples was present at 24 and 48h in at least some animals within the group at all infecting doses of *S. pneumoniae* assessed, with the exception of the lowest dose of $1 \times 10^5$ CFU. By 48h post-infection, bloodstream dissemination was present in 37.5% of mice infected with $2.5 \times 10^5$ CFU and 100% of mice infected with $1 \times 10^5$, thereby suggesting a dose dependent increase in the proportion of mice within a group that developed bacterial dissemination (fig 5.9a). Assessment of survival showed 0% mortality at d1
post-infection for all doses of *S.pneumoniae* tested. The highest mortality rates were actually observed with $7.5 \times 10^5$ CFU (50% at d2), the second highest evaluated dose, followed by $1 \times 10^6$ CFU (25% at d2), $5 \times 10^5$ CFU (12.5% at d2) and $2.5 \times 10^5$ CFU (6.25% at d2). One hundred percent of mice treated with $1 \times 10^5$ CFU dose survived to d3 (fig 5.9b). Weight loss at d2 post infection was greatest in mice infected with $7.5 \times 10^5$ CFU (9.0% reduction from baseline weight), followed by $5 \times 10^5$ CFU (3.4% reduction) and $1 \times 10^6$ CFU (2.4% reduction) with no weight loss observed in mice infected with $2.5 \times 10^5$ CFU, $1 \times 10^5$ CFU or PBS control (fig 5.9c).

**Figure 5.9** Assessment of bacterial loads in bloodstream, survival and weight loss following infection with varying doses of *S.pneumoniae*.

CS7BL/6 mice were challenged with *S.pneumoniae* D39 at doses of $1 \times 10^6$ CFU, $7.5 \times 10^5$ CFU, $5 \times 10^5$ CFU, $2.5 \times 10^5$ CFU and $1 \times 10^5$ or PBS control. (a) Blood samples were harvested by cardiac puncture at 24 and 48h post-infection, serially diluted, plated onto columbia horse blood agar plates and incubated at 37°C for 18h. Counts were performed on plates containing 50-300 colonies and CFU/mL calculated. Individual data points for each animal within group and mean for group shown. (b) Survival curves according to infecting dose of *S.pneumoniae*. Mice were killed when they exhibited signs of severe disease from which recovery was unlikely, as previously described.[387] (c) Mice were weighed daily and % change from pre-infection weight calculated. n=4-6 mice per treatment group. Single experiment only.
5.3.6.2 Evaluation of effect of *S.pneumoniae* dose on airway inflammation

Assessment of cellular airways inflammation at 24h post-infection showed no neutrophilia associated with administration of $1 \times 10^5$ CFU infecting dose but a dose dependent increase in neutrophilia with increasing doses up to $1x 10^6$ CFU (fig 5.10a). Measurement of BAL levels of the anti-bacterial pro-inflammatory cytokine IL-6 showed significantly increased levels in mice treated with $1x 10^6$ CFU *S.pneumoniae* compared to PBS dosed controls and a non-significant trends towards increases with lower infecting doses vs. PBS treatment (fig 5.10b).

Figure 5.10 Assessment of airway inflammation following infection with varying doses of *S. pneumoniae*. C57BL/6 mice were challenged with *S.pneumoniae* D39 at doses of $1 \times 10^6$ CFU, $7.5 \times 10^5$CFU, $5 \times 10^5$ CFU, $2.5 \times 10^5$ CFU and $1 \times 10^5$ or PBS control. BAL was performed at 24h post-infection. Cytospin slides were prepared as described. (a) Neutrophils were differentially counted blind to experimental conditions. (b) IL-6 protein was measured in lavage supernatants by ELISA. Data represent mean (+/- SEM) of 5-6 mice per treatment group (*p<0.05, **p<0.01, ***p<0.001). Single experiment only.

The dose optimisation experiments described above highlighted the difficulty of modelling bacterial colonisation in mouse models using *S.pneumoniae* D39. We observed a narrow threshold between rapid bacterial clearance and minimal inflammation associated with use of low doses such as $1 \times 10^5$ CFU, but evidence of increasing systemic dissemination resulting in mortality observed with doses of $2.5 \times 10^5$ CFU and higher. We considered $1 \times 10^5$CFU to be too low a dose to be used as the lack of a measurable inflammatory response to instilled bacteria observed would prevent assessment of the hypothesised suppressive effects of steroids on anti-bacterial host defence. Consequently, we chose $2.5 \times 10^5$ CFU as the infecting dose of *S.pneumoniae* to be used in further experiments assessing the effect of FP on anti-bacterial immunity and bacterial clearance. This dose led to relatively high
numbers of culturable bacteria in lung tissue at 24h post-infection as well as inducing a mild inflammatory response that could potentially be modulated by ICS administration. We also decided to limit assessment of endpoints to 24h post-infection as the latest timepoint evaluated, since beyond this timepoint a proportion of mice, at all doses assessed (except 1 x 10⁵ CFU), developed systemic dissemination leading to mortality. We therefore reasoned that administration of FP may impair anti-bacterial immunity and therefore potentially lead to even higher mortality.

5.3.7 Effect of FP on low dose *S.pneumoniae* infection

5.3.7.1 Bacterial loads in lung tissue
The effects of intranasal FP (1mg/kg) administration prior to infection with *S.pneumoniae* 2.5 x 10⁵ CFU on lung bacterial loads were assessed by culture of homogenized lung tissue. Mice treated with FP prior to *S.pneumoniae* infection (FP + SP treatment) had increased bacterial loads in lung tissue (~1 log difference) at 8h post-infection compared to treatment with vehicle DMSO prior to *S.pneumoniae* (vehicle + SP). No significant differences in lung tissue bacterial loads were observed between FP + SP and vehicle + SP treatment at 4h or 24h post-infection, although there was a trend towards increased bacterial loads in FP + SP treated mice observed at both time-points (fig 5.11).
Figure 5.11 Effect of FP on lung tissue bacterial loads following *S. pneumoniae* infection. C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO control intranasally and additionally challenged with *S. pneumoniae* D39 2.5 x 10^5 CFU or PBS control, 1h following FP administration. Lung tissue was harvested at the indicated timepoints, homogenized, serially diluted, plated on columbia horse blood agar plates and incubated at 37°C for 18h. Counts were performed on plates containing 50-300 colonies and CFU/mL calculated. Individual data points for each animal within group and mean for group shown. n= 12 mice per treatment group, comprising 2 independent experiments (*** p<0.001, n.s non significant).

### 5.3.7.2 Bacterial dissemination

To assess whether the increased lung tissue bacterial loads associated with FP treatment led to increased systemic bacterial dissemination of *S. pneumoniae*, we measured bacterial loads in blood samples taken by cardiac puncture. There were no significant differences in numbers of CFUs in blood taken from mice treated with FP + SP compared to vehicle + SP at any time-point post-infection, although FP + SP treatment was associated with a trend towards increased bacterial loads in blood vs. vehicle + SP at 24h post-infection (fig 5.12).
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Figure 5.12 Effect of FP on blood bacterial loads following *S. pneumoniae* infection.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO control intranasally and additionally challenged with *S.pneumoniae* D39 2.5 x 10⁵ CFU or PBS control, 1h following FP administration. Blood was harvested by cardiac puncture at the indicated timepoints, serially diluted, plated on columbia horse blood agar plates and incubated at 37°C for 18h. Counts were performed on plates containing 50-300 colonies and CFU/mL calculated. Individual data points for each animal within group and mean for group shown. n= 12 mice per treatment group, comprising 2 independent experiments (n.s non significant).

5.3.7.3 Pattern recognition receptor expression

To identify a mechanism for the increased total bacterial loads measured by 16S qPCR and increased *S.pneumoniae* loads associated with FP administration, we next assessed effects of FP on components of anti-bacterial innate immunity following *S.pneumoniae* infection. The initial recognition of invading pathogens by the host is mediated by PRRs that are activated by PAMPs. Activation of PRRs triggers a signalling cascade that culminates in production of inflammatory mediators.[138] We therefore assessed lung tissue mRNA expression of PRRs TLR2 and TLR4 (which recognise pneumococcal wall component lipoteichoic acid and virulence factor pneumolysin respectively) that have previously been shown to be important in pneumococcal infection.[140, 388, 389] Previous studies in human patients with COPD have shown reduced lung epithelial expression of TLR4 associated with FP treatment and in vitro studies have shown that lung epithelial cell expression of TLR2 is upregulated by dexamethasone administration.[163, 278] In our model, vehicle + SP infection significantly increased TLR2 mRNA expression in lung tissue at 4h post-infection vs.
vehicle + PBS (~3 fold increase). This effect was almost completely inhibited in mice treated with FP + SP (fig 5.13a).

There was no effect of either *S. pneumoniae* or FP, alone or in combination, on *TLR4* mRNA in lung tissue (fig 5.13b).

**Figure 5.13 Effect of FP on pattern recognition receptor expression following *S. pneumoniae* infection.**

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO control intranasally and additionally challenged with *S. pneumoniae* D39 2.5 x 10^5 CFU or PBS control, 1h following FP administration. Lung tissue was harvested at the indicated timepoints. RNA was extracted from tissue and cDNA generated as described. (a) *TLR2* and (b) *TLR4* mRNA expression was assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 6 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, **p<0.01).

### 5.3.7.4 Cellular airway inflammation

Previously, in chapter 4, we showed that FP suppresses induction of several chemokines and proinflammatory cytokines following rhinovirus infection. We therefore assessed the effect of FP on induction of inflammatory parameters by *S. pneumoniae* to determine whether inflammatory responses to bacteria were also impaired by FP.

SP infection led to significantly increased BAL neutrophils compared to vehicle + PBS from 8h and peaking at 24h post challenge. FP treatment suppressed BAL neutrophil number at 8h (complete inhibition) and 24h (~65% suppression) post SP infection (fig 5.14a). SP infection also increased BAL macrophages compared to vehicle + PBS at 4h, 8h and 24h post challenge. FP treatment completely suppressed BAL macrophage numbers at 8h post challenge (fig 5.14b). SP infection did not increase BAL lymphocyte numbers at 4, 8 or 24h post-infection (data not show).
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5.3.7.5 Anti-bacterial chemokines and cytokines

Investigation of antibacterial pro-inflammatory cytokines IL-6 and TNF-α (fig 5.15a&b) and neutrophil chemokines CXCL2/MIP-2 and CXCL1/KC (fig 5.15c&d) revealed them all to be increased in mice treated with vehicle + SP vs. vehicle + PBS at 4h post-challenge. FP suppressed IL-6 (~90% inhibition), TNF-α (~90% inhibition), CXCL2/MIP-2 (~85% inhibition) and CXCL1/KC (~80% inhibition) proteins in BAL at 4h post SP infection (fig 5.15a-d).
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Figure 5.15 Effect of FP on chemokine and cytokine production following *S. pneumoniae* infection.
C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO control intranasally and challenged with *S. pneumoniae* D39 2.5 x 10^5 CFU or PBS control, 1h following FP administration. BAL was performed at the indicated timepoints post-infection. Cytokines (a) IL-6 and (b) TNF-α and chemokines (c) MIP-2/CXCL2 and (d) KC/CXCL1 in lavage supernatants were measured by ELISA. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.01, ***p<0.001).

5.3.7.6 Anti-microbial peptides

AMPs are a further important component of the innate host response to respiratory bacterial infection.[182] Corticosteroids appear to have variable effects on the expression of different AMPs *in vitro* and *in vivo*. [294, 296, 298, 301-303] We assessed the effect of FP on induction of a range of AMPs following *S.pneumoniae* infection. CRAMP protein in BAL was significantly increased by vehicle + SP treatment vs. vehicle + PBS at 8 and 24h post-infection. Suppressed CRAMP protein in BAL (~90% inhibition) was observed in mice treated with FP + SP vs. vehicle + SP at 8h but not 24h post-infection (fig 5.16a), matching the temporal kinetic of increased bacterial loads observed at 8h post-challenge. SLPI mRNA in lung tissue was significantly increased (~3 fold) in vehicle + SP treated mice vs. vehicle + PBS at 4h post-infection. There were no significant differences in lung tissue SLPI mRNA in FP + SP and vehicle + SP treated mice (fig 5.16b). *Surfactant protein-A* mRNA in lung tissue was not
significantly induced by SP and FP had no effects on it’s expression (fig 5.16c). Other AMPs that were assessed including pentraxin-3 protein in BAL and β-defensin-2 mRNA in lung tissue were not detectable at 4, 8 or 24h post-infection (data not shown).

Figure 5.16: Effect of FP on anti-microbial peptides following S. pneumoniae infection. C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO control intranasally and challenged with S.pneumoniae D39 2.5 x 10^5 CFU or PBS control, 1h following FP administration. (a) BAL was performed at the indicated timepoints post-infection and CRAMP protein was measured in lavage supernatants by ELISA. (b&c) Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (b) SLPI and (c) surfactant protein-A mRNA copies were assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 6 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*p<0.05, ***p<0.001).

