Investigating the immune responses of COPD lung tissue explants to viral stimuli

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

2015

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Table of Contents

List of Tables 8
List of Figures 10
Abbreviations 13
Abstract 16
Declaration 17
Copyright Statement 18
Acknowledgements 20

Chapter 1. Introduction 21

1.1. Chronic Obstructive Pulmonary Disease 22
1.1.1. Causes 22
1.1.2. Abnormal Inflammatory Response in COPD 23
1.1.3. Microbiome 24
1.1.4. Treatments 27
1.1.5. Exacerbations 29

1.2. Human Rhinoviruses 31
1.2.1. Classification of Rhinoviruses 31
1.2.2. Entry to Cells 33
1.2.3. Virus Replication 34

1.3. Pattern Recognition Receptors 36
1.3.1. Toll-Like Receptors 37
1.3.2. RIG-I-Like Receptors 42
1.3.3. Important Kinases in PRR Signalling Pathways Relevant to COPD Research

1.4. Mechanisms of Detection of Human Rhinoviruses by Pattern Recognition

Receptors

1.4.1. Adaptive Immune Response to RV

1.5. Human Rhinoviruses and Respiratory Disease Exacerbations

1.5.1. Human Rhinoviruses and Exacerbations Association Studies

1.5.2. HRV Infection Studies

1.5.3. Summary

1.6. Review of RV Infection Models

1.7. Hypothesis and Aims

Chapter 2. Materials and Methods

2.1. Patient Information

2.2. Tissue Culture

2.3. ELISA

2.4. RNA Extraction

2.5. Measuring RNA Concentration

2.6. Complementary DNA synthesis

2.7. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

2.8. Statistics

2.8.1. Time Course

2.8.2. Dose response
2.8.3. Combination of TLR Ligands

2.8.4. Effect of Smoking on Cytokine and IFN Protein and Gene Expression

2.8.5. TNFα Neutralisation

2.8.6. Inhibitors

2.8.7. Rhinovirus Stimulation of BEAS-2B Cells

2.8.8. Comparison Between RV Additional Control Samples


3.1. Introduction

3.2. Materials and Methods

3.2.1. Time and Dose-Response of Lung Tissue to TLR ligands

3.2.2. Simultaneous stimulation of TLR3 and TLR7/8

3.2.3. Statistics

3.3. Results

3.3.1. Secretion of Pro-Inflammatory Cytokines

3.3.2. Gene Expression of Pro-inflammatory Cytokines

3.3.3. Gene Expression of Interferons

3.3.4. Simultaneous stimulation of TLR3 and TLR7/8

3.3.5. The Effects of Smoking Status

3.4. Discussion

3.4.1. Pro-inflammatory Cytokine Secretion

3.4.2. Pro-inflammatory Cytokine Gene Expression

3.4.3. Interferon Gene Expression

3.4.4. Simultaneous activation of TLR3 and TLR7/8
Chapter 4. The effects of TNFα, dexamethasone, p38 MAPK, IKK-2 and IRAK1/4 inhibition on innate immune response in human lung tissue explants.

4.1. Introduction

4.2. Materials and Methods

4.2.1. Neutralisation of TNFα

4.2.2. Inhibition of Inflammatory Response to TLR ligands

4.2.3. Statistics

4.3. Results

4.3.1. TNFα Neutralisation

4.3.2. Anti-Inflammatory Drugs

4.4. Discussion

4.4.1. Effect of TNFα Neutralisation on Pro-Inflammatory Cytokine Production

4.4.2. Effect of Anti-Inflammatory Drugs on Pro-Inflammatory Cytokine Production

4.4.3. Effect of Anti-Inflammatory Drugs on Type I and III IFN Gene Expression

4.4.4. Data Variability

4.4.5. Conclusions

3.4.5. Differences between Smokers and COPD

3.4.6. Data Variability

3.4.7. Conclusions

Chapter 4. The effects of TNFα, dexamethasone, p38 MAPK, IKK-2 and IRAK1/4 inhibition on innate immune response in human lung tissue explants.

5.1. Introduction  

5.2. Materials and Methods  

<table>
<thead>
<tr>
<th>Subsections</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.1. Cell culture</td>
<td>148</td>
</tr>
<tr>
<td>5.2.2. Human Rhinovirus Preparation</td>
<td>148</td>
</tr>
<tr>
<td>5.2.3. Stimulation of BEAS2B Cells with Human Rhinovirus</td>
<td>150</td>
</tr>
<tr>
<td>5.2.4. Stimulation of Lung Tissue with Human Rhinovirus</td>
<td>150</td>
</tr>
<tr>
<td>5.2.5. Statistics</td>
<td>152</td>
</tr>
</tbody>
</table>

5.3. Results  

<table>
<thead>
<tr>
<th>Subsections</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3.1. Stimulation of BEAS-2B cells with RV-16 and RV-1B</td>
<td>153</td>
</tr>
<tr>
<td>5.3.2. Preliminary Time and Dose Response</td>
<td>156</td>
</tr>
<tr>
<td>5.1.1. RV Stimulation of Lung Tissue over Time</td>
<td>161</td>
</tr>
</tbody>
</table>

5.4. Discussion  

<table>
<thead>
<tr>
<th>Subsections</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4.1. Rhinovirus Optimisation Experiments</td>
<td>173</td>
</tr>
<tr>
<td>5.4.2. Effect of RV Stimulation on Pro-inflammatory Cytokine Production in Human Lung Tissue</td>
<td>174</td>
</tr>
<tr>
<td>5.4.3. Effect of RV Stimulation on Type I and III Interferon Gene Expression in Human Lung Tissue</td>
<td>180</td>
</tr>
<tr>
<td>5.4.4. The Effect of UV-Inactivated RV and HeLa Extract on Immune Response in Human Lung Tissue</td>
<td>187</td>
</tr>
<tr>
<td>5.4.5. Data Variability</td>
<td>188</td>
</tr>
<tr>
<td>5.4.6. Limitations</td>
<td>189</td>
</tr>
<tr>
<td>5.4.7. Conclusions</td>
<td>189</td>
</tr>
</tbody>
</table>
Chapter 6. Conclusion

6.1. Limitations

6.1.1. Patients

6.1.2. Technical Limitations

6.1.3. Model Limitations

6.1.4. Analysis Limitations

6.2. Future Work

6.3. Final Conclusion

6.3.1. Meeting Project Aims and Objectives

Appendix I. A summary of studies examining the effects of RV infection in airway cells.

Appendix II. Effect of smoking status on the release and gene expression of cytokines and interferons

Appendix III. Pilot results of poly(I:C)-, R848- and combination-induced activation of p38 MAPK and p65 in human lung tissue.

Appendix IV. Haematoxylin and eosin stain of human lung tissue explants.

References
List of Tables

Table 1-1 Classification of particular RV serotypes according to three different criteria... 32
Table 1-2 Examples of ligands that are recognised by Toll-like receptors (TLRs)......... 39
Table 1-3 Examples of ligands that are recognised by RIG-I-like receptors (RLRs)........ 42
Table 1-4 Summary of studies associating rhinovirus with asthma and COPD exacerbations.
.................................................................................................................................................. 52
Table 2-1 Subject demographics. ................................................................................................. 68
Table 2-2 Components of a typical cDNA synthesis reaction. ...................................................... 72
Table 2-3 List of Taqman probe/primers used and volumes required. ........................................ 73
Table 3-1 Demographics of subjects used in poly(I:C) and R848 time course and dose response experiments. ........................................................................................................................................ 81
Table 3-2 Demographics of subjects used in the combination experiment. ................................. 81
Table 3-3 The effect of smoking status on cytokine release in response to increasing doses of poly(I:C) and R848 from samples derived from Smokers group only. ...................................... 98
Table 3-4 A summary of studies examining poly(I:C) and R848-induced levels of TNFα, CCL5 and IL-6........................................................................................................................................... 101
Table 3-5 A summary of studies examining poly(I:C)- and R848-induced pro-inflammatory cytokine gene expression ........................................................................................................................................ 106
Table 3-6 A summary of studies examining poly(I:C)- and R848-induced type I and III IFN gene expression ........................................................................................................................................... 109
Table 3-7 A summary of studies examining combination effect of poly(I:C) and R848 on pro-inflammatory cytokine release ........................................................................................................... 111
Table 4-1 Demographics of subjects used in TNFα ......................................................................... 118
Table 4-2 Demographics of subjects used in the inhibition ............................................................. 119
Table 5-1 Demographics of subjects used in the RV infection experiment ................................. 152
Table 5-2 A summary of studies that examined TNFα, CCL5 and IL-6 protein expression in response to RV infection.................................................................................................................. 178

Table 5-3 A summary of studies that examined type I and III IFN gene and protein expression in response to RV infection.................................................................................................................. 183

Table 5-4 A summary of studies examining RV-stimulated IFN responses in control vs diseased (asthma or COPD) subjects.................................................................................................................. 186

Table I-1 A summary of selected studies that examined the effects of rhinoviruses on (primarily) airway cells.................................................................................................................. 203

Table II-1 The effect of smoking status on cytokine release in response to poly(I:C) and R848 over time................................................................................................................................. 218

Table II-2 The effect of smoking status on cytokine gene expression in response to poly(I:C) and R848 over time............................................................................................................................. 219

Table II-3 The effect of smoking status on IFN gene expression in response to poly(I:C) and R848 over time............................................................................................................................... 220

Table II-4 The effect of smoking status on cytokine release in response to increasing doses of poly(I:C) and R848 (Smokers and COPD combined).............................................................................. 221

Table II-5 The effect of smoking status on cytokine release in response to increasing doses of poly(I:C) and R848 from patients with COPD only............................................................................... 222

Table II-6 The effect of smoking status on the cytokine release in response to poly(I:C) alone, R848 alone, or the combination in three subgroups: combined Smokers and COPD, Smokers only and COPD only................................................................................................................................. 223
List of Figures

Figure 1-1 Simplified diagram of COPD pathogenesis.................................................. 24
Figure 1-2 Simplified diagram of RV uncoating inside human cells............................... 34
Figure 1-3 A simplified diagram of RV replication in a host cell...................................... 35
Figure 1-4 A simplified diagram of Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) signalling in a human cell................................................................. 41
Figure 1-5 Simplified diagram of rhinovirus recognition by a human cell......................... 48
Figure 1-6 Schematics of various tissue models used for respiratory research.................. 63
Figure 3-1 Production of pro-inflammatory cytokines in TLR-stimulated human lung tissue over time.............................................................................................................. 85
Figure 3-2 Production of pro-inflammatory cytokines from human lung tissue in response to increasing doses of TLR ligands........................................................................ 87
Figure 3-3 Expression of pro-inflammatory cytokines in TLR-stimulated human lung tissue over time.............................................................................................................. 90
Figure 3-4 Expression of type I and III IFN in TLR-stimulated human lung tissue over time.......................................................................................................................... 93
Figure 3-5 Effects of simultaneous activation of TLR3 and TLR7/8 on pro-inflammatory cytokine production in human lung tissue.......................................................... 96
Figure 4-1 Effects of TNFα neutralisation on pro-inflammatory cytokine production from human lung tissue.................................................................................................. 121
Figure 4-2 Effects of dexamethasone on the release of pro-inflammatory cytokines from TLR-stimulated human lung tissue................................................................. 123
Figure 4-3 Effects of p38 MAPK inhibitor on the release of pro-inflammatory cytokines from TLR-stimulated human lung tissue................................................................. 125
Figure 4-4 Effects of IKK-2 inhibitor on the release of pro-inflammatory cytokines from TLR-stimulated human lung tissue. ................................................................. 127

Figure 4-5 Effects of IRAK1/4 inhibitor on the release of pro-inflammatory cytokines from TLR-stimulated human lung tissue. ................................................................................. 129

Figure 4-6 Effects of dexamethasone on the expression of type I and III IFN in TLR-stimulated human lung tissue. ........................................................................................................ 130

Figure 4-7 Effects of p38 MAPK inhibitor on the expression of type I and III IFN in TLR-stimulated human lung tissue. ........................................................................................................ 131

Figure 4-8 Effects of IKK-2 inhibitor on the expression of type I and III IFN in TLR-stimulated human lung tissue. ........................................................................................................ 132

Figure 4-9 Effects of IRAK1/4 inhibitor on the expression of type I and III IFN in TLR-stimulated human lung tissue. ........................................................................................................ 133

Figure 4-10 The effects of inhibitors on TLR3 and TLR7/8 pathway taking into account MDA-5 as an alternative dsRNA sensor. ................................................................. 144

Figure 5-1 Release of pro-inflammatory cytokines from BEAS-2B cells in response to poly(I:C) and a range of RV-16 and RV-1B concentrations. ................................................................. 154