5.3.7.7 Interferons

Previously, in chapter 4, we showed suppression of RV-induced type I and III IFN induction by FP which was causally related to impaired virus control. In addition to anti-viral roles, emerging evidence suggests that IFNs are also an important component of the host response to bacteria including S. pneumoniae.[138] A previous study has shown that mice deficient in IFNα/β receptor
signalling or mice treated with type I IFN neutralising antibody exhibit enhanced development of bacteraemia following primary infection with \textit{S. pneumoniae}.\[154] IFN-\(\gamma\) deficient mice have also been shown to display increased bacterial loads in lung tissue following infection with \textit{S. pneumoniae}.\[155] We therefore assessed whether IFNs were induced by \textit{S. pneumoniae} infection in mice and evaluated the effects of FP administration. \textit{S. pneumoniae} infection increased lung tissue \textit{IFN-\(\beta\)} and \textit{IFN-\(\gamma\)} mRNAs at 4 and 8h (vehicle + SP vs. vehicle + PBS). FP suppressed SP induction of IFNs-\(\beta\) and -\(\gamma\) at 4h post-infection down to similar levels as those observed with vehicle + PBS treatment (fig 5.17a&b). IFN-\(\beta\) and -\(\gamma\) proteins were undetectable in BAL at 4, 8 and 24h post-infection (data not shown). Additionally, SP infection did not increase lung tissue mRNA expression or BAL protein levels of IFN-\(\lambda\) (data not shown).

\begin{figure}[h]
\includegraphics[width=\textwidth]{figure5.17.png}
\caption{Effect of FP on interferons following \textit{S. pneumoniae} infection.}
C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO control intranasally and challenged with \textit{S. pneumoniae} D39 2.5 x 10\(^5\) CFU or PBS control, 1h following FP administration. Lung tissue was harvested at the indicated timepoints post-infection. (a) IFN-\(\beta\) and (b) IFN-\(\gamma\) mRNA copies were assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (*\(p<0.05\), **\(p<0.001\)).
5.3.7.8 Mucins

Mucus hypersecretion in COPD leads to impairment of mucociliary clearance, potentially facilitating increased bacterial growth.[26] We assessed the effect of *S.pneumoniae* +/- FP administration on airway mucin expression. SP infection increased lung tissue *MUC5AC* mRNA at 24h post-infection ~5 fold over vehicle + PBS treatment, an effect that was completely suppressed by FP (fig 5.18a). FP had no impact on expression levels of *MUC5B* mRNA in lung tissue (fig 5.18b).

Figure 5.18: Effect of FP on mucin expression following *S. pneumoniae* infection.

C57BL/6 mice were treated with FP (1mg/kg) or vehicle DMSO control intranasally and challenged with *S.pneumoniae* D39 2.5 x 10⁵ CFU or PBS control, 1h following FP administration. Lung tissue was harvested at the indicated timepoints post-infection. RNA was extracted from tissue and cDNA generated as described. (a) *MUC5AC* and (b) *MUC5B* mRNA copies were assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 5 mice per group from one representative experiment with findings reproduced in at least 2 independent experiments (**p<0.01, ***p<0.001).
5.3.7.9. Phagocytosis

Resident alveolar macrophages in the airways play a key early role in the innate immune response to bacterial infection with direct phagocytic functions contributing to early clearance of pathogens.\[144\] There is evidence that macrophage phagocytosis may be impaired in COPD \[170, 390\] but the effect of corticosteroids on phagocytosis is unclear with some studies showing an impairment of function \[280-282\] and others reporting no effect \[170, 283\]. We therefore assessed phagocytosis of FITC-labelled *S. pneumoniae* *ex vivo* by BAL alveolar macrophages (which represent \(>99\%\) of cells in BAL of naïve mice).

To confirm macrophage phagocytosis, we initially incubated alveolar macrophages from naïve mice with FITC-labelled *S. pneumoniae* D39 and confirmed internalisation of bacteria by mixing with ethidium bromide (to counterstain extracellular bacteria) followed by visualisation by fluorescence microscopy (see methods, section 2.2.10.3). Macrophages that had been incubated with FITC-labelled *S. pneumoniae* with addition of ethidium bromide showed presence of internalised *S. pneumoniae* which fluoresced green (while extracellular bacteria fluoresced red) (fig 5.19a). The relevant controls including macrophages incubated with FITC-labelled *S. pneumoniae* without addition of ethidium bromide and macrophages incubated with unlabelled *S. pneumoniae* with and without ethidium bromide were also assessed (fig 5.19b-d). We concluded that the protocol adopted led to successful *ex vivo* phagocytosis of bacteria by macrophages and we could thus proceed to quantify this effect.

To quantify the effect of FP on macrophage phagocytosis, we incubated BAL alveolar macrophages harvested at 4h post-administration from mice treated with FP 1mg/kg or vehicle control with FITC-labelled *S. pneumoniae* D39 and used flow cytometry to measure the proportion of phagocytosing cells and mean fluorescence intensity, indicating the amount of bacteria taken up by each cell. There was no difference observed in proportion of phagocytosing cells or mean fluorescence intensity between BAL macrophages harvested from mice treated with FP vs. treatment with vehicle DMSO (fig 5.19 e&f) suggesting that FP administration had no effect on the ability of BAL macrophages to phagocytose *S. pneumoniae*. 
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Figure 5.19 Effect of FP on ex vivo phagocytosis of S. pneumoniae by alveolar macrophages. (a–d) BAL was performed in naïve C57BL/6 mice and processed as described. Obtained macrophages were opsonised with mouse serum and incubated with FITC-labelled heat killed S. pneumoniae D39 at 37°C for 30 mins. Ethidium bromide was added to the macrophage/bacteria mixture and the suspension was cytocentrifuged onto a slide and immediately visualised by fluorescence microscopy to confirm phagocytosis. Representative images shown for (a) macrophages + FITC-S. pneumoniae with ethidium bromide (b) macrophages + FITC-S. pneumoniae without ethidium bromide, (c) macrophages + unlabelled S. pneumoniae with ethidium bromide and (d) macrophages + unlabelled S. pneumoniae without ethidium bromide. n= 5 mice pooled/group. (e&f) C57BL/6 mice were dosed intranasally with FP 1mg/kg or vehicle DMSO control and BAL was performed at 4h post administration. Macrophages were again opsonised with mouse serum and incubated with FITC-labelled heat killed S. pneumoniae D39, as described. (e) the proportion of phagocytosing cells and (f) mean fluorescence intensity was assessed by flow cytometry. n= 5 mice pooled/group. Single experiment only.

5.3.8 Dose responsive effects of FP on innate anti-bacterial responses, bacterial loads and airway inflammation

Previously, in chapter 4, the effect of lower FP doses on inflammatory parameters following RV1B infection was assessed. Prior clinical studies have suggested that effects of ICS on pneumonia risk may be dose dependent [266, 267], raising speculation that use of lower doses may be safer in COPD. Furthermore, a previous study that assessed ICS effects on S. pneumoniae in a mouse model showed the opposite effect to our experiments with improved rather than impaired bacterial clearance. However, it should be noted that in this study, doses lower than 1mg/kg were used.[313] We therefore reasoned that lower doses of FP may have different effects on S. pneumoniae infection.
in our model and decided to assess the effects of 0.5mg/kg and 0.1mg/kg doses of FP with the 1mg/kg dose used previously.

5.3.8.1 Effect of FP dose on bacterial loads in lung tissue and systemic dissemination

In contrast to the effects of high dose (1mg/kg) FP which increased bacterial loads in lung tissue at 8h post-infection, administration of 0.1mg/kg FP led to significantly lower CFUs (~7 fold reduction) compared to treatment with vehicle + SP. There was also a non-significant trend towards reduced lung tissue bacterial loads in mice treated with 0.5mg/kg FP + SP vs. vehicle + SP (fig 5.20).

![Figure 5.20 Effect of FP dose on lung tissue bacterial loads following S. pneumoniae infection.](image)

**Figure 5.20 Effect of FP dose on lung tissue bacterial loads following S. pneumoniae infection.**

C57BL/6 mice were treated with FP at doses of 1mg/kg, 0.5 mg/kg, 0.1mg/kg or vehicle DMSO control intranasally and challenged with *S. pneumoniae* D39 2.5 x 10^5 CFU or PBS control, 1h following FP administration. Lung tissue was harvested at 8h post-infection, homogenized, serially diluted, plated on columbia horse blood agar plates and incubated at 37ºC for 18h. Counts were performed on plates containing 50-300 colonies and CFU/mL calculated. Individual data points for each animal within group and mean for group shown. n=7-9 mice per treatment group, comprising 2 independent experiments (*p<0.05; ** p<0.001, n.s non significant).

There were no significant differences in blood bacterial CFU counts between mice dosed with vehicle + SP compared to FP 1mg/kg + SP, FP 0.5mg/kg + SP or FP 0.1 mg/kg + SP treatment at 24h post-infection (fig 5.21).
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Figure 5.21 Effect of FP dose on blood bacterial loads following *S. pneumoniae* infection.
C57BL/6 mice were treated with FP at doses of 1mg/kg, 0.5 mg/kg, 0.1mg/kg or vehicle DMSO control intranasally and challenged with *S. pneumoniae* D39 2.5 x 10^5 CFU or PBS control, 1h following FP administration. Blood was harvested by cardiac puncture at 24h post-infection, serially diluted, plated on horse blood agar plates and incubated at 37°C for 18h. Counts were performed on plates containing 50-300 colonies and CFU/mL calculated. Individual data points for each animal within group and mean for group shown. n= 4-5 mice per treatment group. Single experiment only.

5.3.8.2 Effect of FP dose on innate anti-bacterial responses

Next, we assessed the effect of different doses of FP on components of innate anti-bacterial innate immunity. As previously shown, 1mg/kg FP reduced BAL neutrophilia and BAL protein levels of IL-6 and TNF-α at 8h post-infection compared to vehicle + SP treatment. Lower doses of FP (0.5mg/kg and 0.1mg/kg) also significantly reduced BAL neutrophils, IL-6 and TNF-α protein levels down to similar levels as those observed with 1mg/kg FP (fig 5.22a-c). Therefore, although the lower dose of 0.1 mg/kg FP appeared to have opposite effects on lung tissue bacterial loads compared to 1mg/kg FP, inflammatory suppressive effects were nonetheless retained. This suggested that components of anti-bacterial host defence other than neutrophil recruitment or production of anti-bacterial cytokines IL-6 and TNF-α were differentially affected by lower FP doses.

A previous *in vitro* study has suggested that corticosteroids may increase expression of TLR2 induced by *H. influenzae*.[391] We therefore assessed whether lower doses of FP had similar augmenting effects in our model of *S. pneumoniae* infection. We observed a similar trend towards suppressed TLR2 mRNA in mice treated with high dose 1mg/kg FP + SP compared to vehicle + SP treatment at 4h
post-infection, as observed previously (fig 5.22d). However, lower doses of FP (0.5mg/kg or 0.1mg/kg) did not significantly alter TLR2 expression (fig 5.22c) and thus increased PRR expression does not appear to be the mechanism by which lower doses of FP improve bacterial clearance in our model. We also observed trends towards suppressed MUC5AC mRNA expression in lung tissue in mice treated with either 1mg/kg, 0.5mg/kg or 0.1mg/kg FP + SP compared to vehicle + SP (fig 5.22e) and therefore differential effects on mucin expression also did not appear to be an underlying mechanism for our observations of opposite effects on bacterial clearance with high versus lower doses of FP.
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Figure 5.22 Effect of FP dose on innate anti-bacterial responses following S. pneumoniae infection.

C57BL/6 mice were treated with FP at doses of 1mg/kg, 0.5 mg/kg, 0.1mg/kg or vehicle DMSO control intranasally and challenged with S. pneumoniae D39 2.5 x 10⁵ CFU or PBS control, 1h following FP administration. (a-c) BAL was performed at 8h post-infection. BAL cytospin slides were prepared as described (d&e) Lung tissue was harvested at 4 and 24h post-infection, RNA was extracted and cDNA generated, as described. (a) neutrophils were differentially counted blind to experimental conditions. (b) IL-6 and (c) TNF-α proteins in lavage supernatants were measured by ELISA. (d) TLR2 mRNA expression at 4h post-infection and (e) MUC5AC mRNA copies at 24h post-infection were assessed by Taqman quantitative PCR. Data represent mean (+/- SEM) of 5 mice per group (* p<0.05, **p<0.01, ***p<0.001). Single experiment only.
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5.4 Summary

The single dose elastase mouse model did not accurately reproduce features of impaired anti-
bacterial host-defence believed to be characteristic of human disease. FP administration suppressed
RV induction of some components of anti-bacterial host-defence including expression of TLR2 and
pentraxin-3. Using 16S qPCR, we also showed a transient early increase in bacterial loads in lung
tissue associated with FP administration but a transient early reduction in lung tissue bacterial loads
associated with RV infection in mice.

Attempts to evaluate the composition of lower respiratory microbiota in mice by 16S
pyrosequencing were unsuccessful initially due to likely environmental contamination when BAL was
obtained by normal sampling methods. However, a more sterile sampling protocol was optimised
and showed that BAL samples from mice contain a very low bacterial yield which is likely to be below
the threshold required for pyrosequencing. 16S qPCR showed that lung tissue samples have a higher
bacterial content and pyrosequencing may thus be more feasible in these samples.

Since we were unable to proceed with evaluation of the lower respiratory microbiome in mice, we
adopted a direct approach to modelling lower airways colonisation using S.pneumoniae
administration. Administration of FP at high doses (1mg/kg) prior to S.pneumoniae infection
impaired a number of components of anti-bacterial host defence including attenuated expression of
TLR2 mRNA, suppressed neutrophil recruitment and reduced expression of anti-bacterial mediators
including IL-6, TNF-α, IFN-β and –γ, and CRAMP. Consistent with the observed impairment in
multiple components of anti-bacterial host defence, lung tissue bacterial loads were also increased
by high dose FP treatment, an effect that supports the increased risk of pneumonia associated with
ICS in clinical practice. Conversely, administration of FP at lower doses (0.1mg/kg) retained similar
anti-inflammatory effects to higher dose FP but had the opposite effect on bacterial clearance,
causing reduced bacterial loads in lung tissue.
Chapter 6: Discussion and future work

Using mouse models, we have evaluated the effect of inhaled corticosteroids on viral and bacterial respiratory infection. We were unable to define a model of COPD exacerbation that recreated the deficient anti-viral and anti-bacterial immune responses indicative of human disease and therefore, we subsequently used separate virus and bacterial challenge alone as surrogates for viral exacerbation and bacterial colonisation in COPD and assessed the effects of inhaled FP on a number of parameters.

6.1 Mouse model of COPD

Prior to commencement of the project, the only existing model of RV exacerbation in COPD available in the literature was a protocol comprising of four dose elastase/LPS challenge in mice reported by Sajjan et al.[344] We chose to recreate this model for a number of reasons. Firstly, in terms of maximizing time for the project, this was a relatively short-term model when compared to cigarette smoke exposure and, as discussed in chapter 1, it perhaps represented a more accurate model of patients with severe disease, the sub-population who are most at risk of exacerbations in clinical practice.[25] Secondly, based on the data presented by Sajjan et al [344], four dose elastase + LPS administration in mice appeared to be an accurate model of both stable COPD and, when combined with RV infection, of virus-induced COPD exacerbation.[344] Finally, the reported model also showed deficient IFN and impaired virus clearance that has been reported in human models of experimental RV infection [35] and this feature was critical to our hypothesis that inhaled FP would synergistically combine with COPD and lead to further impairment in IFN responses.

6.1.1 Stable COPD

As discussed in chapter 1, stable COPD is associated with a number of characteristic features including histological emphysema, enhanced airway inflammation, mucus hypersecretion and lung function impairment.[9, 11, 12] Therefore, a representative mouse model of COPD would be expected to mimic these features. We hypothesised that four dose elastase/LPS administration would model representative features of stable COPD, as reported by Sajjan et al. In our hands, administration of four doses of elastase/LPS led to replication of some but not all of the previously reported features, namely histological emphysema, increased BAL macrophages and exaggerated AHR to MCH challenge. However, in contrast to the model reported by Sajjan et al, we failed to observe several features of stable COPD including increased inflammatory cytokine and chemokine expression, increased airway neutrophilia and lymphocytosis and mucus hypersecretion. Therefore,
in our hands, the four-dose elastase/LPS model was only partially representative of stable COPD in humans.

We additionally assessed single dose elastase administration without LPS as an alternative model of stable COPD and found this dosing protocol to be more representative of human disease with increased BAL lymphocytes and macrophages and increased mucin expression compared to PBS-dosed controls. Our findings are in keeping with a study by Inoue et al which assessed airway inflammation in response to varying doses of elastase administration in mice. Administration of a similar dose of elastase to the dose used in our study led to increases in BAL macrophages and lymphocytes and a trend towards increased neutrophils [337], which mirrors our findings.

In contrast to combined four dose elastase/LPS, the single dose elastase model did not show any increased AHR, which is presumably due to the reduced level of lung destruction associated with administration of fewer doses of elastase. However, non-invasive measurements of lung function such as whole body plethysmography used in our study may be less relevant to obstructive airway disorders such as COPD and it is possible that invasive lung function techniques may have revealed more subtle abnormalities associated with a single dose elastase challenge, as suggested by a recent study which directly compared invasive and non-invasive techniques in an elastase-induced model mouse of COPD.[335] In summary, none of the dosing protocols that we assessed accurately mimicked all the key features of stable COPD in humans but single dose elastase was the best model of enhanced cellular airways inflammation and mucus hypersecretion.

6.1.2 RV-induced exacerbation of COPD

6.1.2.1 Airway inflammation and mucus hypersecretion

Respiratory virus infection in COPD is associated with a further amplification of airways inflammation and mucus production above stable state and is also associated with increased inflammation when compared to RV infection in healthy subjects.[31, 35, 91, 392] Therefore, if deemed to be clinically relevant, a model of COPD RV exacerbation should be expected to show increases in these parameters compared to either the baseline COPD model (elastase + UV administration, analogous to stable COPD) or RV infection alone (PBS + RV administration, analogous to RV infection in healthy subjects).

We hypothesised that four dose elastase/LPS administration in combination with RV1B infection would also model features of human RV-induced exacerbation of COPD, as reported by Sajjan et al.[344] However, we found that the four-dose elastase/LPS model mimicked some but not all of the
relevant inflammatory and disease-specific parameters. Similar to the data reported by Sajjan et al [344], we observed that four-dose elastase/LPS led to increased RV induction of IL-13 mRNA in lung tissue. However, in contrast to the published study, we observed reduced, rather than increased expression of TNF-α and no change in MUC5AC mRNA in lung tissue. To further evaluate the model, we also assessed a number of other inflammatory endpoints that were not reported by Sajjan et al but had been reported in previous human studies of COPD exacerbation. For most of the parameters we assessed (except lymphocytic inflammation) including BAL neutrophilia and expression of inflammatory cytokines and airway mucins, four dose elastase/LPS administration failed to enhance responses to RV when compared to PBS + RV treatment. Therefore, as a model of the increased airway inflammation observed in RV infection in COPD vs. RV infection in healthy controls, the four dose elastase/LPS model failed to demonstrate exacerbation of many of the key inflammatory parameters. However, we found this model to be a better representation of exacerbated vs. stable COPD with enhanced neutrophilic inflammation and increased inflammatory cytokine levels observed in elastase/LPS+RV vs. elastase/LPS+UV treated mice.

Some investigators have previously speculated that inducing severe lung damage with very high doses of elastase may lead to impaired inflammatory responses to infectious agents, perhaps explaining our observations with the published four dose elastase/LPS protocol. Inoue et al showed that administration of a very high dose of elastase (12 units, 10 fold higher than doses used in our study) led to severe lung damage and impairment of early BAL neutrophilia and BAL protein levels of TNF-α and CXCL2/MIP-2 induction in response to subsequent challenge with S. pneumoniae.[347] However, we found that RV-induced airway inflammation was not enhanced by reducing the number of doses of elastase/LPS and, therefore, this strategy did not produce a more representative model of COPD exacerbation.

Some reported mouse models of bacterial exacerbation have also successfully used single dose elastase administration alone to induce COPD and have shown enhanced inflammatory responses to challenge with S. pneumoniae and H. influenzae.[348, 349] Repeated LPS exposure may attenuate neutrophil recruitment by inducing a tolerogenic state [338] and additionally, a recent in vitro study demonstrated that LPS administration attenuates RV-induced chemokine production.[393] We therefore reasoned that removal of LPS from the dosing protocol so that only elastase was administered to induce COPD would potentially lead to enhancement of inflammatory responses to RV and therefore produce a more representative model of COPD exacerbation. In contrast to combined elastase/LPS models, single dose elastase administration led to increased RV induction of inflammatory cytokines, mucins and increased cellular airways inflammation and therefore more
accurately modelled the enhanced airway inflammation observed in RV infection in COPD vs. RV infection in healthy controls. Single dose elastase was also a representative model of the enhanced inflammation observed in exacerbated vs. stable disease with increases in several key parameters including BAL neutrophil and lymphocyte numbers, and inflammatory cytokine production observed in elastase + RV vs. elastase + UV treated mice.

6.1.2.2 Airway hyper-responsiveness

Data from the human experimental model of COPD exacerbation showed that RV infection was associated with reduced peak expiratory flow rate following onset of exacerbation compared to stable state with similar reductions not observed in RV infected healthy controls. This would therefore, be expected to be another important feature of a clinically relevant mouse model of COPD exacerbation and we hypothesised that increases in AHR would be observed with four dose elastase/LPS in combination with RV infection when compared to elastase/LPS or RV administration alone. Using invasive measurements of lung function Sajjan et al reported increased AHR at d7 post-infection in elastase/LPS + RV vs. PBS + RV or elastase + sham treated mice. We used an alternative non-invasive technique of whole body plethysmography and observed increased PenH values following MCH challenge with four doses of elastase/LPS + RV vs. PBS + RV treatment but reduced values compared to elastase/LPS + UV treatment. Therefore, in our hands, the four dose elastase/LPS model appeared to be a good model of increased AHR in RV infection in COPD vs. RV infection in healthy controls but a poor model of increased AHR in exacerbated vs. stable COPD. The differences between our findings and those reported by Sajjan et al may be due to the mode of lung function measurement used with invasive techniques more likely to pick up the subtle changes induced when RV1B infection is combined with elastase/LPS while whole body plethysmography perhaps unable to distinguish additional impairment above the severe phenotype induced by multiple elastase/LPS challenges.

Assessment of AHR in our alternative single dose elastase model of RV-induced COPD exacerbation demonstrated increased PenH values following MCH challenge compared to either elastase or RV administration alone. Therefore, although single dose elastase did not increase AHR above treatment with PBS and thus did not accurately model this particular component of stable COPD, single dose elastase in combination with RV infection was a good model of increased AHR in response to RV infection in COPD vs. RV infection in healthy controls (PBS + RV). In contrast to four dose elastase/LPS, single dose elastase + RV infection also accurately modelled increased AHR of exacerbated vs. stable COPD. This may have been due to the fact that less severe lung destruction is induced with single dose elastase compared to four doses of elastase and LPS and, therefore,
additive effects of RV1B infection may be more readily observed, even with use of less sensitive non-invasive lung function techniques.

6.1.2.3 Anti-viral immune responses and virus control

Deficient anti-viral immune responses and impaired virus clearance are also features of human COPD and these were both reported in the four dose elastase/LPS mouse model [344] and also the human experimental RV infection in COPD model [35]. We considered deficient IFN associated with increased virus loads to be an essential feature of a mouse model of COPD exacerbation and we required this feature to be present to enable us to proceed with testing the hypothesis that ICS use in COPD would synergistically impair IFN induction and lead to impaired virus control in vivo. We hypothesised that four dose elastase/LPS administration would suppress IFN responses to RV and impair virus control, as previously reported.[344]

Similar to data reported by Sajjan et al, we observed suppressed RV induction of IFN-β and -λ mRNAs in lung tissue in mice treated with four doses of elastase/LPS. However, this effect was associated with reduced rather than increased virus loads and therefore, rather than being a model of primary IFN deficiency leading to impaired virus control and increased replication, in our hands, four dose elastase/LPS administration produced a model where RV replication was actually reduced and consequently less induction of IFNs occurred. Reducing the number of doses of elastase and LPS had no effect on virus loads with reduced lung tissue RV RNA copies observed in elastase/LPS treated mice regardless of whether 1, 2, 3 or 4 doses were administered. Although single dose elastase combined with RV infection more accurately modelled enhanced airway inflammation and mucus hypersecretion than elastase/LPS, this model also failed to show enhanced virus replication.