Figure 5-2 Release of pro-inflammatory cytokines from BEAS-2B cells in response to poly(I:C) and extended range of RV-16 and RV-1B concentrations. ................................................................. 155

Figure 5-3 Release of TNFα from human lung tissue in response to poly(I:C) and a range of RV-16 and RV-1B concentrations. ................................................................. 158

Figure 5-4 Release of CCL5 from human lung tissue in response to poly(I:C) and a range of RV-16 and RV-1B concentrations. ................................................................. 159

Figure 5-5 Release of IL-6 from human lung tissue in response to poly(I:C) and a range of RV-16 and RV-1B concentrations. ................................................................. 160

Figure 5-6 Release of pro-inflammatory cytokines in response to RV-16 from human lung tissue over time. ........................................................................................................ 162
Figure 5-7 Effects of using UV-RV-16 and HeLa extract on pro-inflammatory cytokine release from human lung tissue................................................................. 164

Figure 5-8 Release of pro-inflammatory cytokines in response to RV-1B from human lung tissue over time........................................................................................................ 165

Figure 5-9 Effects of using UV-RV-1B and HeLa extract on pro-inflammatory cytokine release from human lung tissue........................................................................................................ 167

Figure 5-10 Expression of type I and III IFN in response to RV-16 in human lung tissue over time............................................................................................................................. 169

Figure 5-11 Effects of using UV-RV-16 and HeLa extract on type I and III IFN gene expression in human lung tissue. ........................................................................................................ 170

Figure 5-12 Expression of type I and III IFN in response to RV-1B in human lung tissue over time............................................................................................................................. 171

Figure 5-13 Effects of using UV-RV-1B and HeLa extract on type I and III IFN gene expression in human lung tissue. ........................................................................................................ 172

Figure III-1 Effect of TLR ligands on p38 and p65 activation in the human lung tissue.228

Figure IV-1 WTE samples stained with haematoxylin and eosin........................................ 231
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
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<tr>
<td>FEV1</td>
<td>Forced Expiratory Volume in 1s</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule 1</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IKK</td>
<td>IkappaB Kinase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 Receptor Associated Kinase</td>
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<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-Stimulated Response Element</td>
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<tr>
<td>JNK</td>
<td>c-Jun N Terminal Kinase</td>
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<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>LDLR</td>
<td>LDL Receptor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>MyD88</td>
<td>Myeloid Differentiation Primary Response Gene (88)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-kappa B</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-Binding Oligomerisation Domain</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polyinosinic:Polycytidylic Acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic Acid-Inducible Gene</td>
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<td>RLR</td>
<td>RIG-I-Like Receptor</td>
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<tr>
<td>RV</td>
<td>Rhinovirus</td>
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<tr>
<td>TIR</td>
<td>Toll/Il-1R</td>
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<tr>
<td>TIRAP</td>
<td>TIR Domain Containing Adaptor Protein</td>
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<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
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<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor alpha</td>
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<tr>
<td>TRADD</td>
<td>TNF Receptor Type 1-Associated DEATH Domain Protein</td>
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<tr>
<td>TRAF</td>
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<tr>
<td>TRAM</td>
<td>TRIF Related Adaptor Molecule</td>
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<tr>
<td>TRIF</td>
<td>TIR Domain Containing Adapter Inducing Interferon β</td>
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<tr>
<td>VP</td>
<td>Viral Protein</td>
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<td>WTE</td>
<td>Whole Tissue Explants</td>
</tr>
</tbody>
</table>
Abstract

Rationale Chronic obstructive pulmonary disease (COPD) is one of the leading causes of deaths worldwide. Patients with COPD have episodes of aggravated symptoms called exacerbations caused by pathogens or pollution. Respiratory viruses are associated with a significant number of COPD exacerbations with the most common virus being the rhinovirus (RV). The mechanisms by which RVs trigger COPD exacerbations are still unclear. Using human whole lung tissue explants (WTE), a novel model of RV-induced COPD exacerbations is proposed.

Methods WTE from COPD patients and smokers were initially stimulated with TLR ligands that are known to activate the same receptors as RV: poly(I:C) for TLR3 and R848 for TLR7/8 activation. Pro-inflammatory cytokines and type I and III IFN gene expression was measured by ELISA and qRT-PCR, respectively. A neutralising antibody against TNFα, a corticosteroid, and a panel of inhibitors targeting TLR pathway (p38 MAPK, IKK-2 and IRAK1/4) was applied to the tissue from COPD patients to establish which signalling pathways are responsible for the inflammatory response and IFN release. Explants from COPD patients and smokers was also exposed to two RV serotypes, RV-16 and RV-1B, in order to compare findings with a clinically relevant stimulant.

Results Poly(I:C) and R848 caused a significant increase of protein and gene expression of pro-inflammatory cytokines (TNFα, CCL5 and IL-6). Type I and III IFN gene expression was also significantly increased. Using the two ligands together caused a synergistic release of TNFα and CCL5. Tissue from COPD patients released more pro-inflammatory cytokines and expressed less IFNβ when compared to smokers. TNFα neutralisation inhibited subsequent release of CCL5 and IL-6. Dexamethasone and p38 MAPK inhibitor decreased TLR3- and TLR7/8-induced pro-inflammatory response whereas IKK-2 and IRAK1/4 inhibition had little effect on cytokine release. Dexamethasone and IKK-2 showed limited effect on IFN gene expression whereas p38 MAPK inhibitor significantly decreased and IRAK1/4 inhibition enhanced IFN expression. RV-16 induced modest but significant pro-inflammatory response in lung tissue, whereas RV-1B did not induce cytokine release. Both serotypes induced type I and III IFN gene expression. Tissue from COPD patients showed a lower expression of IFNβ and IFNλ when compared to smokers.

Conclusion This tissue explant was responsive to both synthetic TLR ligands and RV. The release of pro-inflammatory cytokines in response to TLR stimulation was partially inhibited by steroid. p38 MAPK is involved in TLR-induced inflammation but it also further decreases the already impaired IFN gene expression in COPD tissue. The role of IKK-2 and IRAK1/4 in TLR-induced innate immune response remains unclear. This model is a valuable system to study the mechanisms underlying RV-induced COPD exacerbations and also to test new inhibitors in the whole tissue environment.
Declaration

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Dla Rodziców, za wsparcie i pomoc,

i dla Bartka, za motywację i wiarę w moje możliwości.
Acknowledgements

I would like to thank my Supervisory Team: Prof Dave Singh, Prof Mark Lindsay and Dr Sarah Herrick for their guidance and encouragement during the past four years. Thank you Dave for giving me the freedom to explore my own ideas and for supporting my choices throughout the programme. Thank you Sarah for your psychological support during difficult times and for your reassurance in every step of the way. Thank you Mark for always being there for me, for your input in my project and for making the start of my PhD fun. It would not have happened without any of you. Many thanks to my advisor, Prof Dave Thornton, for giving me some special tips.

I would also like to thank the members of Airway Pharmacology Group, especially Simon Lea for his day-to-day availability and help in organising my experiments and (most importantly) statistics! Special thanks to Tabs, Jenny and Jonathan for giving me extra motivation and a helping hand when I needed it most! And lastly, I thank my sponsors: BBSRC and Pfizer.
Chapter 1. Introduction

Chronic Obstructive Pulmonary Disease (COPD) affects millions of people worldwide [95]. It is characterised by abnormal inflammatory response, airway obstruction and remodelling that cause difficulty in breathing [54]. Many patients affected with COPD suffer from intermittent aggravations of symptoms [203, 400]. These sporadic episodes called exacerbations frequently result in patient hospitalisation and may lead to death [400].

Respiratory disease exacerbations can be caused by microorganisms, including viruses, bacteria, fungi and protozoa [313, 350, 401]. Human rhinoviruses (RVs), which are a major cause of the common cold [230], were shown to be one of the most important triggers associated with COPD (approximately 30%) [138, 339] and asthma (66% of cases associated with RV) exacerbations [178]. These findings suggest RVs to be a model organism to study the mechanisms of induction of respiratory disease exacerbations.

The key components of, and events that lead to, respiratory disease exacerbations have been studied using different models, such as cell culture [279], mouse models [27] and human in vivo challenges [233]. However, the mechanisms by which rhinoviruses induce these exacerbations are still poorly understood. This project aims to establish a new human model involving RV infection of human lung tissue. For the first time, this approach will allow for the interaction between various cell types present in lung tissue and give a more physiologically relevant view of the processes that occur during RV infection in COPD.

The following subsections will aim to, firstly, outline the pathogenesis of COPD, describe the human rhinoviruses and describe and explain the complicated pathogen-host interactions. Secondly, RV infection and RV-associated exacerbations will be discussed. Lastly, the methods that could be used to study RV infection will be reviewed.
1.1. Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of death in the world. It is mostly a preventable disease characterised by irreversible airflow limitation and abnormal airway inflammation [137]. According to the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD), COPD is diagnosed with the use of spirometry and should be suspected in patients who have dyspnoea, chronic cough and sputum production. There are also 4 GOLD stages of COPD that classify the severity of the disease as well as provide guidance to physicians on the most appropriate treatments [137].

1.1.1. Causes

The development of COPD is influenced by many factors, both environmental and genetic. Inhalation of noxious gases, particularly cigarette smoke, and the subsequent abnormal inflammatory response to these gases is thought to be the most common situation when a patient develops the disease [407]. However, not all smokers acquire COPD suggesting a major role for genetic predisposition in the development of COPD in susceptible individuals. Similarly, it is not necessary to be a smoker to acquire COPD. Passive inhalation of cigarette smoke can increase the risk of COPD and also smoking during pregnancy may predispose the foetus to the development of COPD later in life [88, 180].

Cigarette smoke is not the only factor associated with the development of COPD. Other noxious gases, including cigar and pipe smoke, and environmental and occupational exposures, e.g. dusts and chemical fumes, may also lead to COPD in predisposed individuals [197, 237, 242, 261, 315, 343, 385]. Indoor and outdoor pollution is another contributing factor, especially in poorer states where burning coal and biomass are the main source of household energy [428]. In addition, respiratory infections and chronic bronchitis also contribute to the decline of lung function and the subsequent development of COPD: childhood infections were shown to
influence lung function in adulthood [177] and persistent mucus production was correlated with an increased risk of developing COPD later in life [390].

These observations show that a variety of factors will influence an individual’s predisposition to COPD. Since COPD is estimated to become the third leading cause of deaths by 2030, and is a significant economic burden to health services worldwide [408], it is necessary to take steps that will limit the progression of disease and thus the associated social and economic burden as well as educate people to prevent the development of COPD in susceptible individuals.

1.1.2. Abnormal Inflammatory Response in COPD

The development of COPD occurs when an individual has an abnormal inflammatory response to inhaled noxious gases. Chronic inflammation in COPD is characterised by an increased number of inflammatory cells in the lung as well as emphysema and tissue fibrosis. The increased number of both macrophages and neutrophils in the lungs have been correlated with the severity of COPD [97, 192]. These cells release pro-inflammatory cytokines (e.g. tumour necrosis factor (TNF) α or interleukin (IL)-6) and proteases which cause structural changes in the lungs that limit the airflow (see Figure 1-1). Epithelial cells also respond to smoke inhalation; they release pro-inflammatory cytokines, including TNFα and CXCL8, which attract macrophages and neutrophils to the site [337]. Macrophages in turn release more TNFα, GM-CSF and CXCL8 and therefore play a role in attracting even more neutrophils [96, 360]. Neutrophils release elastase which seems to be the most important protease in COPD [346, 432]. Longer survival of neutrophils in the airways has also been associated with the increased levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) [82]. Moreover, cigarette smoke may also prolong the lifespan of neutrophils [9, 411]. Therefore the conditions present in the smoker’s lungs favour longer survival of neutrophils and activation of macrophages which in turn release larger quantities of cytokines and elastase and thus contribute to airway inflammation, fibrosis and remodelling.
Increased pro-inflammatory mediators also lead to the development of comorbidities such as ischaemic heart disease or diabetes mellitus. Other cell types, such as eosinophils and lymphocytes, are also increased in COPD lungs however their role in COPD pathogenesis is less well defined.

Figure 1-1 Simplified diagram of COPD pathogenesis.

Cigarette smoke causes increased pro-inflammatory cytokine and chemokine release. Immune cells, e.g. macrophages and neutrophils, receive signals from stressed epithelial cells and migrate to the airway. They also release a plethora of pro-inflammatory signals thus contributing to the inflammation and tissue destruction and recruitment of inflammatory cells. Repeated exposure to smoke makes the structural changes irreversible.