Therefore, despite assessing a number of different dosing strategies, we were unable to define a mouse model of COPD exacerbation that displayed increased virus replication. The precise reasons for differences between our findings in the combined elastase/LPS or single dose elastase + RV mouse models and those of the human experimental RV infection in COPD study with respect to virus control are unclear. One possible explanation may be that the elastase-induced mouse model of COPD is one of severe lung damage representing advanced disease, whereas the human experimental model was carried out in patients with relatively mild airflow obstruction (mean FEV1 % predicted 69.7%). To date, there are no human models of experimental RV infection in severe COPD to draw reference from. We can speculate that, with severe emphysematous lung tissue destruction, as displayed in our mouse model, there may be a reduction in viable airway epithelium required for RV replication to occur. Furthermore, virus replication kinetics differ between human
and mouse experimental RV infection models. In the human model, increased virus levels were only apparent by d3 post-infection, by which time replication has ceased in the mouse.\[35, 367\] It must be also noted that evidence for increased RV replication and impaired IFN induction in COPD is based on only one human and one mouse study to date and in vitro, air liquid interface cultured bronchial cells from patients with moderate to severe COPD have demonstrated enhanced virus replication but increased rather than decreased interferon induction \[83\].

### 6.1.3 Limitations of elastase-induced mouse model of COPD

There are a number of limitations of the elastase model of COPD that may limit the validity and relevance of findings to human disease. Unlike models involving cigarette smoke exposure, elastase models do not use the primary disease-causing agent in humans. Although smoke exposure models are acknowledged to require prolonged administration and do not induce severe emphysematous changes consistent with advanced disease \[325\], such approaches may be considered to more closely related to human disease. It could be argued that elastase administration is more consistent with a model of acute lung injury rather than the chronic repetitive tissue injury associated with prolonged cigarette smoking in humans. Alternative models of COPD in mice including ozone exposure \[394\] and transgenic strains such as IL-13 over-expression \[322\] have been used previously but can also be criticised for their lack of relevance to human disease.

Another potential limitation of the elastase model is with the use of the intranasal dosing route. This approach was chosen because it was the method employed in the four dose elastase/LPS model previously reported by Sajjan et al. \[344\] Although intranasal dosing is a recognised method of administration to lung tissue in mice, a proportion of the elastase dose may theoretically have been absorbed by the nasal mucosa, potentially leading to some degradation of the nasal epithelium. Histological evaluation of nasal tissue following elastase administration was not carried out in this model but would have allowed a direct evaluation. It is possible that the reduced rhinovirus loads observed with elastase models may have been directly related to destruction of nasal epithelium preventing subsequent effective infection with rhinovirus. Notably, some previously reported elastase administration models have used the intra-tracheal exposure route \[337, 349\] which would avoid the potential issue of degradation of nasal epithelium and thus may represent a better approach.

### 6.1.4 Summary

In summary, as a model of RV exacerbation in COPD compared to either RV infection in healthy controls or stable COPD, four dose elastase/LPS + RV administration failed, in our hands, to produce
an exacerbation of a number of features of human disease and impaired virus control that the existing literature suggests are indicative of human COPD exacerbation. The precise reasons for our failure to replicate the findings from the study by Sajjan et al [344] are unclear. We contacted the authors to enquire whether there were any additional methodological factors that we had not considered and also to clarify reagents and techniques used. However, we could not identify any potential areas of inconsistency. We therefore made the decision to try to optimize the model and assessed a number of alternative dosing protocols, as discussed. Single dose elastase administration, in combination with RV infection, as an alternative model, more accurately modelled the enhanced airway inflammation and mucus hypersecretion that have been reported in human studies. This strategy was also a better model of increased AHR in exacerbated vs. stable disease. However, similar to combined elastase/LPS, the model also failed to show impaired virus control.

6.2 Effect of FP on RV1B infection
Since we were unable to define a representative model of RV infection in COPD that displayed the key feature increased virus replication, we did not proceed to assess the effects of ICS administration in this model, as set out in our aims. Instead, primary RV1B infection in mice was used as a surrogate for virus-induced exacerbation of COPD. Previous studies have used the mouse RV infection model as a surrogate for studying disease mechanisms relevant to exacerbations of asthma and COPD [56, 69] and therefore this represents a valid approach but it should be emphasised that the relevance of any findings from the mouse model must be interpreted with caution and require validation in human models of disease.

6.2.1 Anti-viral innate immune responses
We hypothesized that ICS would suppress anti-viral innate immune responses in vivo and this was confirmed by our finding that high dose FP (1mg/kg) administered intranasally prior to RV1B infection led to impaired lung tissue mRNA expression of IFN-β and -λ and BAL protein levels of IFNs –α, -β and -λ. These findings are in keeping with in vitro studies that have used a range of different corticosteroid compounds in a number of different cell types and shown suppressed IFN responses.[246-248] However, our data provides the first in vivo confirmation of these potentially detrimental effects of ICS. Consistent with these effects, we also showed that RV induction of a number of ISGs, which encode proteins with specific anti-viral roles, was significantly suppressed by FP. NK cells also play an important role in the innate immune response to virus infections by directly eliminating virally infected cells [61] and have been shown to be dependent on type I IFN responses
during RV infections [56]. In keeping with the effect of FP on IFN induction by RV, we also observed suppressed numbers of NK cells in BAL and activated NK cells in lung and BAL and this represents a further impairment of innate anti-viral immune responses.

6.2.2 Adaptive immune responses

In addition to impairment of innate anti-viral immune responses, we also observed impairment of adaptive immune responses to RV infection by FP including reduced numbers of total CD4+ and CD8+ cells in BAL and reduced numbers of activated CD4+ and CD8+ T cells in lung and BAL. T cells possess cytotoxic effector functions and may therefore contribute directly to virus control and effective clearance from the airways and, additionally, CD4+ cells provide assistance to B cells and are thus indirectly involved in the production of antibody responses.[64, 395, 396]. A number of studies have shown previously that ICS can suppress numbers of CD4+ and CD8+ cells in the lungs of patients with stable COPD.[233, 235, 236] Although no existing studies have assessed the effects of ICS on T cell responses during COPD exacerbations, one study examined ICS administration in a human model of experimental RV infection in asthma.[67] This study reported no effect of 4 weeks inhaled budesonide therapy on CD4+ or CD8+ T cell numbers in the lamina propria or epithelium from bronchial biopsies following experimental infection with RV16.[67] Our findings are in contrast to the minimal effects on T cell responses observed in this human study. The reasons for this are not entirely clear but may relate to the underlying asthmatic status of patients and/or potentially the likely lower doses used in the human study.

In addition to suppression of T cell responses, FP administration in our model also led to reduced production of RV-specific IgG and neutralizing antibodies in blood. Neutralising antibody responses to RVs provide protection against infection and symptoms in humans and, as well as accelerating virus clearance during the first naturally acquired infection with a given serotype, also protect against re-infection with the same serotype.[71, 72, 397] RV infection in the mouse is a poor model of the protective antibody responses observed in man, since sequential infection with the same RV serotype does not detectably improve virus control during the second RV infection (G.McLean, unpublished observations). However, our observation that FP impairs production of antibody responses following RV infection in the mouse model suggests that use of these drugs during acute RV exacerbations in patients with COPD may potentially impair protective responses against future RV infections, an effect that would further limit their efficacy in reducing disease exacerbations.

Yerkovich et al followed a group of 60 patients with COPD for 1 year and showed that those who had at least one exacerbation had lower serum RV-specific IgG1 levels in stable state than those who were exacerbation free. They also showed a trend towards increased ICS use in the exacerbation-
prone group and this indirectly suggests that ICS use may be associated with reduced antibody responses in humans.[85] However, there are a number of other potential confounding factors such as disease severity and comorbidities that may be contributing and were not accounted for in the study.

6.2.3 Virus control

Whether FP suppression of innate and adaptive immune responses to RV is associated with impairment of virus control and increased virus replication has previously been unclear. Studies have reported that administration of systemic corticosteroids prior to experimental RV infection in healthy subjects or use of intranasal FP during naturally occurring colds can increase virus loads.[249, 250] Farr et al also reported a non-significant trend towards prolonged virus shedding in healthy subjects treated with combined inhaled beclomethasone and oral prednisolone during experimental infection with RV39.[398] These studies have raised speculation that corticosteroids may interfere with virus control in the airways and thus potentially worsen exacerbation severity.

In our study we provided further support for this hypothesis by showing that FP administration significantly increases RV loads in the lungs, measured by either Taqman quantitative PCR or titration assay. This effect is in contrast to a study by Singam et al where intranasal FP administration (alone and in combination with salmeterol) following RSV infection in a mouse model of ovalbumin-induced allergic asthma led to reduced rather than increased virus loads.[399] A number of factors may explain the differences observed between this study and ours, including the different viruses used and different mouse strains used (RSV in BALB/c mice vs. RV in C57BL/6 mice in our study) and differences in the ICS dosing strategy used (multiple administrations of low dose FP compared to a single higher dose of FP used in our study). Other studies have assessed systemic corticosteroid administration in mouse models of respiratory virus infection with one study showing increased virus replication when hydrocortisone was administered during pneumovirus infection [251], and another showing that dexamethasone had no effect on virus loads following influenza infection.[253] However, it is difficult to directly compare these models of systemic corticosteroid therapy with our model of direct administration of FP into the airways.

In a clinical context, our finding that FP administration increases virus loads in vivo imply that ICS use may render patients with COPD more prone to RV-induced exacerbations and potentially question the uncritical use of these medications during acute exacerbations. As discussed, COPD is believed to be associated with an inherent deficiency of IFN responses [35] and therefore, use of ICS may further suppress innate anti-viral responses and synergistically impair virus control and increase virus loads.
Whether strategies such as cessation of ICS at the onset of exacerbation or targeting of therapy to sub-groups based on underlying immune profile could be beneficial in limiting these potentially adverse effects requires further investigation in human models of disease. The increased virus loads associated with FP administration may also potentially have adverse effects on other clinical disease parameters in COPD such as airway remodelling and FEV1 decline that could not be measured in our mouse model.

### 6.2.4 Airway inflammation

To assess whether increased virus replication had any disease-relevant effects in the context of acute exacerbations of COPD, we assessed the effect of FP administration on virus-induced airway inflammation in our model. Although corticosteroids have extensively been shown to be potent anti-inflammatory agents in the context of stable airways disease [233-236], there has been speculation that their effects may be limited in the context of acute exacerbations.[57] This hypothesis is based on the relatively poor effect of ICS on reducing COPD exacerbation frequency in clinical trials [217, 239, 240] and also experimental virus infection studies in human and animal models that have shown limited effect of ICS on inflammation.[67, 254]

We therefore hypothesised that the increased virus loads observed with FP administration in our model would outweigh the anti-inflammatory effects of ICS on inflammatory chemokine and cytokine expression and thus lead to enhanced rather than reduced airway inflammation. However, our findings disproved this hypothesis as we observed that FP led to near complete inhibition of protein levels of neutrophil and lymphocyte chemokines (including CXCL1/KC, CXCL2/MIP-2, CCL5/RANTES and CXCL9/MIG) and proinflammatory cytokines IL-6 and TNF-α in BAL. We also observed that FP administration led to a profound impairment in numbers of neutrophils and lymphocytes recruited to the airways in response to RV infection. Therefore, the adverse effects of FP on virus control did not appear to impact upon the ability of the drug to suppress inflammatory responses to RV. These findings have important clinical implications as suppression of inflammation may be one mechanism of potential benefit of ICS on exacerbations. Although clinical trials report only a modest effect of these drugs on exacerbations, their use is still associated with a ~20-25% reduction [217, 239] and this may be due to the fact that anti-inflammatory effects are preserved despite adverse effects on virus control. It is possible that suppressed inflammation associated with ICS use leads to a greater frequency of unnoticed exacerbations in which virus infection occurs but absence of an airway inflammatory response leads to failure of the patient to report symptoms. The long term sequelae of the occurrence of such events are unclear but studies that have employed diary cards to longitudinally follow patients with COPD have revealed that around 50% of
exacerbations are unreported but still associated with similar falls in peak flow to reported events.[24]

Although RV induction of many inflammatory chemokines was suppressed by FP, interestingly, opposite effects were observed with regards to the Th2 lymphocyte chemokines CCL22/MDC and CCL17/TARC which were enhanced rather than suppressed. Primary RV infection in the mouse model is dominated by a Th1 mediated response with limited Th2-mediated inflammation (no eosinophilia and limited expression of Th2 cytokines IL-4, -5 and -13). Th2-mediated inflammation is classically associated with asthmatic airways disease, although some studies have also implicated these processes in COPD.[400-402] Whether the effects of FP on increasing Th2 lymphocyte chemokine induction would lead to enhanced Th2 mediated inflammation in mouse models of asthma and/or in patients with asthma or COPD during acute RV exacerbations would be of interest.

6.2.5 Airway mucins

To assess whether FP had any other adverse effects on disease-relevant features, we also measured major airway mucins MUC5AC and MUC5B in the model. In contrast to the potentially beneficial effects on reduced RV-induced airway inflammation, we observed that administration of FP led to increased MUC5AC lung tissue mRNA and BAL protein at d7 post-infection and increased MUC5B BAL protein at d14 post-infection. Impaired mucociliary clearance with mucus hypersecretion is a recognised feature of stable COPD [11] and RV can further stimulate mucus production from the airway epithelium and thus potentiate sputum production during acute exacerbations [368, 369].

Our finding that FP administration increases RV-induced mucin expression further suggests that use of these therapies in the context of acute RV exacerbations may have potentially detrimental effects in COPD, if similar effects were applicable in human disease.

Previous studies that assessed the effect of corticosteroids on mucin expression have reported variable findings. In vitro studies in a range of cell types have reported suppression [306, 403, 404] or no effect [309, 405] of corticosteroids on constitutive mucin expression. Other in vitro studies have shown that corticosteroids suppress mucin expression in response to LPS [406], H. influenzae [308] or P.aeruginosa in airway epithelial cells [307]. In vivo studies in animal models have reported reduced MUC5AC expression in response to nebulised budesonide in an ovalbumin model of asthma in guinea pigs [407] and in response to intraperitoneal dexamethasone in a mouse model of H. influenzae challenge [308]. In humans, ICS treatment had no effect on the expression of MUC5AC or MUC5B in airway biopsy samples from asthmatic patients.[310] Therefore, the majority of existing studies report that corticosteroids have either no effect or cause a reduction in mucin expression.
This is in contrast to our finding that FP increases mucins in the context of RV infection. The only existing study that is supportive of our findings reported that dexamethasone administration increased $MUC5AC$ mRNA and protein expression in response to recombinant IL-13 administration in bronchial epithelial cells.[311] Ours is the first study to specifically assess the effect of ICS on virus-induced mucins. The signalling pathways involved in the induction of mucins are poorly understood but the differences between our study and the majority of studies discussed above may be due to the fact that different signalling pathways are likely to be involved in virus-induced mucin expression compared to constitutive expression or in response to bacterial stimuli. A previous in vitro study demonstrated that RV induction of $MUC5AC$ occurs through a distinct epidermal growth factor receptor (EGFR) pathway mediated through TLR3 [369], a pathway that would therefore be specific to double stranded RNA viruses and may be directly affected by ICS.

As discussed, FP also increased RV induction of Th2 lymphocyte chemokines CCL22/MDC and CCL17/TARC. Th2 cytokines, particularly IL-13 play a key role in pathways leading to MUC5AC expression, particularly in animal models of allergic airway inflammation.[408] However, as discussed previously, the mouse model of RV1B infection induces a predominantly Th1 mediated inflammatory response. We were unable to measure any increases in IL-13 mRNA expression in lung tissue or protein levels in BAL with either FP treatment and/or RV1B infection in the model and therefore, increased IL-13 does not appear to be playing a mechanistic role in the increased expression of mucins observed in our model.

6.2.6 Dose responsive effects of FP

The dose selected for use in our model of 1mg/kg was based on advice from an industry collaborator who had prior experience of the use of ICS in mouse asthma models. It is important to note that the dose that was used represents one that is higher than those used in clinical practice, which are typically around 1000μg per day (which equates to ~0.014 mg/kilogram for a 70kg human, ~70-fold lower than the 1mg/kg dose used in our model). Comparison of intranasal dosing in mice with inhaled dosing in humans is difficult because it is unclear exactly how much effective drug is delivered to lung tissue via either method. Intranasal delivery in the mouse is likely to lead to relatively higher tissue drug concentrations than delivery via inhaler devices in humans, which have been shown to be around 1-10nM for a 1000μg FP dose administered via volumatic spacer.[409] Use of mass spectrometric techniques would allow a similar characterisation of lung tissue concentrations in the mouse model of intranasal FP administration and enable direct comparisons to be made. Nonetheless, we evaluated the effects of 0.5mg/kg and 0.1mg/kg doses of FP on similar
inflammatory and immune parameters to assess whether similar effects were observed with lower, perhaps more clinically realistic, doses.

We found that a 50% dose reduction (0.5mg/kg) had similar effects on anti-viral innate responses and virus control to the 1mg/kg dose with a trend towards suppression of RV-induction of IFN-λ and significantly increased virus loads. We also observed a similar suppression of virus-induced airway inflammation. By contrast, at 90% dose reduction (0.1mg/kg) many of the effects of FP on RV infection were lost with no significant changes in RV-induced inflammation, a non-significant trend towards lower IFN-λ mRNA in lung tissue and minimal effect on virus loads.

We were unable to identify a dose of FP which retained potentially beneficial anti-inflammatory effects but did not display the adverse effects on virus control. However, at 50% dose reduction, a similar level of anti-inflammatory effect was observed to the higher 1mg/kg dose but the effect on increased virus loads was less pronounced with ~6 fold increase in RV loads observed with 1mg/kg FP administration and only ~3 fold increase observed with 0.5mg/kg. A 90% dose reduction (0.1mg/kg) led to loss of both anti-inflammatory effects and adverse effects on virus control. Therefore, it is theoretically possible that a dose between 0.1mg/kg and 0.5mg/kg may potentially have beneficial anti-inflammatory effects without having detrimental effects on anti-viral immune responses, although time constraints prevented a more extensive dose response from being assessed.

Although the doses at which we observed detrimental effects on virus control in the mouse model are likely to be higher than those used in humans, it is also worth noting that we observed increased virus loads in a model where minimal replication (no more than 24h) occurs. In human RV infection, where replication is much more robust and of longer duration, it may be possible that daily administration of lower doses of ICS may still exert similar adverse effects. This is supported by studies of naturally occurring colds where increased RV culture positivity was observed at d7 post onset in patients treated with intranasal FP vs. placebo. These human studies suggest that detrimental effects of ICS on anti-viral responses may occur at lower doses. Additionally, since COPD is associated with an inherent deficiency in IFN responses, effects of ICS may be even more pronounced in disease states than in healthy subjects.

6.2.7 Effect of recombinant IFN-β on FP suppressed responses
To further confirm the importance of FP suppressed IFN responses and assess whether this effect was causally related to the enhanced virus loads observed, we evaluated whether recombinant IFN-β administration could reconstitute suppressed responses and improve virus control. IFN-β
administration in combination with FP led to reconstitution of suppressed innate responses to RV infection with increased expression of ISGs. Addition of IFN-β also improved the impaired virus control associated with FP. These data suggest that FP suppression of IFN responses does play a major mechanistic role in the adverse effects observed on virus control. We also demonstrated that recombinant IFN-β did not alter the effect of FP on suppression of RV-induced airway inflammation. Therefore, recombinant IFN-β was able to improve virus control without reducing the beneficial effect of FP in suppressing inflammation.

However, IFN-β administration had no impact on the increased mucin expression and suppressed adaptive immune responses associated with FP administration and therefore could not directly improve these other potentially detrimental effects. The fact that recombinant IFN improved virus control but could not improve other adverse disease-relevant effects questions the potential benefit of using recombinant IFN-β as a therapeutic agent during virus-induced exacerbations of airways disease in ICS-users. In a recently published study assessing the effect of inhaled IFN-β therapy in asthmatic patients taking ICS who developed an exacerbation, a similar enhancement of ISG expression and a trend towards reduced virus loads was observed [410] as seen in our mouse model. These effects did not correlate with any improvements in the primary endpoint of asthma symptom scores [410] which mirrors our finding that recombinant IFN-β in the mouse model had no impact on airway inflammation or mucin expression. However a sub-analysis did reveal some benefit in patients with more severe disease in this study.[410] IFN-β therapy also improved morning PEF recovery, thereby suggesting that reduced virus loads may have beneficial effects other than symptomatic improvements [410] but such parameters cannot be easily measured in our mouse model.

6.2.8 Components of interferon signalling pathways affected by FP

To further characterise the effect of FP on suppression of IFN, we attempted to define which components of IFN signalling were disrupted. The type I IFN response is biphasic with initial release of IFN related to virus detection by endosomal receptors TLR3, 7 and 8 or cytosolic receptors MDA5 and RIG-I.[50-52] Secreted type I IFN then binds to IFNAR and stimulates further release and induction of ISGs.[55] Using a luciferase reporter assay in BEAS2B cells, we observed that FP suppressed TLR3-agonist (Poly(I:C)) induced IFN-β promoter activity but conversely had no effect on MDA-5-agonist (transfected Poly(I:C)) induced IFN-β promoter activity, thereby suggesting that FP acts on the TLR3 but not MDA5 mediated virus detection pathway. Matsukura et al similarly reported that FP administration suppressed Poly(I:C) induced mRNA and protein expression of ISG CXCL10/IP-10 in BEAS2B cells.[411] Conversely, in a mouse model of intranasal Poly(I:C)
administration, FP treatment had no effect on IFN-β or CXCL10/IP-10 protein in BAL.[412] However a dose of 0.125 mg/kg FP was used in this study which is similar to the 0.1mg/kg dose used in our in vivo model that also had no effects on innate responses to RV infection. With use of higher doses, suppressive effects on Poly(I:C) induced IFN responses, in keeping with our in vitro experiments, may have been observed.

Time constraints prevented us from assessing the effect of FP on other virus sensing pathways including TLR 7/8 and RIG-I signalling and the secondary component of IFN signalling via IFNAR using specific agonists within a similar in vitro system. However, evaluation of the relevant control groups from the in vivo recombinant IFN-β administration experiment allowed analysis of the IFNAR pathway component. We observed that FP suppressed IFN-β induction of the ISGs OAS and IFN-λ mRNA in lung tissue and CXCL10/IP-10 protein in BAL, thereby suggesting that ICS may also impair the secondary component of IFN signalling via IFNAR.

6.2.9 Limitations of FP and RV1B infection mouse model

A limitation of the FP mouse model was that it involved a single dose of intranasal FP administration immediately prior to infection. This contrasts with the typical daily inhaled dosing that occurs in humans and it could be argued that a more representative model of disease would involve daily administration of FP prior to and/or during infection, as employed in a previous mouse model.[399]

The validity of the mouse model of RV1B infection may also be questioned as it involves use of a human pathogen rather than a mouse adapted strain and therefore relatively high infecting doses are required to produce inflammatory responses that are representative of human disease. Therefore, separate rhinovirus batches were required for each individual experiment and, despite use of similar virus TCID50 in each experiment, variations in inflammatory responses between experiments were frequently observed. Use of a mouse adapted strain may have improved inter-experimental variability. Additionally, there is an obligate requirement for use of minor group RV1B in the mouse model due to lack of the cellular receptor ICAM-1 in mouse lung preventing use of major group serotypes. This makes it difficult to make direct comparisons between findings from this study and those of human experimental models of disease, which commonly use major group RV serotypes [35, 67]. Whether inflammatory responses associated with minor group serotypes are comparable with those of major group RVs in humans is unclear.
6.2.10 Summary

We have shown that FP can suppress innate and adaptive immune responses to RV infection and also impair virus control *in vivo*. Although the hypothesised increase in RV-induced inflammation with FP was not evident, we did observe an increase in mucin expression associated with FP administration in this model. In a clinical context, our results suggest potentially beneficial and detrimental effects of ICS in the context of RV-induced exacerbations of COPD with the desirable effect of reduced inflammation but the potentially adverse effect of increased mucus production which may theoretically lead to protracted symptoms.

Additionally, the reduced adaptive immune responses associated with FP administration may also impair protective responses to reinfection with the same RV strain and thus limit the efficacy of FP in reducing exacerbation frequency. We have also shown that administration of recombinant IFN-β reconstituted FP suppressed immune responses, improved virus control but did not have any beneficial effects on adaptive immune responses or mucin expression. We must be cautious in directly extrapolating effects observed in an otherwise healthy mouse to exacerbations of complex chronic lung disorders. Therefore, confirmation of the applicability of our findings to COPD through translation into human models of disease is warranted.

6.3 Effect of FP on bacterial infection

Having evaluated the effects of FP on anti-viral immune responses following RV infection, we next sought to investigate our hypothesis that ICS administration would suppress RV induction of anti-bacterial mediators and thus alter the lower respiratory microbiota leading to proliferation of colonising PPMs and increased risk of secondary bacterial pneumonia.

6.3.1 Anti-bacterial host defence mechanisms in the single dose elastase COPD model

COPD is associated with a deficiency in certain anti-bacterial host responses to respiratory virus infection and, in the human model of experimental infection in COPD, RV was shown to directly precipitate secondary bacterial infection defined by sputum culture or culture-independent methods in a significantly greater proportion of patients with COPD than healthy controls.[122, 190] Given that single dose elastase administration was the most representative model of RV-induced COPD exacerbation that we assessed, we measured a number of anti-bacterial factors in this model. Similar to observations in human studies, single dose elastase administration was associated with increased numbers of BAL macrophages and addition of RV infection also led to further increases in BAL neutrophils and pro-inflammatory cytokine TNF-α compared to RV infection in PBS dosed
controls. Since these components all have recognised anti-bacterial effector functions, the observed increase would be expected to be associated with enhancement rather than impairment of host defence against bacteria. Whether the function of macrophage and neutrophils in our model is associated with similar impairments as those reported in human disease [170, 172-174] is unknown. Ganesan et al reported that macrophage phagocytosis of *H. influenzae* was impaired in the four dose elastase/LPS COPD mouse model [350] but it is unclear whether similar abnormalities are induced with single dose elastase administration.