CCL – chemokine (C-C motif) ligand; CXCL – chemokine (C-X-C motif) ligand; GM-CSF – granulocyte-macrophage colony-stimulating factor; IL – interleukin; MMP – matrix metalloproteinase; TNF – tumour necrosis factor.

1.1.3. Microbiome

Recently it has been demonstrated that lungs are not a sterile environment as previously thought but in fact are colonised with resident bacteria and fungi; and these, along with possible resident viral species, form what is now considered the lung microbiome [100]. Moreover, changes in lung microbiome are thought to play a role in COPD exacerbations [250]. Therefore
the understanding of the microbiome and its role in disease is pivotal in elucidating the mechanisms that lead to development of chronic respiratory illnesses, including COPD.

To date, there have been several attempts to identify particular bacterial and fungal species of the normal human lung microbiome [73, 74, 84, 111, 257]. Several bacterial phyla, including Bacteroidetes, Firmicutes and Proteobacteria were shown to be the most prominent members of the healthy lung microbiota. In addition, Aspergillus spp. and Candida spp. were among the most common fungal species found in the healthy human lung [74]. The majority of studies used either BAL or lung explant samples and showed that there are distinct species resident in the upper and lower airways within the same donor [257]. In addition, it was also observed that there can be multiple microenvironments within the same lung [111], suggesting that local infections or other kind of damage (perhaps caused by cigarette smoke) may produce a favourable setting for particular bacterial strains to thrive in. For example, in one lung sample Haemophilus spp. were most abundant, whereas Pseudomonas spp. were most commonly found in a sample from a different location (both taken from the same lung) [257].

Cigarette smoking is one of the most important causes of COPD development. Interestingly, smoking may also influence the microbiome of the airways. Charlson et al. found significant differences in the composition of airway microbiome between non-smokers and current smokers [73]. This study compared the bacterial species found in nasopharynx and oropharynx. Others, however, did not find significant differences in the composition of the microbiome either in BAL samples or in oral washes [111, 257]. It has been suggested that early changes in the microbiome in smokers with normal lung function may be one of the factors that will later lead to COPD [111]. This hypothesis does require more longitudinal studies to assess if and how changes in bacterial composition of the airways may contribute to the development of COPD.
Bacterial infections are common in COPD patients. However, studies examining COPD lung microbiota suggest that even when there are no symptoms of infection, there is evidence of increased *Haemophilus* spp. colonisation of the lungs [111]. In addition, *Pseudomonas, Streptococcus* and *Prevotella* were commonly found in the lungs of patients with COPD and changes in the composition of the microbiome were associated with exacerbations [60, 250, 296]. For example, in severe COPD patients at exacerbation there was a 20% increase in several bacterial genera, including *Haemophilus, Streptococcus, Pseudomonas* and *Neisseria* [250]. These observations suggest that there may be a link between changes in bacterial composition of the lung microbiome and the occurrence of exacerbation. It has been proposed that acquisition of new bacterial strains may be the cause of bacteria-induced exacerbations and perhaps studies of the microbiome will elucidate the mechanisms of these exacerbation [101]. Interestingly, it was also found that rhinovirus exposure causes an increase in *Proteobacteria* in COPD patients, but not in healthy controls [253]. In addition to the bacterial studies above, there is also increasing evidence of fungal colonisation of COPD lungs. For example, in severe cases of COPD there is an increase in *Pneumocystis* colonisation [84, 256]. These studies suggest that there is a link between microbiome and chronic respiratory disease and that interaction between various classes of microorganisms may influence the development and progression of COPD.

In summary, the role of microbiome in COPD and other chronic respiratory diseases is poorly understood. There seems to be a link between specific microorganisms and COPD however whether these are acquired because COPD has already developed or whether these pathogens played a role in COPD development is still to be elucidated. Nevertheless, the existence and possible influence of microbiome in COPD should be appreciated in any COPD research.
1.1.4.  Treatments

1.1.4.1.  Bronchodilators

There are several classes of drugs currently in use for the treatment of COPD [137]. There are two types of bronchodilators commonly used: β₂-adrenoceptor agonists and anticholinergics. β₂-agonists act on the β₂-adrenoceptor which is present in many cell types found in the human lung [92]. The action of the drug is most desired on airway smooth muscle cells where it leads to muscle relaxation [207]. Anticholinergics, however, act on muscarinic receptors on nerve cells where they compete with acetylcholine to, as in the case of β₂-agonists, relax the airway smooth muscle [19]. Another class of drugs – methylxanthines – are also bronchodilators but they have some anti-inflammatory properties as well. The most known and commonly prescribed methylxanthine is theophylline which relaxes airway smooth muscle and decreases pro-inflammatory signals via the inhibition of phosphodiesterases (PDEs) [294, 358]. Methylxanthines, however, are not the primary recommended treatment for COPD as they have numerous side effects and are less efficient at airway relaxation than the newer drugs [21, 344].

1.1.4.2.  Glucocorticosteroids

Glucocorticosteroids (glucocorticoids, corticosteroids, GCs) are another class of drugs used in COPD therapy. Glucocorticoids are hormones naturally produced in the human body by the adrenal glands. They are released as a counteractive mechanism during inflammation/injury to aid the restoration of homeostasis. Due to anti-inflammatory nature of their action, synthetic corticosteroids have been developed. Corticosteroids bind to the cytoplasmic glucocorticoid receptor (GR) which is expressed by the majority of cells [269]. In cytoplasm, GR exists as a homodimer and is stabilised by several molecules, including heat-shock proteins [297].

Corticosteroids bind to its receptor in the cytoplasm which causes the dissociation of the heat-shock proteins and the GC-GR complex is able to move freely to exert its anti-inflammatory effects. Free GC-GR complex will translocate to the nucleus where it will bind to
glucocorticoid response elements (GREs) and (1) initiate the transcription of anti-inflammatory signals, e.g. IL-10 (transactivation) and (2) bind to GREs present at sites where pro-inflammatory transcription factors will bind thus blocking the transcription of pro-inflammatory cytokines (classical transrepression) [2, 20]. The GC-GR complex can also interact with pro-inflammatory transcription factors, such as nuclear factor (NF)-κB, and prevents them from starting transcription of their target genes (also transrepression) [2, 20, 213, 306]. Corticosteroids were also shown to interact with various proteins and receptors outside of the nucleus (e.g. direct activation of phosphatidylinositol-3 kinase or Akt to activate endothelial nitric oxide synthase) (non-genomic effects) [146].

The results of GC action are not always anti-inflammatory: high doses of GCs or prolonged use have been shown to produce side-effects such as osteoporosis and increased risk of heart disease [89, 357]. Glucocorticoids are the most commonly prescribed anti-inflammatory treatment for COPD. However, evidence shows that stable COPD patients are unresponsive to steroid treatment, possibly due to cigarette smoke interfering with corticosteroid action [166, 418]. Although the exact mechanisms of GC resistance are still unclear, it is thought that oxidative stress caused by smoking can decrease the histone deacetylase (HDAC) activity causing increased transcription of pro-inflammatory genes [166]. Other mechanisms responsible could be genetic predisposition or phosphorylation of GR by kinases involved in the inflammatory processes (e.g. p38 MAPK) [24].

Steroid treatment in COPD patients does not reduce the rate of FEV1 decline, however, some studies show that patients taking inhaled corticosteroids had reduced exacerbation and mortality rates [56, 64]. In addition, evidence suggests that eosinophils and their chemoattractants are steroid-responsive and that eosinophilia in COPD patients is a good predictor of the successful steroid therapy [14, 15]. This is important in the context of patient responses during exacerbations: patients who receive steroid therapy are therefore less likely to develop symptoms when challenged with a pathogen that would normally cause exacerbation.
1.1.4.3. **Taylored Therapies for COPD**

Current treatment for stable COPD depends on the severity of the disease. Treatment of mild disease may consist of occasional use of bronchodilators, i.e. short-acting $\beta_2$-agonists (salbutamol) or anticholinergics (ipratropium bromide). As the disease progresses and becomes more severe, long-acting $\beta_2$-agonists (salmeterol) or anticholinergics (tiotropium) are introduced. If this therapy is not effective, inhaled corticosteroid (budesonide) or phosphodiesterase-4 inhibitor (roflumilast) in addition to bronchodilator therapy or methylxanthines (theophylline) may be used. Moreover, smoking cessation and vaccination against respiratory infections is recommended in all stages of COPD [137].

Other therapies for COPD, e.g. oxygen therapy, antibiotics or mucolytic agents, will not be discussed here in more detail.

1.1.5. **Exacerbations**

A COPD exacerbation is defined as an abnormal increase of respiratory symptoms “beyond the normal day-to-day variation and leads to change in medication” [137]. COPD exacerbations frequently require patient hospitalisations and can accelerate the progression of disease. Current treatments for COPD exacerbations are almost the same as for the management of stable disease. However, antibiotics and ventilation support are more often used.

Exacerbations of COPD seem to be underreported as patients are used to day-to-day fluctuations of symptoms [341]. However, exacerbations lead to long-lasting (3 months) decline in lung function which is not reversible in all cases [342]. In addition, symptoms of common cold were associated with longer recovery times [342]. COPD exacerbations were also correlated with an increased risk of myocardial infarction and stroke [104]. It is therefore necessary to
educate patients about disease exacerbations so that they can seek appropriate treatment early on to avoid the serious complications.

There are several causes of COPD exacerbations, including pathogens and environmental triggers. However, the most common cause of COPD exacerbations seems to be viral infections [138, 339]. At exacerbations, levels of pro-inflammatory cytokines in sputum, such as IL-6 and CXCL8 are increased when compared to stable COPD, thus the chronic inflammation already present in COPD airways is further enhanced [38].

Exacerbations contribute to disease progression and therefore to accelerated decline in lung function that leads to hospitalisations and death. As viral infections are the most common cause of COPD exacerbation it is necessary to understand how they induce exacerbations in the human lung.
1.2. Human Rhinoviruses

Human rhinoviruses (RVs) are estimated to be responsible for half of the episodes of common cold [152] and a significant number of asthma and COPD exacerbations [121, 339]. RVs mainly invade the upper respiratory tract although they have also been shown to be able to infect lower airways [278].

RVs are single-stranded RNA viruses and belong to the family of Picornaviridae [139]. Their genome of approximately 7200 bases is known to be surrounded by an icosahedral capsid formed from multiple copies of four proteins: VP1, VP2, VP3 and VP4 [320].

1.2.1. Classification of Rhinoviruses

There are over one hundred serotypes of human rhinoviruses known [276] and they can be divided into subgroups based on the following criteria: (1) the type of attachment receptor they use, (2) their genetic composition and (3) their susceptibility to a specific class of antiviral drugs (see Table 1-1). Currently, only the first two classifications are used to describe human rhinoviruses.

According to criterion 1, RVs can be divided into two groups: major and minor. The major group rhinoviruses (e.g. RV-16) gain entry to the cells via the intercellular adhesion molecule (ICAM)-1 receptor [139, 359]; whereas the minor group rhinoviruses (such as RV-1B) use the low density lipoprotein (LDL) receptor to enter the host [157].

Recently a new distinction based on genotyping of RVs (criterion 2) has been proposed and three species were formed: RV-A, RV-B and RV-C. To date, every attempt to infect cell lines with a member of RV-C species has been unsuccessful and, because of this, little is known about the mechanisms by which they can infect humans. However, in a recent study, Bochkov et al. reported that they have successfully infected a human mucosal organ culture with an RV-C strain [44]. They also established that the attachment receptor used by this RV-C is neither
ICAM-1 nor LDL receptor. Consequently, the preferred attachment site for RV-C species remains unknown. In addition, a bioinformatics-based model of RV-C structure was developed by Basta et al. and suggested that this species has a closed hydrophobic pocket (site of drug binding in rhinoviruses A and B) [30]. Increasing evidence suggests that RV-C serotypes are more virulent and more capable of inducing asthma exacerbations [176, 251]. It is therefore important to identify the mechanisms that underlie RV-C infections.

Table 1-1 Classification of particular RV serotypes according to three different criteria.

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Classification according to criterion 1 (C1), 2 (C2) and 3 (C3). M – major group, m – minor group, A – group A, B – group B. 1 – virus more susceptible to WIN 51711, 2 – virus more susceptible to R 61837. Serotypes RV-9, RV-13, RV-49, RV-52, RV-54, RV-81, RV-89 have several subtypes which are classified into the same groups. Adapted from Palmenberg et al. [275].