Mucus hypersecretion was one feature that was observed in the single dose elastase model that could be associated with impaired anti-bacterial host-defence. Increased mucus production in COPD is believed to contribute to impaired mucociliary clearance with increased particle and pathogen retention in the airways precipitating defective bacterial clearance and potentially contributing to lower airways colonisation and development of bacterial exacerbations.[98] We observed increased mucins and PAS staining scores in the baseline elastase model with further increases observed when elastase was combined with RV compared to elastase or RV administration alone.

We also assessed a number of other factors that have been reported to be impaired in human studies, including expression of PRRs TLR2 and 4. We found no difference in expression of either TLR2 or TLR4 in elastase + UV vs. PBS + UV treated mice. Although this contradicts findings from some human studies which have reported reduced TLR2 and TLR4 expression in stable COPD [162, 163] others have conversely shown increased expression [164, 165]. Therefore the precise expression pattern in human lung tissue is unclear and thus modelling these features accurately in mice is difficult. We found that elastase administration suppressed TLR-2 induction by RV but increased TLR4 expression. The dynamics of bacterial PRR expression during RV-induced exacerbations of COPD have not been studied in human models and therefore, the relevance of these observations to secondary bacterial infection in COPD is difficult to evaluate.

We also measured AMPs SLPI, *surfactant protein A*, *β-defensin* 2 and pentraxin-3 and also found no difference in expression between elastase + UV vs. PBS + UV treated mice and expression of *surfactant protein-A* and *β-defensin* 2 was increased rather than reduced following RV infection in elastase treated mice compared to mice treated with PBS + RV. Very few human studies have characterised the dynamic changes of AMP expression from stable state to RV-induced exacerbation and, as with PRRs, it is therefore difficult to determine whether the observed effects are representative of human disease. Regardless of this, our model failed to demonstrate deficiencies in these particular components of anti-bacterial host defence.
Due to time constraints and methodological problems (discussed in section 6.3.3) we were unable to assess whether any of the measured anti-bacterial deficiencies in the single dose elastase model affected the lower respiratory microbiota. Therefore, whether this model accurately recreates the chronic bacterial colonisation associated with stable COPD and whether RV infection in the model leads to dysregulation of the microbiota with increased proliferation of PPMs, as described in human models [122], could not be characterised.

### 6.3.2 Anti-bacterial host defence mechanisms in the FP and RV1B infection model

In the INSPIRE study, pneumonia was shown to occur more frequently following an unresolved exacerbation in COPD patients taking ICS.[317] This has raised speculation that ICS may increase the risk of virus-induced secondary bacterial infection and thus increase pneumonia frequency. Although time constraints prevented evaluation of the effect of FP administration on combined RV1B and *S. pneumoniae* challenges, we assessed whether ICS administration led to impairment of RV induction of anti-bacterial mediators. We assessed a number of parameters in the high dose (1mg/kg) FP + RV1B mouse model and observed a number of features that were suppressed by FP that may theoretically increase risk of development of and/or worsen the course of secondary bacterial infection.

In our model, FP suppressed RV-induction of IL-6, TNF-α, neutrophil chemokines and BAL neutrophils, effects that have also been reported in a number of previous *in vitro* studies in response to viral stimuli.[246, 247, 413] This effect might be expected to increase the risk of acquisition of secondary bacterial infection, particularly in patients with COPD who are chronically colonised with bacteria in the lower airways since all these factors have been shown to have roles in anti-bacterial host defence.[147-149] Studies that have assessed sequential infection with RV and *H. influenzae* have shown impaired neutrophil responses and delayed bacterial clearance associated with RV infection prior to bacterial infection [209] and therefore an initial robust inflammatory response to one infection may dampen responses to a second pathogen and impair clearance. Suppression of inflammatory responses to RV by FP may interfere with this dynamic and thus theoretically improve the immune response to a secondary bacterial infection. Therefore it may be possible that ICS increase the risk of development of pneumonia through suppression of anti-bacterial mediators and proliferation of PPMs in the lungs of patients with COPD but paradoxically improve the course of a secondary bacterial infection by limiting the initial immune response to virus infection and thus increasing the response to the secondary infection. This hypothesis is supported by existing clinical data which reveal an increase in risk of development of pneumonia [217, 256, 257] but an improvement in pneumonia-related adverse outcome associated with ICS use in COPD [274, 275].
Another effect of FP in the RV1B infection model that may impact upon secondary bacterial infection is the observed alteration in mucin expression. We found that FP suppressed BAL MUC5B protein at early timepoints but led to increases in MUC5AC and MUC5B proteins at later timepoints post-infection. Interestingly, although the quantity of mucus produced in the airways of patients with COPD is increased overall, expression of MUC5B has been shown to actually be impaired in the airway epithelial surfaces of smokers.[414] Roy et al showed that MUC5B deficient mice had impaired bacterial functions and accumulation of bacterial species in the lungs while MUC5B overexpressing mice did not have similar abnormalities and actually showed improved bacterial phagocytosis.[152] Therefore, our finding that FP suppresses RV-induced MUC5B at early timepoints may potentially be the more important effect in terms of impairment of anti-bacterial host-defence than the increased mucin expression observed later in the course of infection. However, these processes are poorly understood and firm conclusions are therefore difficult to make.

The effect of FP on IFN responses and implications in terms of anti-viral host defence has already been discussed in detail in section 6.2. However, there is evidence that IFNs may also play important roles in anti-bacterial host defence. In contrast to the adverse effects on virus control associated with deficient IFN responses to primary RV infection, deficient type I IFN signalling appears to protect against secondary bacterial challenge as IFNAR−/− mice infected with influenza have been shown to have improved bacterial clearance and survival following secondary challenge with S. pneumoniae.[211, 415] Therefore, IFN suppression by FP might be an effect that would be expected to improve rather than impair subsequent bacterial infection.

Other parameters that were suppressed by FP in the RV1B infection model include the AMP pentraxin-3. Levels of pentraxin-3 have been shown to be increased in the sputum of patients with COPD compared to control subjects following experimental infection with RV [190] and in vitro, corticosteroids suppress pentraxin-3 release by LPS in dendritic cells.[416] In the human model of experimental RV infection in COPD, secondary bacterial infection also correlated with reduced levels of AMPs SLPI and elafin.[190] However, we observed no effect on SLPI expression by FP and/or RV infection in the mouse model and were unable to measure elafin due to lack of a mouse homolog.

Other innate anti-bacterial components that were assessed in the FP + RV model include the expression of PRRs. We found that TLR2 mRNA expression in lung tissue was upregulated by RV infection but suppressed in the presence of FP. TLR2 is a PRR that plays a key role in pattern recognition of gram-positive bacteria and activation of innate immune responses. A previous study by Saba et al showed that TLR2 was required for RV-induced proinflammatory cytokine responses,
since bone marrow derived macrophages treated with TLR-2 blocking antibody or taken from TLR-2 deficient mice had impaired cytokine responses to RV1B.[417]. Therefore, the suppressive effect of FP on induction of TLR2 by RV1B may contribute to the suppressed inflammatory responses that were observed in our model and thus directly contribute to increased risk of secondary bacterial infection following RV infection.

6.3.3 Evaluation of the lower respiratory microbiota in mice

To assess our hypothesis that the lower respiratory microbiota in mice contained PPMs relevant to human disease, we initially attempted to perform 16S rRNA pyrosequencing in BAL samples from wild-type untreated mice. When samples were sequenced, we found that they were dominated by Herbaspirillum, a bacterial genus that is typically found in plants [382] and has not been described in previous studies that have assessed the airway microbiota in either humans or mice [116, 122, 355, 356]. Using BAL performed by a more sterile technique we found that the 16S rRNA yield was extremely low and thus unsuitable for pyrosequencing. Studies have also reported that pooling multiple BAL samples or sampling the upper respiratory tract by nasal lavage can allow successful characterisation of the mouse airway microbiota [95, 99] but we found low 16S qPCR copy number for both these approaches and were thus also unable to sequence such samples. A study by Goulding et al successfully characterised the mouse microbiota in BAL samples by clone library analysis.[199] This technique is recognised to be more sensitive than 16S pyrosequencing methods but suffers from limitations of cloning bias and is labour intensive and relatively more expensive.[118] A recent study by Barfod et al described successful characterisation of the mouse microbiome in BAL samples using pyrosequencing. In order to circumvent the difficulties of low bacterial 16S rRNA content in BAL samples, they performed an additional PCR step to increase amplification.[355] Although this appeared to allow successful 16S pyrosequencing, the additional PCR step is very likely to increase the chance of contamination and the authors do not present any sequencing data from negative controls to rule out the possibility of universal contamination. It is notable that they reported the presence of several bacterial genera including Polaromona, Schlegella and Brochothrix that are considered to be environmental bacteria and have not been reported in samples from human studies.[355]

Having observed low 16S rRNA yields in airway lavage samples from mice, we next focussed on harvested lung tissue samples. We found that lung tissue had a higher bacterial yield than BAL with ~2 log increase in 16S qPCR copy numbers in lung tissue samples, regardless of the quantity of tissue used to extract DNA from. Although the minimum threshold of qPCR copies required to allow 16S pyrosequencing is currently unclear, these data suggest that use of lung tissue may be a more
feasible approach to characterisation of the lower respiratory tract microbiota in mice than using BAL samples. In the study by Barfod et al described above, successful characterisation of the microbiota in lung tissue was also reported, using a similar protocol to that used for BAL samples.\textsuperscript{355} However, as the authors discuss, use of lung tissue samples may be complicated by the occurrence of non-specific binding of 16S primers due to low amounts of bacterial DNA with large amounts of host eukaryotic nucleic acids and this adds yet another level of complexity to this technique.

6.3.4 Evaluation of bacterial loads in lung tissue following administration of FP and/or RV1B infection

Although we were unable to proceed with 16S pyrosequencing of samples in the FP + RV1B model to directly test our hypothesis that FP administration in combination with RV1B infection would precipitate disturbance of the respiratory microbiome, we were able to use the 16S qPCR assay to assess effects on the total bacterial loads in lung tissue following these manipulations. We observed an early and transient fall in lung tissue bacterial loads in mice challenged with RV1B compared to UV–RV1B treated controls at 8h post-infection. This early effect correlates temporally with the peak expression of many anti-bacterial mediators following RV infection in the mouse model. Therefore, it is possible that, through up-regulation of these mediators, RV infection reduces bacterial loads in the lung. We did not, however, observe any differences between RV1B and UV-RV1B challenged mice at timepoints later than 8h. Molyneaux et al reported that experimental RV infection in patients with COPD led to a 6 fold rise in 16S copy numbers in sputum but saw no difference in bacterial loads associated with RV infection in healthy controls.\textsuperscript{122} Molyneaux et al did not assess 16S qPCR copies at earlier timepoints than d15 post-infection \textsuperscript{122} and so it is unclear if a similar early reduction in bacterial loads occurs in human models. Notably, mediators such as IL-6 and TNF-α peak around d9 in the human model of RV infection (P.Mallia, unpublished observations) and therefore assessment of bacterial loads at earlier timepoints may have demonstrated similar reductions to those observed in the mouse model.

Assessment of bacterial loads following FP administration in our model revealed a trend towards a transient increase in bacterial loads at 8h with significant increases observed when FP was combined with RV infection. This raises speculation that FP suppresses anti-bacterial components of host-defence and thus directly alters the microbiota, increasing bacterial loads in the lung. Although we observed no effect of FP and/or RV infection on total bacterial lung loads at later timepoints, it should be noted that these treatments could still be causing alterations in the bacterial community composition without having any effect on the total quantity of bacteria within the lungs.
6.3.5 Exogenous administration of *S. pneumoniae* to model bacterial colonisation

Requirement of a change in sequencing platform prevented progression of work to characterise the lower respiratory microbiota in mice. We therefore employed an alternative strategy of instilling low dose *S. pneumoniae* to directly model bacterial lung colonisation in mice. Optimisation experiments to define the lowest possible infecting dose at which bacteria could be reliably recovered from lung tissue by culture whilst inducing a mild inflammatory response suggested that $2.5 \times 10^5$ CFU was the most appropriate dose. However, we found that there was a narrow threshold between rapid clearance of bacteria at low infecting doses of $1 \times 10^5$ CFU and systemic dissemination with mortality observed in a proportion of mice infected with $2.5 \times 10^5$ CFU and above. A recently published study, which was not available at the time we conducted our optimisation experiments has shown that a number of factors including the strain of *S. pneumoniae*, the volume in which the dose is administered and the strain of mouse used can all influence whether lower airways colonisation can be reliably established.[352] This study showed that *S. pneumoniae* D39, the pneumococcal strain used in our study, was not suitable for accurate modelling of colonisation and use of an alternative strain LGst215 in CBA/Ca mice led to 90% of mice infected having recoverable bacteria in the lungs at 7d post-infection.[352] Therefore, adopting such approaches may have enabled us to establish a more representative colonisation model. Other approaches such as encasing bacteria in agar beads to restrict in vivo phagocytosis and thus prevent rapid clearance from the airways have also been reported to successfully achieve a representative colonisation model [353, 354] but these approaches are more technically difficult.