* RV-87 was identified and reclassified as enterovirus (EV)-68 [42, 165].
Another classification of human rhinoviruses (by criterion 3) divides them into two groups based on their susceptibility to antiviral drugs such as WIN 51711 (disoxaril) and R 61837. Andries et al. showed that various RV serotypes respond differently to various classes of antiviral drugs, despite the fact that WIN 51711 and R 61837 bind to the same hydrophobic pocket on the viral capsid [8]. It has been suggested, however, that the exact shape of this pocket is responsible for the differences in drug binding [30]. This classification is based on the following correlation: rhinoviruses more susceptible to WIN 51711 were called “group A” whereas those more susceptible to R 61837 were denoted “group B”. These groups are represented in Table 1-1 as “1” and “2”, respectively. It can be noted that all of the RV-B serotypes are more susceptible to WIN 51711 whereas the vast majority of RV-A serotypes are more susceptible to R 61837. In addition, minor group rhinoviruses are found only within RV-A serotypes. These observations suggest that the RV characteristics are very closely related to the viral genome and, therefore, the viral structure.

1.2.2. Entry to Cells

Major group rhinoviruses gain entry to the cells via the ICAM-1 receptor (located on the plasma membrane) which belongs to the immunoglobulin (Ig) family [359]. Viral infection was shown to upregulate the expression of ICAM-1 which promotes the spread of the virus [281]. Lymphocyte function associated molecule (LFA)-1, a natural ligand for ICAM-1, is known to promote cytolysis of the cells infected with viruses [238] which can explain why ICAM-1 expression is increased following infection. Major group rhinoviruses are believed to bind to ICAM-1 via the conserved sequences found in the canyons of their capsids [274]. Once bound, the whole complex is thought to be internalised into endosomes where low pH aids the uncoating of the viral capsid [135] and promotes the release of viral RNA (Figure 1-2) [122].

Minor group rhinoviruses use the LDL receptor which is known to be expressed on the plasma membrane only to a minor extent. The exact mechanisms of internalisation of the RV-
LDL receptor complex are not yet fully understood but it is believed that, as in the major group, it is taken into endosomes where the virus particles uncoat and release their RNA [125].

**Figure 1-2** Simplified diagram of RV uncoating inside human cells.

Major and minor group RV bind to their attachment receptors (ICAM-1 and LDLR, respectively) expressed on plasma membrane. Once virus is bound to the receptor it internalises into an endosome. Acidic environment in endosomes leads to virus uncoating and its ssRNA genome is released.

ICAM-1 – intracellular adhesion molecule 1; LDLR – low-density lipoprotein receptor

### 1.2.3. Virus Replication

Once the virus is inside the cell and has uncoated its positive-strand ssRNA genome, it then uses the host cell machinery to replicate (see Figure 1-3). Viral genome encodes four capsid proteins and several non-structural proteins, including proteases and RNA polymerase required for viral replication [170, 379]. Viral replication occurs in the cytoplasm in the viral replication complex and is dependent on the viral polymerase [10]. At the 5’-UTR virus genome has a binding site for eukaryotic ribosomes which begin the translation of viral RNA into one
polyprotein chain [170]. Virus encoded proteases then cleave the polyprotein into capsid proteins that are then used for the assembly of new virus particles [243].

Figure 1-3 A simplified diagram of RV replication in a host cell.

Virus enters host cell using its attachment receptor. Then it uncoats and releases its genome into the cytoplasm. Viral ssRNA is then translated and transcribed in the cytoplasm to obtain new viral proteins and RNA. Proteins are translated as one polyprotein which is cleaved into separate proteins by virus-encoded enzyme. Then, new virus particles are packaged and once these are ready they egress the host cell. Adapted from Whitton et al. [406].

The following section will introduce the concept of pattern recognition receptors as one of the major components of the innate immune system, and will describe the receptors and pathways that are primarily involved in the recognition of human rhinoviruses.
1.3. Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are one of the recently discovered features of the innate immune system and can detect conserved molecular structures or pathogen-associated molecular patterns (PAMPs) present on various types of pathogens, e.g. lipopolysaccharide [295]. These PAMPs are usually the building blocks fundamental to the survival of the pathogen (e.g. cell wall components or nucleic acids) [191]. Consequently, pathogens cannot change their structural components to evade the host recognition system without compromising the stability of their structure and their chance of survival. PRRs can also detect the damage- (or danger-) associated molecular patterns (DAMPs) which can be released by damaged or necrotic host cells, for example formyl peptides from the mitochondria [429]. PRRs are therefore important components of the innate immune system since they can rapidly recognise and induce an inflammatory response to a variety of signals associated with infection and injury.

There exist several families of these receptors, including Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs) [191]. TLRs were shown to recognise ligands from a variety of organisms (cf. Table 1-2). NLRs and RLRs were shown to bind bacterial and viral components, respectively [72, 163, 424, 425]. Finally, CLRs are able to recognise carbohydrates from a variety of organisms [106, 236]. It has been proposed that different receptors from different families may cooperate in detecting components from the same pathogen [83], e.g. different receptors can detect viral components at different stages of infection. It is therefore important not only to understand the mechanisms that drive the response to a particular structural component, but also to identify the ways in which PRRs cooperate with each other to orchestrate an appropriate response to an invading pathogen.

Evidence to date suggests that TLRs and RLRs are the two PRR families responsible for sensing human rhinoviruses [393], and therefore they will be described in more detail below.
Currently, no CLR s have been discovered that are specific for HRV recognition. However, NLR s play an important role in maturation of several inflammatory cytokines, e.g. conversion of pro-IL-1β to IL-1β, and therefore may have an indirect role in the response to HRVs [240]. Due to their lesser involvement in HRV recognition, CLR s and NLR s will not be discussed further.

1.3.1. Toll-Like Receptors

Toll-like receptors (TLRs) were discovered less than 20 years ago [245] but, despite their relatively recent emergence, investigation of their structure and mechanisms of activation have already greatly extended our understanding of how the innate immune system responds to a variety of pathogens. TLRs, which gained their name from the resemblance to Toll receptor found on Drosophila [7], are expressed in the cells of the innate immune system [201, 322] as well as on mucosal epithelium [69, 70, 384]. The TLR family has 10 known human members but they can recognise components derived from numerous human pathogens, including viruses, bacteria, fungi and protozoa. Ligands recognised by TLRs include microbial cell wall components, e.g. lipopolysaccharide (LPS) or lipoteichoic acid (LTA), as well as nucleic acids, e.g. viral single-stranded RNA (cf. Table 1-2).

All TLRs share the same simple structure, namely type I glycoproteins containing 3 domains: 1 – a horseshoe-shaped-like extracellular leucine-rich repeat (LRR) domain that recognises PAMPs, 2 – a transmembrane domain and 3 – an intracellular domain, called Toll/IL-1R homology (TIR) domain which is responsible for downstream signalling [48]. Of the 10 known human TLRs, six are expressed on the plasma membrane and the remaining four are expressed on the membranes of intracellular components such as endosomes or lysosomes [191]. TLRs found on the plasma membrane are usually responsible for recognising PAMPs found on external surfaces of the pathogen, e.g. lipids, whereas receptors found intracellularly bind mainly nucleic acids (see Table 1-2). The strategic location of TLRs on the outside or inside of the cell
therefore increases the chances of the receptor recognising and binding their ligand. The ligand(s) that each TLR responds to and their location are listed in Table 1-2.

The six TLRs expressed on the cell surface are TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10. TLR4 and TLR5 are believed to form homodimers upon the recognition of the ligand [149, 199] whereas TLR2 forms heterodimers with TLR1 [175] and TLR6 [181]. As most of the studies examining TLRs have been conducted in mouse models, TLR10 is the least extensively examined TLR found in humans due to the fact that its murine homologue is inactivated by retroviral insertion; however TLR10 is thought to be another extracellular TLR and it was shown to homodimerise [268] as well as form heterodimers with TLR1 and TLR6 [148]. In addition, experiments conducted recently by Lee et al. demonstrate that TLR10 is up-regulated and plays a role in pro-inflammatory response to influenza virus [217].

The remaining four TLRs (TLR3, TLR9, TLR8 and TLR9) are found on intracellular membranes and are mainly responsible for the recognition of the non-self nucleic acids [3, 32, 141, 153, 182, 225, 235]. There are mechanisms in place which prevent self DNA from being recognised by these TLRs [29], however their occasional failure may trigger the onset of such autoimmune disorders as systemic lupus erythematosus [26].

1.3.1.1. TLR Signalling Pathways

The activation of plasma membrane TLRs by PAMPs usually result in the release of inflammatory cytokines (e.g. IL-6) via the activation of nuclear factor-κB (NF-κB), whereas intracellular TLRs induce the release of both inflammatory cytokines and type I and III interferons (IFNs) via the activation of NF-κB and interferon regulatory factors (IRFs), respectively [348, 433]. The only exception to this pattern is TLR4 which, under certain conditions and upon recruitment of different adaptor molecules, can be incorporated into an endosome and work analogously to intracellular receptors inducing the release of cytokines and IFNs [116]. TLR4 (and possibly other TLRs too) is therefore able to adjust its signalling...
pathways depending on the type of ligand it recognises. A simplified diagram of TLR signalling is depicted in Figure 1-4.

Table 1-2 Examples of ligands that are recognised by Toll-like receptors (TLRs).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand(s) (examples only)</th>
<th>Cellular location</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>Lipoteichoic acid [338]</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td></td>
<td>GPI anchor from <em>Trypanosoma cruzi</em> [65]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zymosan from <em>Saccharomyces cerevisiae</em> [329]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipomannan from mycobacteria [391]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haemagglutinin from paramyxovirus [41]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-glucan from fungal cell wall (when additional receptor – dectin-1 recruited) [124]</td>
<td></td>
</tr>
<tr>
<td>TLR2-TLR1 dimer</td>
<td>Triacylated lipopeptides [175]</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>TLR2-TLR6 dimer</td>
<td>Diacylated lipopeptides [181]</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double-stranded RNA (and its synthetic analogues, e.g. poly(I:C)) [3]</td>
<td>Membrane of intracellular compartments</td>
</tr>
<tr>
<td></td>
<td>Small interfering RNAs [182]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Double-stranded RNA from replicating single-stranded viruses [141]</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>Lipopolysaccharide (LPS) [160]</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td></td>
<td>Fusion protein from respiratory syncytial virus (RSV) [210]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibrinogen [354]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Taxol [59]</td>
<td></td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin [134]</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>TLR7 and TLR8</td>
<td>Single-stranded viral RNA, e.g. HIV [153]</td>
<td>Membrane of intracellular compartments</td>
</tr>
<tr>
<td></td>
<td>Small interfering RNAs [158]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNA derived from bacterial species (only on TLR7 expressed on conventional dendritic cells) [235]</td>
<td></td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG DNA (abundant in bacteria and viruses in contrast to the mammalian cells) [32]</td>
<td>Membrane of intracellular compartments</td>
</tr>
<tr>
<td></td>
<td>Viral DNA, e.g. from herpes simplex virus (HSV) [225]</td>
<td></td>
</tr>
<tr>
<td>TLR10, TLR10-TLR1 complex, and TLR10-TLR6 complex</td>
<td>Influenza virus [217]</td>
<td>Thought to be plasma membrane</td>
</tr>
</tbody>
</table>

There are several adaptor molecules involved in TLR signalling that determine which inflammatory mediators are expressed following TLR activation. These adaptor molecules
include: MyD88, TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM) and TOR domain containing adaptor protein (TIRAP). MyD88 is used by almost all TLRs except for TLR3 which uses solely TRIF [413]. TLR5, TLR7, TLR8 and TLR9 recruit MyD88 directly [51], whereas TLR2-TLR1, TLR2-TLR6 and TLR4 bind MyD88 via TIRAP adaptor [412]. TLR4 can also use TRIF via TRAM to activate IRFs [116]. There are therefore two major ways via which TLR can act, namely MyD88-dependent and MyD88-independent pathways, which will be described in more detail below.

**MyD88-Dependent Signalling Pathway**

TLRs that use MyD88 as the primary adaptor molecule are able to induce the expression of inflammatory cytokines via the NF-κB. When a ligand binds to a particular TLR, the receptor recruits MyD88 which in turn recruits IL-1 receptor-associated kinases (IRAKs) [404]. This leads to the activation of NF-κB and mitogen-activated protein kinase (MAPK) which promote the transcription of the target genes, i.e. IL-6, tumour necrosis factor (TNF)-α, and other regulatory molecules (for example those that modulate the actions of NF-κB) [79]. However, the signalling pathway via the intracellular TLRs not only results in NF-κB activation but also induces the expression of type I and III IFNs [126, 334, 381]. Stimulation of type I IFN expression via MyD88 adaptor has been extensively studied in plasmacytoid dendritic cells (pDCs) and it has been shown that IRF7 binds to MyD88 and then forms a complex with other signalling molecules (such as IRAKs) [190].