6.3.6 Effect of FP on low dose *S. pneumoniae* infection

6.3.6.1 Bacterial loads in lung tissue and systemic dissemination

Administration of high dose FP (1mg/kg) prior to infection with $2.5 \times 10^5$ CFU *S. pneumoniae* led to significantly increased bacterial loads in lung tissue at 8h post-infection with a non-significant trend towards an increase also observed at 4 and 24h post-infection. We also observed a non-significant trend towards increased systemic dissemination (bacterial CFU counts in blood) at 24h post-infection. Our findings suggest that ICS use could potentially lead to increased proliferation of existing PPMs such as *S. pneumoniae* in the lungs of patients with COPD who are chronically colonised and thus directly precipitate pneumonia. The relevance of this mechanism to human disease is based on the assumption that the model of low dose *S. pneumoniae* we used is representative of lower airways colonisation in COPD. However, it could be argued that our model is one that is more in keeping with an acute bacterial pneumonia, since systemic dissemination and death occurs in a proportion of infected animals. If indicative of an acute pneumonia model, then
our findings would actually contradict those from some human studies, which have reported that ICS use during established pneumonia in patients with COPD is associated with reduced pneumonia-related complications and improved outcomes.[269, 274-276] However, these studies could not directly assess the effect of ICS on bacterial loads in patients with pneumonia and it is possible that the observed beneficial effects may be due to suppressed inflammatory responses rather than effects on bacteria clearance as reduced systemic inflammatory responses have been reported with ICS use in community-acquired pneumonia.[418] Our finding that high dose FP increases bacterial loads in lung tissue is in keeping with other studies that have assessed the effect of ICS administration in mouse pneumonia models. Patterson et al reported that nebulised FP administration increased bacterial loads in lung tissue and blood following Klebsiella pneumoniae challenge in mice.[283] Wang et al also showed increased bacterial loads in lung tissue associated with nebulised budesonide following challenge with P. aeruginosa in a mouse model of ovalbumin-induced asthma.[303] Neither of these studies accurately characterised the key underlying mechanism of how FP increased bacterial loads, although they report some similarities to our observations of suppressed anti-bacterial mediators including neutrophil chemokines, IL-6 and CRAMP (discussed in sections 6.3.5.2 and 6.3.5.4).

6.3.6.2 Anti-bacterial cytokines and inflammatory cell recruitment and function

The induction of anti-bacterial cytokines and recruitment of inflammatory cells was assessed in the FP + low dose S. pneumoniae mouse model. We observed suppression of the early induction of proinflammatory cytokines and chemokines including IL-6, TNF-α, CXCL1/KC and CXCL2/MIP-2 and suppression of macrophage and neutrophil numbers by FP. Studies have reported similar effects of corticosteroids on inflammatory responses following stimulation by bacterial products in vitro [287-290] and following challenge with live bacteria in vivo.[283, 312] These are all components that are involved in the innate response to bacterial respiratory infection. IL-6 plays a role in delaying neutrophil apoptosis and enhances neutrophil cytotoxic function [419] and mice deficient in IL-6 show increased lung tissue bacterial loads and impaired survival [148]. TNF-α is also a key cytokine in pneumococcal pneumonia and is required for neutrophil recruitment [420] and anti-TNF-α antibody administration in a mouse model of S. pneumoniae infection has been shown to be associated with reduced neutrophil counts and accelerated death [147]. The direct involvement of neutrophils in bacterial clearance has been demonstrated in a mouse model where selective neutrophil depletion increased lung tissue bacterial loads.[149] Therefore, some or all of these suppressed responses may contribute to the increased bacterial loads observed in our model.
In addition to assessment of macrophage numbers, we also evaluated the ability of alveolar macrophages to phagocytose FITC-labelled *S. pneumoniae* ex vivo to assess whether ICS had any effects on this function of alveolar macrophages to explain the effects on bacterial loads observed. We observed no significant difference in %phagocytosis or mean fluorescence intensity (indicating the amount of bacteria taken up by each cell) between alveolar macrophages from FP and vehicle treated mice, suggesting that FP does not affect phagocytosis of bacteria by macrophages. Patterson *et al.* used eGFP-labelled *Klebsiella* to quantify the effect of FP on in vivo phagocytosis in a mouse pneumonia model. They observed no difference between % macrophages that had engulfed bacteria and a trend towards increased mean fluorescence intensity associated with FP treatment.[283] Other studies that have assessed the effect of corticosteroids on macrophage phagocytosis ex vivo report conflicting results with some showing an impairment [280, 281] and others, in keeping with our findings, showing no effect [170].

**6.3.6.3 Pattern recognition receptors**

We also assessed the effect of FP on expression of PRRs by *S. pneumoniae*. Similar to RV infection, we observed that *S. pneumoniae* infection induced TLR2 expression, which was supressed by FP administration. Chu *et al.* reported very similar findings in a mouse model of *M. pneumoniae* infection.[312] TLR2 is an important PRR that recognises lipoteichoic acid and lipoprotein components of the pneumococcal cell wall [138] and our finding that its expression is suppressed by FP is potentially important as this may lead to reduced bacterial recognition and suppressed initiation of anti-bacterial immune responses which could be contributing to the increased bacterial loads we observed in the model. The critical role of TLR2 signalling in host defence against *S. pneumoniae* infection has been highlighted in mouse strains deficient in TLR2 which show impaired bacterial clearance following challenge with *S. pneumoniae*. [139]

**6.3.6.4 Anti-microbial peptides**

The effect of FP administration on expression of a number of AMPs was also evaluated in the low dose *S. pneumoniae* model. Of those assessed, CRAMP was the only expressed AMP that was affected by FP administration. We observed near complete inhibition of CRAMP protein in BAL at 8h post-infection, which matched the temporal kinetic of increased bacterial loads we observed. Cathelicidins are released by neutrophils and epithelial cells [182] and therefore, our finding that FP suppressed levels of CRAMP in the mouse model is perhaps unsurprising as neutrophil recruitment was also significantly suppressed at the same timepoint. CRAMP deficient mice have been shown to have reduced neutrophil recruitment and impaired bacterial clearance following *P. aeruginosa* infection.[421]
6.3.6.5 Interferons

The potentially protective effects of suppressed type I and II IFN induction on virus-induced secondary bacterial infection were discussed previously in section 6.3.2. Similar to the RV model, we also observed that FP suppressed the induction of IFN-β and –γ by S. pneumoniae infection. However, where this effect may protect against secondary bacterial challenge following RV infection, the opposite relationship has been reported for primary bacterial infection. Mice that lack type I IFN signalling (IFNAR -/-) have been shown to exhibit enhanced development of bacteraemia following intranasal pneumococcal infection, while maintaining comparable bacterial numbers in the lung, an effect that may be related to changes in expression of PAFR altering transmigration of bacteria across the lung.[154]

However, our finding that FP increases bacterial lung loads with only minimal effect on systemic dissemination perhaps suggests that the effects we observed are not mediated by suppression of IFN-β. In contrast, IFN-γ deficient mice have increased bacterial loads in lung tissue following infection with S. pneumoniae and therefore type II IFN suppression by FP may potentially be more important in our model.[155] Mancuso et al showed that IFNAR -/- mice had reduced survival following systemic challenge with S. pneumoniae.[422] We could not assess this parameter in our study as we limited endpoint assessment to 24h post-infection.

6.3.6.6 Dose responsive effects of FP on bacterial infection

As discussed in section 6.2.6, the dose of 1mg/kg FP used in the mouse model is likely to be considerably higher than those used in clinical practice. Observational studies have suggested that the risk of pneumonia associated with ICS use in COPD may be dose-dependent with higher doses associated with greater risk.[221, 266, 267, 270] Additionally, a mouse model study which used the same method of dosing via the intranasal route as adopted in our studies, showed the opposite effect with reduced bacterial loads associated with administration of a lower dose of FP (10µg per mouse, 20 fold lower than the doses used in our study).[313] We therefore assessed whether lower doses of FP had differential effects on anti-bacterial responses and bacterial loads following S. pneumoniae infection.

In contrast to high dose (1mg/kg) FP administration, administration of 0.1mg/kg FP prior to S. pneumoniae infection reduced bacterial loads in lung tissue compared to vehicle + S. pneumoniae treatment with a trend towards reduction also associated with 0.5mg/kg FP administration. However, the ability of lower doses of FP to suppress induction of anti-bacterial cytokines IL-6 and TNF-α and neutrophil recruitment was no different to high dose FP. A previous study reported that
dexamethasone enhances *H. influenzae* induced upregulation of TLR2 in an ear epithelial cell line [391] and this has led to the hypothesis that glucocorticoids may prime the innate immune system for rapid activation and enhancement of the acute phase response[423]. We therefore assessed whether lower doses of FP also increased TLR2 expression in our model but did not find this to be the case. In a clinical context, these data provide some mechanistic insight into how ICS may increase pneumonia risk in COPD and also suggest that lower doses of ICS may potentially retain beneficial anti-inflammatory effects without causing detrimental effects on bacterial clearance.

### 6.3.7 Limitations of FP and *S.pneumoniae* infection mouse model

A major limitation of the *S.pneumoniae* infection model was that, even at the low infecting doses used, it was more representative of a phenotype of severe pneumonia/sepsis rather than lower airways colonisation. This prevented formal testing of the hypothesis that ICS administration would lead to proliferation of colonising pathogenic species and thus predispose to increased risk of pneumonia and limits extrapolation of findings to human disease. This model also suffers from similar limitations regarding the differences in intranasal FP dosing method employed in mice compared to daily inhaled dosing in humans as previously discussed with the FP and RV1B infection model (see section 6.2.9).

### 6.4 Summary / Conclusions

- Four dose elastase/LPS administration in mice does not accurately model the inflammatory changes or anti-viral immune responses associated with RV-induced exacerbation of COPD in humans.
- Single dose elastase administration in mice more accurately models the inflammatory changes of RV-induced exacerbation but is also not representative of the impaired virus control that is thought to be indicative of human disease.
- Administration of inhaled FP impairs type I and III IFN responses to RV infection which is causally related to impaired virus control.
- Administration of inhaled FP has the potentially beneficial effect of suppressing virus-induced airway inflammation but also impairs adaptive immunity and increases mucin expression, which may have detrimental effects in the context of RV exacerbations of COPD.
• Inhaled FP suppresses a number of components of anti-bacterial host defence in the lungs following infection with *S. pneumoniae* including TLR2 expression, anti-bacterial cytokine and CRAMP production, airway neutrophil recruitment and type I and II IFN expression.

• High dose FP increases 16S bacterial copy numbers in the lung of naïve mice and also increases lung tissue bacterial loads following challenge with *S. pneumoniae*.

• Lower doses of FP have the opposite effect to high dose FP, causing reduced lung tissue bacterial loads following challenge with *S. pneumoniae*.
6.5 Future work

6.5.1 RV-induced exacerbation of COPD model

In chapter three, we presented an assessment of a number of different protocols for modelling RV-induced exacerbation of COPD. Although we report a novel model of single dose elastase challenge in combination with RV1B infection that mimics a number of key aspects of human disease, we were unable to define a model that recreated all the key elements of human disease.

6.5.1.1 Further characterisation of single dose elastase model

There are a number of additional features of stable and exacerbated COPD that were not assessed in the single dose elastase model that we established that are relevant to human disease and, if present, would increase the clinical validity of the model. Characterisation of lymphocyte sub-populations by flow cytometry would allow a more detailed assessment of the observed enhancement in lymphocytic inflammation and permit an assessment of whether the model mimics the increased CD8+ T cell recruitment that has been reported in the human experimental model of RV infection in COPD.[84] Other disease-relevant features of human COPD that could be evaluated in the model would include measurement of markers of oxidative stress, which have been reported in cigarette-smoke exposure models.[329] Additionally systemic inflammation [424] and extrapulmonary disease manifestations such as skeletal muscle atrophy and cardiac dysfunction are features that have also been reported in cigarette smoke exposure models [424-427] but not extensively studied in elastase models of COPD.