**MyD88-Independent (TRIF-Dependent) Signalling Pathway**

Similarly to the MyD88 adaptor, TRIF also recruits other molecules to induce the expression of inflammatory mediators and IFNs [330]. Upon the recognition of a ligand, TLR recruits TRIF which forms a complex with TNF receptor-associated factor (TRAF) 6 (and other regulatory molecules, such as TNF receptor type 1-associated DEATH domain protein (TRADD)) [112, 291, 330]. This complex then activated NF-κB and MAPK which in turn induce the expression of inflammatory cytokines [330]. However, when TRIF forms a complex
with TRAF3 (and, similarly as above, other molecules, e.g. IκB kinase (IKK)-i) it activates and promotes nuclear translocation of IRF3. This induces the expression of type I IFNs [270].

**Figure 1-4** A simplified diagram of Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) signalling in a human cell.

Most of extracellular TLRs, i.e. TLR1, TLR2, TLR4, TLR6 recruit MyD88 via TIRAP to activate IRAKs and then NF-κB and MAP kinases. TLR5, however, directly recruits MyD88. This is also done by intracellular receptors TLR7, TLR8 and TLR9 but in addition to activating NF-κB and MAP kinases they also activate IRF3 and IRF7. TLR3 signals only via TRIF which seems to be IRAK-independent. TRIF then complexes with TRAFs to activate pro-inflammatory and interferon pathways. In addition, TLR4 can recruit MyD88 via TRAM or signal via TRIF independently of MyD88. Adapted from Cell Signaling Technology [71]. TLR10 was not included in this diagram as its signalling pathway remains unclear.

Intracellular RNA helicases, i.e. RIG-I, MDA5 and LGP2 recognise pathogen-derived nucleic acids in the cytoplasm. Upon recognition, they associate with IPS-1 located on the mitochondria and signal via TRAF3 to induce the expression of pro-inflammatory cytokines and type I and III IFNs.

CCL5 – chemokine (C-C motif) ligand 5; IFN – interferon; IKK – IκB kinase; IL – interleukin; IPS-1 – interferonβ promoter stimulator-1 (also known as MAVS/VISA/Cardif); IRAK – IL-1 receptor associated kinase; IFN – IFN regulatory factor; LGP2 – laboratory of genetics and physiology 2; LPS – lipopolysaccharide; LTA – lipoteichoic acid; MDA-5 – melanoma differentiation associated protein-5; MyD88 – myeloid differentiation primary response 88; NEMO - NF-κB essential modulator; NF-κB – nuclear factor-kappaB; RIG-I – retinoic acid inducible gene 1; TAK1 – TGFβ-activated kinase 1; TBK1 – TANK binding kinase 1; TLR – Toll-like receptor; TIRAP – Toll/Interleukin 1 receptor (TIR) domain containing adaptor protein; TRAF – TNF receptor associated factor; TRAM – TRIF-related adaptor molecule; TRIF – Toll/IL-1 receptor-domain-containing adapter-inducing IFNβ.
In this section, TLRs and the pathways they employ have been extensively described. The
great variety of TLRs and the PAMPs they recognise, as well as the number of different adaptors
that facilitate downstream TLR signalling suggests that these pathways have been minutely
tailored to ensure that the correct molecules are produces in an attempt to clear a specific type
of pathogen or its components. Next, the family of cytoplasmic helicases (RLRs) will be
introduced.

1.3.2. RIG-I-Like Receptors

RIG-I-like receptors (RLRs) are mainly responsible for sensing nucleic acids (especially
RNA) from a variety of viruses and synthetic RNA analogues such as poly(I:C) (see Table 1-3)
[187]. This family of cytoplasmic RNA helicases consists of only three members: retinoic acid-
inducible gene (RIG)-I, melanoma differentiation-associated gene (MDA)-5 (also known as
interferon induced with helicase (IFIH)-1) and laboratory of genetics and physiology (LGP) 2.
In response to viral infections they activate the mechanisms which induce type I and type III
IFN (IFNα/β and IFNλ, respectively) and inflammatory cytokines, e.g. IL-1β or IL-18 [292]
(see Figure 1-4).

| Table 1-3 Examples of ligands that are recognised by RIG-I-like receptors (RLRs). |
|------------------|------------------|------------------|
| **Receptor** | **Ligand(s) (examples only)** | **Cellular location** |
| **RIG-I** | RNA containing 5’triphosphate (5’ppp RNA) [159] | Cytoplasm |
| | Double-stranded RNA [85] | |
| | Viral double-stranded RNA [424] | |
| | RNA species from negative-sense RNA viruses, e.g. hepatitis C virus, Sendai virus, influenza virus [187] | |
| | Short RNA sequences [33, 188] | |
| **MDA-5** | Poly(I:C) [187] | Cytoplasm |
| | Double-stranded RNA from picornaviruses [187] | |
| | Herpes simplex virus [247] | |
| **LGP2** | Cytosolic DNA [293] | Cytoplasm |
| | Intracellular bacterial DNA (e.g. *Listeria monocytogenes*) [293] | |
| | Viral DNA (e.g. from vaccinia virus) [293] | |
RIG-I and MDA-5 possess two caspase-recruitment domains (CARDs) which are responsible for downstream signalling. They also have a DEHD/H-box helicase domain which recognises RNA species [425]. LGP2, however, lacks the CARD domains and is thought to be a negative regulator of RIG-I [425]. Recently, Pollpeter et al. showed that LGP2 plays a role in recognising DNA from bacteria and viruses [293]. In addition, RLRs were shown to be the most important PRRs in viral detection in conventional dendritic cells, macrophages and fibroblasts [186]. In sum, these receptors have an important role in recognising nucleic acids from pathogens, especially viruses.

1.3.3. Important Kinases in PRR Signalling Pathways Relevant to COPD Research

Currently, the most common treatment to limit inflammation associated with infections in COPD patients are corticosteroids [137]. As the knowledge of the signalling pathways of PRRs expands, specific drugs that will inhibit the action of signal-transducing kinases are being developed. Such drugs will therefore be able to prevent, or at least significantly reduce, the activation of specific pro-inflammatory molecules without affecting other ones. Several kinases involved in PRR signalling are considered as potential targets to reduce chronic and infection-associated inflammation in several diseases, including COPD. They will be described in more detail below.

1.3.3.1. p38 MAP kinase

p38 belongs to the family of mitogen-activated protein (MAP) kinases which are activated in response to various stimuli, including stress signals such as UV light or infections, e.g. response to LPS [427]. Both TLRs and RLRs can activate p38 kinase in their downstream signalling cascade [12]. There are four p38 variants known: α, β, γ, and δ [394]. The former two are ubiquitously expressed in the majority of cells in humans, whereas the latter two are expressed in specific tissues [394].
Activation of p38 MAPK plays an important role in inflammation and apoptosis [427]. p38 MAPK is known to up-regulate the expression of pro-inflammatory cytokines (e.g. TNFα or IL-6) and adhesion molecules (such as VCAM-1 and ICAM-1) [206, 290, 302]. p38 also plays a role in apoptosis when, depending on the signal, it can lead to cell death or promote cell survival [5, 61, 67, 283, 328, 395]. In the cell cycle, increased expression of p38 was shown to decrease cell proliferation [102, 162]. On the other hand, several groups reported that activation of p38 MAPK may lead to increased cell proliferation [103, 228].

The link between p38 MAPK and various inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease, has been established [114, 375]. Evidence also suggests that p38 MAPK expression is increased in patients with COPD and therefore p38 can be one of the factors responsible for the systemic effects of COPD [123, 308]. Although tobacco smoking was shown to decrease pro-inflammatory cytokine release in vitro, smokers with COPD have significantly elevated levels of activated p38 when compared to smokers with normal lung function [75, 308]. These observations have therefore led to extensive research of p38 MAPK in COPD and several p38 inhibitors are now in clinical trials [223, 226, 351].

1.3.3.2. **IKK-2**

IκB kinase (IKK)-2, also known as IKKβ, is a kinase involved in PRR signalling upstream to NF-κB [372]. In the non-activated state, the transcription factor NF-κB remains in the cytoplasm where its active site is masked by IκB protein [46, 347]. Upon signal recognition, PRR signalling can lead to the activation of the IKK complex which will phosphorylate IκB protein thus detaching it from NF-κB. IκB protein is then degraded whereas NF-κB is free to translocate to the nucleus and activate the expression of its target genes, including pro-inflammatory cytokines [184].

Due to the role NF-κB plays in the inflammation, inhibitors of IKK-2 are thought to be a potential treatment to limit the release of pro-inflammatory cytokines [17, 23, 25]. Research
shows that IKK-2 inhibition may be useful in treating chronic diseases e.g. rheumatoid arthritis (IKK-2 inhibition blocks joint degradation) [244]. On the other hand, there are concerns that inhibition of IKK-2 will lead to increased susceptibility to infections [214]. Since NF-κB is an important transcription factor in PRR signalling pathways, IKK-2 inhibition could also be used as treatment during COPD exacerbations.

There exists a link between increased NF-κB activity and stable COPD as well as during COPD exacerbations as demonstrated in studies using macrophages and bronchial biopsies [52, 68, 98, 420]. However data also show that NF-κB activation is also increased in smokers with normal lung function indicating that this effect could be smoking-dependent [52]. Interestingly, cigarette smoke was shown *in vitro* to decrease the pro-inflammatory response (including NF-κB activation) to TLR2 and TLR4 ligands but not to poly(I:C) in alveolar macrophages [75]. On the other hand, recent mouse model study conducted by Rastrick et al. suggested that cigarette smoke does not increase NF-κB activation [305]. Others suggested that components of NF-κB are increased following smoke exposure, but only in aged mice [255]. These observations underscore a still elusive role of NF-κB in COPD development however there is a clear of NF-κB involvement in pathogen-induced exacerbations [68].

**1.3.3.3. IRAKs**

Interleukin-1 receptor-associated kinases (IRAKs) play an important part in TLR (except for TLR3) and in IL-1R signalling. It is thought that activation of IRAKs is required for the pro-inflammatory cytokine gene and protein expression in response to TLR ligands [117]. When the ligand binds to a TLR receptor, IRAK4 and IRAK1 are recruited to the intracellular domain of the receptor and associate with MyD88 through TIR domains [272, 404]. IRAK1 is then activated by phosphorylation and detaches from the IRAK/MyD88 complex to interact with TRAF6 and, subsequently, with TAK1 to activate NF-κB and MAPK signalling [220, 262, 423]. Currently, there are four members of IRAK family known: IRAK1, IRAK2, IRAKM, and IRAK4.
It is thought that only IRAK1 and IRAK4 have kinase activity. Details of IRAK1 and IRAK4 signalling in the TLR pathway still remain to be elucidated. Evidence suggests that these IRAKs are required for IFN expression [366, 383, 419]. Although some of the cytokines (e.g. TNFα) can be decreased in models with deficient expression or activation of IRAKs, NF-κB activation is dependent on IRAKs’ adapter rather than kinase function [241, 367]. IRAK2 and IRAKM are also thought to compensate for the lack of IRAK1 as an adapter molecule in the signalling cascade [221, 405]. Others have also shown that inhibition of IRAK1 may enhance type I IFN expression suggesting that IRAKs can have a different role in innate immunity [6].

In COPD, IRAK signalling was implicated in TLR4-mediated response (increased IL-8 secretion) to cigarette smoke extract in monocyte-derived macrophages [183]. Thus smoke via TLR4 can directly contribute to the recruitment of neutrophils to the lungs. The role of IRAKs in innate immunity is still actively investigated and inter-relations of IRAKs are becoming more complex. Interestingly, rhinovirus infections were shown to increase the expression of IRAK2 and IRAKM and to degrade IRAK1 resulting in decreased cytokine release to other infections [16, 386]. Therefore further research is required to fully understand the involvement of IRAKs during COPD exacerbations.

Other kinases such as JNK and ERK MAPKs also convey signals from TLRs, however, in this thesis the focus is on the three pathways described above.
1.4. Mechanisms of Detection of Human Rhinoviruses by Pattern Recognition Receptors

Previous sections have introduced human rhinoviruses (RVs) as the most frequent cause of common cold and provided the description of proposed virus entry to the cells. The pattern recognition receptors, i.e. Toll-like and RIG-I-like receptors have been shown to play a major role in sensing rhinoviruses. This section will describe how and when viral components are recognised by these innate receptors.

Once viral particles have entered the cells and begin to replicate, the virus’ single stranded- and double stranded-RNA species are then available for the recognition by various pattern recognition receptors expressed either on intracellular membranes (such as TLR3, TLR7 and TLR8) or those present freely in the cytoplasm (e.g. MDA-5) (see Figure 1-5).

Although RVs are single stranded RNA viruses TLR3 has been implicated in the mediation of the response to RV [353]. As already mentioned, TLR3 is able to recognise dsRNA and, in this case, it recognises the RNA that is produced during viral replication. Hewson et al. examined the responses of BEAS-2B cells (human bronchial epithelial cell line) to several serotypes of RV as well as poly(I:C) and found that following TLR3 inhibition, BEAS-2B cells showed a markedly different response to RVs and poly(I:C) [154]. During TLR3 inhibition, RV infection led to an increase in the release of proinflammatory cytokines (e.g. IL-6 and CCL5), whereas stimulation with poly(I:C) resulted in the decrease in the release of these cytokines. They concluded that inhibition of TLR3 leads to an increase in viral replication which in turn activates inflammatory pathways that are independent of TLR3. In addition, TLR3 is proposed to be the receptor responsible for fine-tuning of the antiviral response.
Figure 1-5 Simplified diagram of rhinovirus recognition by a human cell.

Viral capsid can be recognised by TLR2 expressed on the plasma membrane. Virus particles can also bind to their attachment receptors also located on the plasma membrane. Once these are internalised into the endosome and the virus uncoats, its ssRNA genome can be recognised by TLR7. Then the virus begins replication and dsRNA species will be formed as a result. These in turn will be recognised by TLR3. Viral dsRNA will also be released into the cytoplasm where MDA5 will be able to recognise it. All of the aforementioned receptors will activate their respective signalling pathways leading to the translocation of transcription factors to the nucleus and subsequent transcription of their target genes. Pro-inflammatory cytokines and type I and III IFNs will be released from the infected cells.

Moreover, two RNA helicases (members of RLR family), retinoic acid-induced gene (RIG)-I and melanoma differentiation-associated gene (MDA)-5 have been implicated in the mediation of immune response to rhinoviruses. Kato et al. conducted a mouse model experiment (RIG-I and MDA-5 knock-out) and found that MDA-5 is an important mediator in the response to
positive sense single stranded viruses (including those belonging to Picornaviridae family – encephalomyocarditis virus) [187]. They also established that MDA-5 plays a major role in increasing production of type I interferons following the stimulation with poly(I:C). Additionally, TLR3 was shown to be essential for the production of proinflammatory cytokines (e.g. IL-12) in response to poly(I:C). Wang et al. with the use of siRNAs against MDA-5 and RIG-I determined that in BEAS-2B cells MDA-5 and not RIG-I has a major role in recognising RV in addition to TLR3 [393]. These findings correspond to those of Hewson et al. (as described above) that also identified TLR3 as a key receptor in response to RV infection.

In a more recent study, Triantafilou et al. showed that three other TLRs: TLR2, TLR7 and TLR8 as well as MDA-5 are important mediators in the immune response to RV-6 in primary human bronchial epithelial cells [380]. They established that viral capsid is recognised by TLR2, whereas viral RNA is identified by TLR7 and TLR8. They did not report TLR3 as an important receptor involved in RV-6 infection. TLR2 was also shown to mediate replication-independent inflammation in bone marrow-derived macrophages from BALB/c mice [321].

Most of the studies described above underscore the importance of MDA-5 in sensing dsRNA produced during RV replication in the mediation of innate immune response either in mouse models or in bronchial epithelial cells. Overall, from the evidence provided to date, it can be concluded that RV is recognised by multiple receptors, including plasma membrane and intracellular TLRs, depending on the stage of infection. The following subsection will briefly describe the adaptive immune response to viruses and its importance in viral research.

1.4.1. Adaptive Immune Response to RV

Adaptive immune response is another important aspect of antiviral immunity. It is highly specific to particular pathogens and is acquired following infection: antigen presenting cells display pathogen-derived antigens on their surface and therefore activate T and B lymphocytes. In addition, infected cells, e.g. epithelial or endothelial cells, secrete chemokines such as IP-10
which attract lymphocytes to the site of infection [397]. T cells were shown to infiltrate lung mucosa following experimental RV challenge [120]. Although it may take a few weeks to acquire specific immunity against a pathogen, memory cells ensure that subsequent infection is dealt with quickly and without severe clinical symptoms [4, 18]. There is evidence of presence of memory cells and resident T cells in the respiratory mucosa and therefore, if secondary infection occurs, these cells will protect lung tissue by either early virus opsonisation or direct killing of virus-infected cells [222, 325].

There are over 100 known rhinovirus serotypes but cross-protection is very limited: acquired immunity to one serotype will not confer protection against a different one [136]. When conducting human in vivo challenges using RV, researchers will test the volunteers’ blood for the presence of RV-specific antibodies and any patients whose blood is positive for these antibodies will not take part in the study [120, 233, 234, 368, 370]. On the other hand, adaptive immunity is not involved when using primary cells of one type, e.g. epithelial cells. However adaptive immunity may play a role in the tissue models: in the human lung, there are a plethora of different cell types, including lymphocytes and memory cells. In addition, there is evidence of lymphoid tissue being present in the lungs [311]. Therefore it is possible that adaptive immunity will have an impact on the in vitro tissue responses to RV. Ideally, if not using single cell type model, tissue donors’ blood should be tested to ensure that all of the patients are naïve to the particular RV serotype used.
1.5. **Human Rhinoviruses and Respiratory Disease Exacerbations**

Previous sections introduced human rhinoviruses as a common human respiratory pathogen and described the ways by which the virus can be detected. However, infections, especially viral, are proposed to be one of the most important triggers of respiratory disease exacerbations [266, 340, 373]. Rhinoviruses are particularly significant in worsening the symptoms in asthmatic patients [178] and in patients with COPD [340].

1.5.1. **Human Rhinoviruses and Exacerbations Association Studies**

There are many studies that associate rhinoviruses with disease exacerbations however particular virus serotypes were not reported (see Table 1-4). Introduction of more sensitive detection methods, such as RT-PCR, resulted in the discovery that viral infections are more prevalent during asthma exacerbations than initially thought [169]. Studies prior to RT-PCR reported virus detection rate during exacerbations of asthmatic patients at approximately 10-50% [285]. However Johnston et al. conducted a study among children with asthma and showed that viruses can be detected in around 80-85% of exacerbation episodes with rhinoviruses detected in two thirds of these episodes [178]. Another study by Nicholson et al. examined exacerbations in asthmatic adults and found that virus can be detected in 57% of episodes and 64% of these were associated with rhinoviruses [266]. Similar observations were made by Khetsuriani et al. who reported that viruses were detected in 63% of episodes of asthma exacerbations and 60% of them were correlated with the presence of RVs [196]. In a recent study, Kennedy et al. observed that nasal washings of 57% and 56% of wheezing children and children with allergic rhinitis, respectively, were positive for rhinovirus [193].
Table 1-4 Summary of studies associating rhinovirus with asthma and COPD exacerbations.

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Virus association %</th>
<th>RV association %</th>
<th>Method</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASTHMA</strong></td>
<td></td>
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</tr>
<tr>
<td>Children</td>
<td>Virus detected in 80-85% exacerbation samples (percentage dependent on symptoms)</td>
<td>67% cases of virus detection included rhinovirus</td>
<td>Nasal aspirates and blood samples taken; RV detected by cell culture and PCR</td>
<td>Johnston et al. [178]</td>
</tr>
<tr>
<td>Adults</td>
<td>Samples from 57% exacerbations episodes tested positive for non-bacterial pathogen</td>
<td>64% of samples tested positive for rhinovirus</td>
<td>Nasal and throat swabs; RV detected by cell culture and PCR</td>
<td>Nicholson et al. [266]</td>
</tr>
<tr>
<td>Children</td>
<td>Asthma exacerbations were associated with virus in 63% of cases</td>
<td>60% cases of virus exacerbations linked with RV</td>
<td>Nasal and throat swabs; RV detected by PCR</td>
<td>Khetsuriani et al. [196]</td>
</tr>
<tr>
<td>Children</td>
<td>Patients with asthma and rhinitis tested for RV presence</td>
<td>56-57% of samples tested positive for RV</td>
<td>Respiratory specimens (nasal and throat) taken; RV detected by PCR</td>
<td>Kennedy et al. [193]</td>
</tr>
<tr>
<td><strong>COPD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults</td>
<td>Exacerbations associated with virus in 39% of cases</td>
<td>58% of virus-induced exacerbations linked with RV</td>
<td>Nasal aspirates and induced sputum tested for RV by PCR</td>
<td>Seemungal et al. [339]</td>
</tr>
<tr>
<td>Adults</td>
<td>Viruses found in 56% of exacerbation episodes</td>
<td>36% of samples tested positive for RV</td>
<td>Nasal lavage and induced sputum tested for RV by PCR</td>
<td>Rohde et al. [317]</td>
</tr>
<tr>
<td>Adults</td>
<td>Viruses detected in 19% of respiratory illness episodes in COPD patients</td>
<td>24% of virus associated illness linked with RV</td>
<td>Respiratory specimens and blood samples taken, RV confirmed by cell culture and PCR</td>
<td>Greenberg et al. [138]</td>
</tr>
<tr>
<td>Adults</td>
<td>Samples tested for RV and other bacterial pathogens</td>
<td>53% of samples collected at exacerbations were positive for RV</td>
<td>Sputum samples analysed by PCR</td>
<td>Goerge et al. [128]</td>
</tr>
</tbody>
</table>
Studies examining the effects of viral infections in patients with COPD also identify rhinovirus as a significant marker of disease exacerbation. However, the virus detection in COPD episodes is lower than in asthma. Seemungal et al. examined 83 patients with COPD exacerbations and reported that 39% of exacerbations were associated with viral respiratory infections and that 23% of total COPD episodes (or 58% of episodes involving viruses) were associated with rhinoviruses [339]. Rohde et al. conducted a case-control study and reported that respiratory viruses are more common in patients with a COPD exacerbation (found in 56% of patients) as opposed to individuals with stable COPD (19%) [317]. In addition, viruses from family Picornaviridae (in this case rhinoviruses and enteroviruses) constituted 36% of all viruses detected in 42 patients. In yet another study, Greenberg et al. also found that a quarter of viruses associated with COPD are rhinoviruses [138]. Moreover, George et al. found that increased respiratory symptoms were associated with increased viral titres present in sputum and frequent exacerbators had an increased risk of contracting RV-induced exacerbation [128].

1.5.2. HRV Infection Studies

The above observations have established a major link between rhinoviruses and asthma/COPD exacerbations. Therefore it is of great importance to study the mechanisms that trigger these episodes. The next section will summarise the studies that examined the actions of rhinoviruses using in vitro and in vivo infection models. Selected studies have been described in more detail in Table I-1 in Appendix I.

1.5.2.1. Rhinoviruses Can Infect Lower Airways

Rhinoviruses have been long known to infect the upper airways and cause common cold. However, approximately 10 years ago, several research groups confirmed that RVs are also able to infect lower airways. Mosser et al. infected primary cells of upper and lower respiratory tract ex vivo and showed that RVs are able to infect both types of cells [259]. However, the infection
rate was low – only 10% of cells were infected. In another study, Papadopoulos et al. established that rhinoviruses are as capable of replicating at 37°C as they are at 33°C (thought to be the optimal temperature for RV replication), suggesting that RV can as easily replicate in the lower as in the upper respiratory tracts [277]. To confirm that rhinoviruses are able to infect lower airways \textit{in vivo}, they inoculated 7 healthy and 3 asthmatic volunteers and found that 100% of them had upper airways infected, and 50% of them also developed a lower respiratory tract infection [278]. These findings were corroborated in other studies [130, 336]. Additionally, it was found that when rhinovirus infects the lower airway the severity of symptoms increase [409].

1.5.2.2. \textit{Rhinoviruses Trigger Potent Inflammatory Response}

Experimental HRV infection models have established that rhinoviruses initiate a potent inflammatory response. In the next sections, a description of studies examining the effects of RV infection on various aspects of innate immune system will be offered, including the migration of granulocytes, release of inflammatory mediators and tissue remodelling.

\textbf{Epithelial Cells}

Zhu et al. showed that infection with RV causes a significant increase in IL-8 production (neutrophil chemoattractant) from A549, MRC-5 and primary human bronchial epithelial cells [434]. They also showed that the production of IL-8 is at least partially mediated by NF-\kappaB. Terajima et al. have studied primary human tracheal epithelial cells and also observed increased production of IL-1\beta, IL-6, IL-8 and TNF\alpha [374]. Schroth et al. showed that the production of CCL5 (a potent chemoattractant of eosinophils and monocytes) was significantly upregulated in bronchial epithelial cells infected with RV [336]. This was confirmed in the study by Papadopoulos et al. which reported the increased production of CCL5 along with IL-6, IL-8 and IL-16 [278]. In another study, Papadopoulos et al. examined the expression of a number of
chemokines and cytokines and found that the production of CCL5, IL-8 and eosinophil-specific chemoattractant eotaxin was increased in RV infected BEAS-2B cells [279].

**Neutrophils**

Number of circulating and airway resident neutrophils also increase following RV infection. Jarjour et al. infected asthmatics and showed that an increase in CXCL8 and G-CSF correlated with an increased number of peripheral and bronchial neutrophils [172]. These findings were also confirmed by other groups, both in humans and mice, indicating that RV infection will cause neutrophil influx to the site of infection [132, 229, 318]. In a pivotal study, Bartlett et al. established an RV-1B infection mouse model and showed that rhinovirus triggers influx of inflammatory cells (including neutrophils) and the release of pro-inflammatory cytokines (e.g. TNFα and IL-6) and interferons, especially IFNλ [27].

**Eosinophils**

Eosinophils were shown to play an important antiviral role during RV infection. Avila et al. reported that the presence of eosinophils prior to rhinovirus infection delays the onset of the disease and reduces the severity of symptoms [13]. Additionally, eosinophils expressing ICAM-1 were also implicated as antigen-presenting cells that are able to activate RV-specific T cells which leads to T cell proliferation and release of interferons [147].

**Macrophages and Monocytes**

The above studies confirm that RVs are able to infect human epithelial cells of the respiratory system triggering the chemotaxis of neutrophils and eosinophils to the site of infection. However, it was also shown that RV are able to enter and, sometimes, replicate in monocytes and macrophages. Johnston et al. examined peripheral blood mononuclear cells (PBMCs) during RV infection and found that PBMCs increased their production of IL-8 [179]. They also demonstrated that although RV replication was not seen in PMBCs, it was observed
in THP-1 monocyte cell line in vitro. In a more recent study, Laza-Stanca et al. examined macrophages derived either from THP-1 cells or from primary human monocytes and found that RV is capable of replicating only in THP-1 cells [215]. These findings seem to be in accordance with the earlier study conducted by Gern et al. who established that RV are able to enter, but not replicate inside human monocytes and macrophages, thereby confirming that RV replication in human monocytes and macrophages is limited [129]. In addition, viral titres measured in mouse-derived macrophages following RV infection also confirm that rhinoviruses do not replicate inside macrophages, however both mouse and human macrophages do respond by releasing pro-inflammatory cytokines and chemokines [185, 321].

**Airway Remodelling**

More recently, rhinovirus was shown to activate the production of factors responsible for airway remodelling. Leigh et al. reported that RV infection up-regulates the production of amphiregulin, activin A and vascular endothelial growth factor (VEGF) in primary human bronchial epithelial cells and BEAS-2B cell line [219]. All of these factors are known to play a role in airway tissue remodelling. However, there is some disagreement as some research groups confirm that VEGF production is increased in BEAS-2B cells and fibroblasts during rhinovirus infection [300, 392] and others report that RV does not influence the level of VEGF production in either primary bronchial cells or primary lung fibroblasts [93]. This seems surprising as all studies were conducted using the most commonly employed major group rhinovirus – RV-16; therefore more analyses need to be carried out to confirm the role of rhinovirus on VEGF production.

Extracellular matrix (ECM) deposition is also a hallmark of airway remodelling. Kuo et al. reported that the production of perlecan (an ECM protein), collagen V and matrix-bound VEGF were significantly increased in human bronchial epithelial cells and fibroblasts following RV exposure [209]. They also established that the increase in ECM deposition is mediated via
the TLRs underscoring their importance in the RV infection. This study gives further evidence that rhinoviruses are responsible for triggering the production of VEGF as described in the previous studies.

**Induction of Type I and III Interferon**

Gene and protein expression of type I and III Interferon (IFN) have been examined in numerous studies investigating the effects of RV in the human cells. Interferons are expressed in response to viral infections, although intracellular bacteria may also trigger their release [288, 304]. The downstream signalling leads to the expression of interferon-stimulated genes (ISGs) e.g. MyD88 or TRADD (involved in TLR signalling) or interferon regulatory factors (IRFs) which convey antiviral and pro-inflammatory signals (e.g. increased expression of TNF, CCL5, and IL-6) [94]. Most of the cells are responsive to IFNs, however different cell types may not produce all types of IFN. In addition, recent studies suggest that TNFα may play a role in IFN induction or suppression and the imbalances in these cytokines may lead to the development of autoimmune diseases [66].

The two type I IFNs most commonly examined in response to viral infections are IFNα and IFNβ. Both types signal via IFNAR1 and IFNAR2 receptors whose activation leads to the expression of genes containing interferon-stimulated response element (ISRE) sequence upstream to the target gene [168]. Type III IFNs, called IFNλ, act through different receptors (IL28R1 and IL10R2) to induce the expression of genes with ISRE; however the exact mechanisms are still to be elucidated [431].

Gene and protein expression of type I and III IFNs were examined in multiple cell types, including primary human epithelial cells and airway epithelial cell lines, in fibrocytes, monocyte-derived macrophages, human peripheral blood monocytes and BAL fluid [90, 107, 164, 234, 298, 303, 335, 368-370]. Rajan et al. investigated IFNα in PBMC and bronchial epithelial cell co-culture in response to RV-14 and RV-16 and showed that epithelial cells do not produce the
cytokine. Only in the presence of PBMCs, IFNα was detected after RV-14 stimulation [303]. In a study conducted on primary human epithelial cells Sykes et al. did not observe any induction of IFNα in response to rhinovirus but reported a significant increase of IFNβ and IFNλ [370]. In another study, however, Sykes et al. showed that IFNα and IFNβ release was increased following RV-16 exposure in PBMCs and BAL cells [368].

Other groups have confirmed an abundant release of both IFNβ and IFNλ in response to rhinovirus in numerous cell types [63, 110, 164, 370, 410]. For example, Calven et al. showed a significant increase in IFNβ gene expression in bronchial smooth muscle cells following infection with RV-1B [63]. Wu et al. reported a 100-fold increase in IFNβ gene expression after 4 and 24h following RV-16 infection (10^4 TCID_{50}/ml) in NCI-H282 cells [410]. Type III IFNs are emerging to be the most ubiquitously expressed IFN in response to viral respiratory infections [43, 273, 370]. Parsons et al. reported a 1000-fold increase in of IFNλ release from primary bronchial epithelial cells in response to RV1B [284]. Sykes et al., however, observed over 100-fold increase in RV1B-induced-IFNλ protein production [370].

The role of type III IFNs in regulating immune response to viruses has also been demonstrated, e.g. RV-induced expression of epidermal growth factor receptor was shown to attenuate the activity of IRF1 and expression of IFNλ thus increasing RV infection [382]. Another experiment conducted in a mouse model showed that mice lacking receptor for IFNλ (IL-28R) were more susceptible to influenza virus [254]. The levels of viral titers in IL-28R knockout mice matched the levels in IFNAR1 (type I IFN receptor) knockout mice, however double knockout of these receptors showed an even higher susceptibility to virus infection. These data taken together show that type III IFNs are important in antiviral immunity, can act in complement with type I IFNs and therefore should be included in further research of antiviral immunity.
1.5.2.3. **Mechanisms of RV-Induced Exacerbations of COPD**

There are not many studies available that examined the mechanisms of RV infection and RV-induced exacerbation of COPD. Mallia et al. have recently developed a human *in vivo* model of RV exacerbation of COPD which could be used to test novel drugs in final stages of clinical trials [234]. Mallia et al. showed that COPD patients have diminished IFN response to rhinovirus. Other groups also started to investigate the factors that contribute to RV-induced exacerbations of COPD. Proud et al. have examined the effect of cigarette smoke extract (CSE) on RV-induced cytokine release and found that CSE markedly suppressed pro-inflammatory and antiviral responses in BEAS-2B cells, including CCL5 and interferons [299]. In another study, Quint et al. examined whether vitamin D deficiency plays a role in RV-induced COPD exacerbations [301]. They did not find any association of vitamin D deficiency with frequency of exacerbations. In addition, Suri et al. examined whether expression of bronchial platelet-activating factor receptor (PAFR – used to attach by bacteria infecting lower airways [87]) is modulated by RV infection and therefore increases the frequency of exacerbation [365]. Although PAFR expression was increased in both smokers and smokers with COPD when compared to non-smokers, RV did not further enhance PAFR in bronchial epithelial cells. These studies are pilot attempts to elucidate the mechanisms that govern RV-induced exacerbations in COPD. There is clear evidence that cigarette smoke decreases antiviral responses in the airway cells, however these are not enhanced in patients with obstructed airway disease. Evidence also suggests that the expression of IFNs may be impaired during virus-induced COPD exacerbations. Therefore further studies are required to increase our understanding in this subject area.

1.5.2.4. **Asthma Phenotype Predisposes to RV Infection**

Since, as indicated, RV infection is a significant factor in asthma exacerbations, it has initiated a proliferation of studies examining the response of asthmatic airways to rhinoviruses. There are, however, scarce data examining the rhinovirus-induced exacerbations of COPD.
Since both asthma and COPD are syndromes, many of the symptoms overlap and insight gained from experiments conducted with asthmatic cells may be helpful in understanding the mechanisms that govern RV-induced COPD exacerbations. This section will therefore briefly summarise studies that examined RV infections in asthma.

**ICAM-1 Up-Regulation**

Wegner et al. reported that the expression of ICAM-1 is up-regulated in monkeys following an antigen challenge and that inhibition of ICAM-1 production reduces airway inflammation and hyper-responsiveness [402]. However, ICAM-1 levels in stable asthmatics were shown to be similar to levels in normal volunteers [396]. Two separate studies conducted by Bianco et al. showed that mediators such as IL-4 or IL-5 associated with T-helper 2 cells increase the expression of ICAM-1 in human bronchial epithelial cell line (NCI-H292) and human nasal epithelial cells suggesting that asthmatic phenotype predisposes to RV infections [39, 40]. This seems to be in accordance with the findings by Corne et al. who reported that asthmatic patients have prolonged and more severe symptoms than the individuals not affected by the disease – the existing inflammation caused by allergens is enhanced by the RV infection [81]. Interestingly, treatments currently in use for asthma and COPD, i.e. long-acting β2-agonists, long-acting anti-cholinergics and inhaled corticosteroids were shown to decrease the expression of ICAM-1 as well as decrease the overall inflammation associated with RV infection [414-416].

**Differences in Response to RV Infection**

Numerous studies have compared the responses of asthmatic and normal cells of the respiratory tract to RV infection in order to find a possible preventative means for disease exacerbations. For instance, Fraenkel et al. reported that eosinophilic infiltration associated with rhinovirus infection was found in both asthmatic and normal patients, however only in asthmatics did the increased number of eosinophils persist for up to 8 weeks following the initial
RV infection [120]. Similar to normal patients undergoing RV infection, asthmatic individuals also have an increased number of neutrophils or IL-8 [142].

Thomas et al. reported that individuals with asthma have higher expression of tumour growth factor-β (TGF-β) which reduces the production of interferons thus predisposing them to viral infections [376]. This is in accordance with the experiment conducted by Contoli et al. that reported that deficient production of type III interferons was correlated with the severity of cold symptoms [78]. These findings, along with the ones made by Bartlett et al. and Bullens et al., underscore the importance and the protective role of IFNλ during RV infection [27, 55].

Papadopoulos et al. conducted a study on peripheral blood mononuclear cells infected with rhinovirus and found that cells from asthma patients had reduced levels of IFNγ and IL-12, and increased levels of IL-10 in comparison to normal subjects [280]. In addition, asthmatic cells produced IL-4 (which was not detected in normal cells). Wark et al. reported that levels of IL-6 and CCL5 were similar in bronchial epithelial cells derived from normal and asthmatic patients; however asthmatic cells have decreased production of IFNβ [396]. In addition, they observed increased rate of RV replication in asthmatic cells. Interestingly, when cells were treated with exogenous IFNβ, they were able to clear the infection faster. These findings were confirmed by Bedke et al. who infected human bronchial fibroblasts with RV and reported that IFNβ plays an important role in viral clearance [34].

1.5.3. Summary

It can be inferred from the above observations that rhinoviruses can trigger a potent inflammatory response which can persist for longer in patients with atopic and chronic airway disease. Rhinoviruses can also initiate or potentiate pathways that contribute to airway remodelling. Interferons have an essential role in clearing RV infection. All of these, including the defective IFN production mechanisms present in asthma and COPD patients can not only impede the progress of convalescence but also contribute to disease exacerbations.
As stated throughout this introduction, the exact mechanisms that trigger respiratory disease exacerbations are not fully understood. It is therefore important to examine the pathways activated by RVs that lead to these exacerbations. The models that have been or could be used to investigate the events causing respiratory disease exacerbations will be summarised in the next section.
1.6. Review of RV Infection Models

While immune responses to RVs have been extensively examined in animal models and single cell-type monolayer cultures, there is scarce data relating to whole lung tissue models. So far three different whole respiratory organ tissue models have been employed, namely: (1) air-liquid interphase model using nasal mucosa [171], (2) lung fragment model [145], and (3) lung slice model [361]. The three models will be briefly explained below, and their advantages and disadvantages will be summarised.

Nasal turbinate mucosa organ culture was used by Jang et al. to investigate rhinovirus-caused pathophysiology in the human airway [171]. They divided the tissue using 4mm biopsy punch and placed the sample into a 24-well plate so that the apical surface remained in contact with air, whereas the distal surface was embedded into the gelfoam and surrounded by media (see Figure 1-6, A). Rhinovirus was placed directly onto the apical surface to resemble the natural course of infection.

Figure 1-6 Schematics of various tissue models used for respiratory research.
(A) The air-liquid interphase system where tissue is placed on a gelfoam inside the well. Rhinovirus is then put apically on the tissue surface. Adapted from Jang et al. [171].
(B) The lung fragment model where several whole tissue explants are submerged in the tissue culture medium. Rhinovirus is then introduced into the medium for uptake by the tissue fragments.
(C) The precision-cut lung slices where a thin slice of tissue is submerged in the tissue culture medium. Rhinovirus is placed in the culture medium for uptake by the tissue.
The main advantage of the air-liquid interphase model is that it mimics the natural course of infection in the human respiratory system. The pathogen, in this case human rhinovirus, is placed on the surface it would normally be in contact with *in vivo*. However, it has one main disadvantage – it would require the lung samples to contain a sufficient surface airway area to perform biopsy punches. Therefore this model could only be used in experiments that required a small number of samples due to limitations of tissue sample size.

The second method that used whole-lung organ culture was described by Hackett et al. [144]. In this model, the lung parenchyma was cut into small fragments and “weighed out... to normalise cytokine output”. Approximately 30mg of tissue (submerged in media) was used per well (on a 24-well plate) (see Figure 1-6, B). Tissue samples were then stimulated with lipopolysaccharide (LPS) to study the immune response to bacteria-mediated exacerbations in COPD patients. To date, RV has not been used in this setting.

This model does not require any specific orientation or composition of tissue. This is its principal advantage and its main disadvantage, since while it does not resemble the natural way of infection, it is easy to set up and maintain tissue samples in this culture system. Moreover, because of the lack of specific tissue type requirement, it could be used for experiments that require a large number of samples.

The lung slice model as described by Sturton at al. involved more lung processing than the previous two models [361]. Briefly, ultra-low melting point agarose was first injected into the tissue to stabilise it. Then, the tissue containing small airways was cut into slices of approximately 250µm thickness and placed into 12-well plates filled with culture medium (see Figure 1-6, C). After the samples were heated to 37°C, they were washed every 30 minutes to remove the injected agarose from the tissue. Finally, the samples were incubated overnight and used for designated experiments the next day.
This lung slices model can be seen as a combination of the previous two, in that it does not allow for the air-liquid interphase and, at the same time, it requires specific types of tissue to be present within the samples. The ability to study small airways in multiple conditions seems to be the model’s greatest advantage. Its main disadvantage, however, is that it requires a significant amount of tissue preparation.

All of the above methods could potentially be used to develop a model to study RV infection in the human lung. While each of the methods has both advantages and disadvantages, the second model, i.e. tissue fragments, is the easiest one to set up and does not require large amounts of tissue to test the model’s efficiency and reproducibility. Therefore, the small lung fragments method could be used initially to optimise the required virus dose and incubation time. Once established, it would be more appropriate to use the optimised conditions in the air-liquid interphase model, which would allow for a more natural course of rhinovirus infection in the human lung.
1.7. Hypothesis and Aims

The studies discussed above describe the events that occur during RV infection both in subjects with normal lung function and those affected with asthma/COPD. However, cell studies do not provide a full view of the mechanisms that are implicated in the course of infection and subsequent exacerbations. Infecting human volunteers, in turn, has its ethical limitations and, as in cell studies, it is not possible to picture all the complex interactions that may have taken place over the course of infection. Mouse models seem to have more advantages – as it is possible to observe all of the effects in response to RV. Nonetheless rodent animal models differ from humans quite considerably (e.g. lack of ICAM-1 attachment receptor).

Since rhinovirus is the most important viral trigger of COPD exacerbations, there is a need to develop new treatments and prophylactic medicines for acute exacerbations of COPD to limit the rate of hospitalisations and decrease the disease progression in those affected with frequent exacerbation episodes. Others have shown that TNFα levels are increased in COPD, both in stable state [192] and after TLR4 stimulation [144]. TNFα is an inflammation-amplifying cytokine and is an actively investigated target in the management of COPD; its role in TLR signalling will also be explored in this project. In addition, evidence suggests that patients with COPD exhibit a resistance to corticosteroid treatment that targets the enhanced inflammation [86, 349]. Small molecule inhibitors against kinases downstream to TLRs (such as MAPK, IKK and IRAK kinases) could help reduce the elevated inflammation and limit the number of exacerbations. A selection of several small molecule inhibitors will also be utilised here to assess their effectiveness in reducing the levels of pro-inflammatory cytokines.
In light of the described limitations and observations a novel virus infection model – using human lung tissue explants – is proposed. This project addresses the hypothesis that a human lung tissue explant model will be a reliable reflection of *in vivo* RV infections and will serve as a new platform to test novel molecules aiming to treat acute exacerbations. It is also hypothesised that patients with COPD, as in the case of RV *in vivo* challenges, will exhibit increased pro-inflammatory response and attenuated IFN gene expression compared to tissue from patients with normal lung function.

The principal aim of this project is to establish a reliable and repeatable virus infection model of whole lung tissue explants and to assess its role as a potential platform to test novel inhibitors for acute exacerbations of COPD. Specific aims and objectives are included in Chapters 3-5.
Chapter 2. Materials and Methods

This chapter outlines only the general methods used in the current project. Detailed methods relating to particular experiments are described in Chapters 3-5.

2.1. Patient Information

Tissue from 60 different donors, undergoing surgery for confirmed or suspected lung cancer, was used in the experiments presented in this thesis. Tissue was obtained from donors with or without COPD and all had history of smoking (see Table 2-1). All of the patients gave informed consent and the study was approved by local ethics committee (03/SM/396, NRES Committee North West – Greater Manchester South). In general, patients were classified as COPD when their forced expiratory volume in 1s (FEV1) % predicted for their age was less than 80% and/or their % ratio of FEV1 to forced vital capacity (FVC) was less than 70%. Patients without airway obstruction were classified as smokers and include both current and ex-smokers.

Table 2-1 Subject demographics.

<table>
<thead>
<tr>
<th></th>
<th>COPD (n=36)</th>
<th>Smokers (n=24)</th>
</tr>
</thead>
<tbody>
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<td>Sex (M/F)</td>
<td>19/17</td>
<td>13/11</td>
</tr>
<tr>
<td>Age</td>
<td>69±7</td>
<td>67±8</td>
</tr>
<tr>
<td>FEV1</td>
<td>1.82±0.54</td>
<td>2.54±0.69</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>73.0±16.9</td>
<td>99.4±18.9</td>
</tr>
<tr>
<td>FEV1/FVC % ratio</td>
<td>58.9±11.5</td>
<td>75.1±6.8</td>
</tr>
<tr>
<td>Smoking status (Current/Ex)</td>
<td>22/14</td>
<td>10/14</td>
</tr>
<tr>
<td>Smoking history (pack-years)</td>
<td>44 (13-126)</td>
<td>36 (2-71)</td>
</tr>
<tr>
<td>ICS use? (Y/N)</td>
<td>9/27</td>
<td>0/24</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD or mean (range).

Samples that were used for optimisation or trial experiments are not included in this table.
Samples from patients used in the experiments are subject to patient-to-patient variations, e.g., patients within a subject group had mixed smoking status (some of them were current and some were ex-smokers). Also, within the COPD group, 9 of 36 patients had a history of inhaled corticosteroid use. It must be noted that different sets of patients were used in different experiments in Chapters 3-5 and demography tables for separate experiments can be found in subsequent chapters.

2.2. **Tissue Culture**

Human lung tissue was prepared using modified protocol described before [144]. Briefly, using two sterile scalpels 1-5 grams of tissue were cut into small (approximately 1mm³ fragments) and known as whole tissue explants (WTE). Subsequently, WTE were put into a 5ml plastic bijou and rinsed 3 times with 3ml of RPMI-1640 medium (Sigma-Aldrich, Dorset, UK) supplemented with 10% (v/v) foetal bovine serum (Invitrogen, Paisley, UK), 100U penicillin/0.1mg streptomycin (Sigma-Aldrich, Dorset, UK) and 2mM L-glutamine (Invitrogen, Paisley, UK). Tissue (30±3mg) was weighed out in pre-weighed 1.5 or 2ml sterile eppendorf tubes and approximately 5-7 randomly chosen explants were needed to obtain the required weight. Samples were then placed in 24-well plates with 800μl of supplemented RPMI medium per well and incubated at 37°C, 5% (v/v) CO₂ overnight. If the tissue was prepared for subsequent protein extraction, only 784μl of medium were added to each well (see Appendix II for WTE culture for protein extraction). Samples were stimulated with TLR agonists or human rhinovirus the following day (see sections 3.2.1. and 5.2.3. for more details).

Tissue was processed within 2 hours of sample collection if received before 5pm. If the sample was collected after 5pm, the processing was delayed until the following day to ensure that processed tissue derived from all subjects was in culture for the same amount of time. If the tissue was not processed immediately, it was kept at 4°C in a container filled with supplemented RPMI medium.
2.3. ELISA

TNFα, CCL5 and IL-6 were chosen as outputs for the ELISA assays. ELISA was used to measure the levels of these cytokines, however, if costs were not an issue, there are high-throughput or multiplex methods that could be used to expand the cytokine panel measured (e.g. luminex or cytokine bead array). In addition, the levels of interferon could be measured from the supernatants. Due to the cost of ELISA it was decided that IFNs were to be measured using qRT-PCR (see section 2.7).

Three pro-inflammatory cytokines were measured in this thesis: TNFα, CCL5 and IL-6. They were chosen because of their involvement in COPD and/or were shown before to be released in response to RV. TNFα has been implicated in the inflammatory processes in human disease, including COPD [192]. TNFα is an inflammation-amplifying cytokine and therefore can trigger the subsequent release of other cytokines [144]. CCL5 is a cytokine that has been previously shown to be release from human cells in response to RV [202, 336]. It is an eosinophil chemoattractant but evidence suggests that it can also attract basophils, monocytes and lymphocytes [77, 208]. The release of CCL5 therefore causes an additional influx of the immune cells. Interestingly, eosinophilia in blood of COPD patients was shown to improve their response to steroid treatment [15]. IL-6 was shown to be increased in COPD patients and rhinovirus can further upregulate the levels of this cytokine [38, 362, 433]. Polymorphisms in the IL-6 gene were also correlated with defective lung function [150]. The role of IL-6 in COPD is still unclear and requires further research.

Cytokine protein levels in supernatants were measured by ELISA (R&D Systems Europe, Abingdon, UK) according to manufacturer’s protocol. Briefly, 96-well plate was coated with capture antibody in 1x Dulbecco’s phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK) and incubated overnight. The following morning plate was blocked using reagent diluent (1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, Dorset, UK)) and was incubated for 1 hour. Subsequently, samples, standard and blank samples were loaded onto the plate and
incubated for another 2 hours. Samples, if required, and standard were diluted in supplemented RPMI medium, whereas the blank samples consisted of medium only. Next, detection antibody in reagent diluent was put onto the plate. After another 2 hour incubation, streptavidin-horseradish peroxidase (HRP) diluted in reagent diluent was added to the plate and incubated for 20 minutes. Finally, reaction substrate A and B (R&D Systems, Abingdon, UK) (volume ratio 1:1) was added to each well and the colour change was allowed to develop for up to 20 minutes. After each of these steps, 3x wash with washing buffer (1x PBS with 0.05% (v/v) Tween20 (Sigma-Aldrich, Dorset, UK)) was performed. All incubations were done in the dark at room temperature. Once colour change was developed, the reaction was stopped using 1M sulphuric acid (Sigma-Aldrich, Dorset, UK) and the absorbance was read using OPTIMA Plate Reader (BMG Labtech, Aylesbury, UK) at 450nm with 544nm wavelength correction. The detection limit for TNFα and CCL5 was 15.625pg/ml, whereas for IL-6 it was 9.375pg/