We used non-invasive lung function measurement techniques to show evidence of AHR to MCH challenge in the model. AHR has previously been regarded as a hallmark of asthma but is also observed in COPD.[428] However, we failed to demonstrate any baseline abnormalities (without MCH challenge) with either elastase administration alone or in combination with RV infection and therefore, we were unable to demonstrate the key feature of fixed airflow obstruction in our model of COPD. As discussed previously, invasive techniques may be more sensitive for detection of baseline abnormalities induced by elastase administration and would allow a more comprehensive assessment of single dose elastase as a model of airflow limitation.

6.5.1.2 Alternative models of COPD exacerbation

We were unable to define a mouse model that accurately replicated the deficient anti-viral responses and impaired virus control that is believed to be indicative of human disease. Development of such a model would be desirable to enable formal testing of our hypothesis that combined use of ICS in COPD leads to synergistic suppression of IFN leading to increased RV
replication. Additionally, such a model could also be used to test novel anti-viral therapies with the potential for future use in clinical practice.

One possible explanation for our observation of reduced rather than increased virus loads in all models that we assessed is that the initial inflammatory response evoked by elastase administration primes the immune system so that when RV1B infection occurs 10d later, the virus can be cleared more rapidly. Other previously reported COPD exacerbation models have used increased intervals of up to 21d between elastase administration and bacterial challenge and shown impaired rather than improved pathogen clearance.[348, 349] Therefore, the model may be improved by increasing the interval between elastase and RV challenges to allow characterisation of a more accurate model of deficient anti-viral immune responses and impaired virus control in COPD.

Finally, it may be possible that elastase-induced emphysema in mice is too severe a model to allow similar features to those reported in the human model of RV-induced COPD exacerbation which was conducted in patients with early stage disease.[35] In the absence of human experimental infection studies in patients with more severe disease to draw reference from, a more representative mouse model of early stage COPD, such as cigarette smoke exposure in combination with RV1B infection may potentially offer a more accurate representation of then deficient anti-viral immune responses reported in the existing human model of RV infection in mild COPD. Gualano et al showed that short-term (4 days) cigarette exposure increased virus loads following challenge with H3N1 influenza A [341] and a similar dosing protocol could be attempted with RV1B infection.

As discussed previously (section 6.1.3), a major limitation of the mouse model of COPD exacerbation is the limited validity and relevance of an acute exposure model to human disease. Therefore progression of previous work involving human experimental RV challenge in patients with early stage COPD [35] to a human infection model in more advanced disease would represent the optimum future approach towards characterising the precise anti-viral and inflammatory responses following RV infection in severe COPD.

6.5.2 FP and RV1B infection model

As we were unable to define an accurate model of the impaired virus control associated with human COPD, we used RV infection in wild type (otherwise healthy) mice as a surrogate for an acute exacerbation of COPD to evaluate the effects of ICS administration. However, in clinical practice, healthy patients who develop RV infections are not routinely treated with ICS. Therefore, to increase the validity of our findings to human disease, it would still be of interest to evaluate the effects of ICS administration in our single dose elastase model of COPD exacerbation. Although this may not
represent a valid approach to studying the combined effects of COPD and ICS on anti-viral immune responses, it may be useful for assessment of other parameters that are adversely affected by FP administration such as mucin expression, which was also increased in the single dose elastase + RV model.

We present limited in vitro data on characterisation of the specific components of IFN signalling impaired by FP. It would be interesting to assess the effects of FP on IFN expression in response to other specific agonists of other receptors involved in initial virus sensing and induction of release of type IFN including TLR7 and 8 (R848) and RIG-I (5’ppp₃RNA). Similarly, the effect of FP on IFN-β induced ISG expression in BEAS2B cells could also be evaluated to assess whether ICS also impair the secondary component of IFN signalling, which was suggested by evaluation of control groups from our in vivo recombinant IFN-β administration experiments. Use of small interfering RNAs (siRNAs) to knock down specific genes involved in these signalling pathways (such as adaptor molecules MAVS or TRIF) would further allow a more detailed characterisation of the components that are specifically affected by FP. Such information would be useful to give a clearer understanding of the effects of ICS on innate anti-viral immune responses and potentially facilitate the development of novel agents that preserve the beneficial anti-inflammatory effects of ICS but avoid detrimental effects on anti-viral immune responses. Such agents could be more useful than currently available therapies for prevention of exacerbations and/or as treatment during an acute exacerbation.

Our observation that FP increases RV-induced mucins in the mouse model may be of particular importance in clinical practice, if similar effects were to occur in humans. We were, however, unable to define the precise mechanism of how FP increases the late expression of MUC5AC and MUC5B following RV infection but showed that it was not related to the increased virus loads observed as the reduced virus loads associated with recombinant IFN-β administration had no impact on mucin expression. Previous studies using in vitro models have shown that EGFR-dependent cascades are important in the upregulation of MUC5AC by RV.[368, 369] Using a similar protocol to that described by Hewson et al [368], NCL-H292 cells could be treated with FP and stimulated with either RV or specific agonists such as Poly(I:C) and effects on MUC5AC mRNA expression could be measured by quantitative PCR and EGFR expression assessed by Western blot analysis. A previous study has shown that inhibition of EGFR inhibits TLR3-mediated MUC5AC expression in vitro [369] and therefore, it is possible that FP may increase TLR3- or RV-mediated EGFR expression and thus augment MUC5AC expression. Such mechanistic studies may facilitate development of alternative therapies that have suppressive effects on both inflammation and mucins, which may be more beneficial for the treatment of COPD than existing therapies such as ICS.
We performed a limited assessment of the dose responsive effects of FP in the RV1B mouse model. We were able to identify a dose (0.5mg/kg) which had lesser effects on impairment of anti-viral immune responses but equivalent anti-inflammatory effects to the higher dose (1mg/kg) that was evaluated. However, at the lowest dose we assessed (0.1mg/kg) anti-inflammatory effects were lost. Therefore, future studies in the model could be aimed at performing a more extensive dose evaluation which may lead to identification of a specific dose that retains anti-inflammatory effects but has no effect on anti-viral host responses. This may suggest that similar doses exist in the context of ICS use in humans, although the relative ineffectiveness of these drugs in COPD due to reduced expression of HDAC-2 [229], with usual requirement for use of higher doses in clinical practice may prevent this concept being directly applicable to human disease.

In the longer term, it would be interesting to evaluate the effects of ICS administration in a human model of experimental RV infection in COPD to assess whether similar effects are seen as those observed in the mouse model of primary RV infection. Specifically, it would be important to assess whether inhaled ICS at clinically relevant doses suppresses induction of type I and III IFNs and leads to impaired virus control. Clinically relevant endpoints that may be affected by ICS therapy that could be assessed in a human model would include symptom scores, lung function and airway mucin production. This would allow a more detailed disease-relevant evaluation of the potential adverse effects of ICS therapy during virus-induced exacerbations of COPD.

6.5.3 FP and bacterial infection

Future studies on the effects of FP on bacterial infection would focus on progression of work to characterise the microbiome in the FP + RV1B model and further evaluation of the effects of FP in the low dose S. pneumoniae model.

6.5.3.1 Respiratory microbiota

Our preliminary data showed that RV infection in mice transiently reduced 16S bacterial loads while FP administration, alone or in combination with RV infection, transiently increased bacterial loads. This raises speculation that both RV infection and FP administration may have direct effects on bacterial communities within the lung and potentially lead to proliferation of PPMs, as we hypothesised. Formal characterisation of the specific composition of lung microbiota using 16S pyrosequencing of lung tissue samples from our model of combined FP and RV infection will allow detailed characterisation of the effects of these manipulations. Furthermore, it will be interesting to assess whether immune replacement strategies such as administration of recombinant IFN-β or other recombinant proteins of mediators that were found to be suppressed by FP and may be
relevant to virus-induced secondary bacterial infection, such as pentraxin-3 would be able to positively modulate the microbiota and reduce expansion of PPMs. It would also be useful to assess whether single dose elastase administration models the disordered microbial communities that has been reported in human studies of COPD [114, 116, 429] and also assess the effects of combined FP and RV infection on the lower respiratory microbiota in this model as stated in our original aims.

### 6.5.3.2 Effect of FP in *S. pneumoniae* model

A number of future experiments could be carried out to further study the anti-bacterial effects of ICS in the *S. pneumoniae* model we established. We identified a number of potential mechanisms by which FP suppressed anti-bacterial innate host responses and may thereby lead to the increased bacterial CFUs in lung tissue observed in the model. In a similar fashion to our assessment of recombinant IFN-β in the FP+RV1B model, the FP + *S. pneumoniae* mouse model could be directly used to test cause and effect by assessing whether administration of ICS suppressed mediators can restore anti-bacterial immune responses and reduce lung tissue bacterial loads. Similar to its beneficial effects on virus control in the FP+RV1B model, recombinant IFN-β administration may also have a beneficial effect on the increased bacterial loads associated with FP administration in the *S. pneumoniae* model, especially since a recent study showed that IFN-β can reduce systemic dissemination of bacteria.[154] Additionally, we observed a large inhibitory effect of FP on CRAMP protein levels in BAL, an effect that matched the temporal kinetic of increased bacterial loads observed. Evaluation of the ability of recombinant CRAMP to improve anti-bacterial innate responses would also be of interest and such approaches have been proposed as potential therapeutic strategies in other conditions associated with chronic bacterial infection such as cystic fibrosis.[430]

As with the FP + RV1B mouse model, we performed a limited dose assessment to evaluate the effect of lower doses of FP on *S. pneumoniae* infection. We found that lower doses of FP have opposite effects on bacterial control in the lungs but were unable to define by what mechanism this occurs as the components that we assessed were all similarly suppressed by high and lower doses of FP. A more detailed evaluation of the effect of lower dose FP on the other anti-bacterial components that were assessed with high dose FP such as *ex vivo* assessment of macrophage phagocytosis and CRAMP protein levels in BAL may allow identification of a specific component that is inhibited by high dose FP but enhanced by lower doses. This may also allow us to identify which of the many anti-bacterial responses suppressed by FP is most relevant to the observed increase in bacterial lung loads.
Some studies have suggested that budesonide, the other commonly used ICS in COPD may be associated with lower risk of pneumonia but this finding is controversial.[222, 264, 265] A direct evaluation of comparable doses of budesonide and FP on anti-bacterial host defence and bacterial clearance in the *S. pneumoniae* mouse model would therefore be particularly informative. Additionally, a few studies have shown that LABAs such as salmeterol can also have effects on components of anti-bacterial host defence.[431, 432] Since ICS are frequently prescribed in conjunction with LABAs in COPD, it would also be interesting to assess whether co-administration of salmeterol with FP in our model of bacterial infection would have beneficial or detrimental effects.

In the longer term, evaluation of the effects of commencing ICS on airway bacterial loads and the microbiota in patients with COPD that are colonised and non-colonised with PPMs would allow a more direct and disease-relevant approach. The difficulty with such a study would be identification of an appropriate cohort of patients who are ICS naïve. Alternatively, a recently described approach of experimental *S.pneumoniae* colonisation in healthy human subjects [433] could be adopted to directly study the effects of ICS administration on bacterial infection in patients with early stage COPD. This experimental approach would also allow other important questions and hypotheses to be addressed including whether bacteria can directly trigger exacerbations in COPD, which remains a controversial and widely debated issue [434].

**6.5.3.3 Alternative co-infection models**

An alternative approach to assessment of the effects of FP and/or RV1B infection on the lower respiratory microbiota in mice would be to directly model secondary bacterial infection by sequential infection with RV1B and *S. pneumoniae*. We were unable to define an accurate model of bacterial colonisation using *S. pneumoniae* D39 and therefore had to limit assessment of endpoints to 24h post-infection. As discussed previously, a recent study demonstrated use of an alternative *S. pneumoniae* strain in CBA/Ca mice which more accurately represented lower airways colonisation.[352] Combination of RV infection with this model of bacterial colonisation may allow a more representative investigation into the effects of ICS on RV-induced secondary bacterial infection in COPD. Development of such a model would require considerable optimisation with a number of factors to consider including the specific timing of challenge with RV and bacteria. Previous studies have administered *H. influenzae* 48h after infection with RV1B and showed delayed bacterial clearance in mice.[209] Alternatively, studies in influenza mouse models have administered *S. pneumoniae* at later timepoints after viral challenge.[211, 385] The majority of suppressed anti-bacterial effects in our model of FP + RV1B infection occurred at 8-24h post-infection and therefore this may represent the optimum timepoint to assess, but the literature on influenza and the late
effects on mucin expression observed in our model might suggest that longer intervals should also be assessed. A virus/bacteria confection model could also be combined with our single dose elastase COPD model which would then allow us to directly assess the effect of ICS administration on RV-induced secondary bacterial infection in COPD and thereby directly test our overall hypothesis.
Chapter 7: References


Chapter 7: References


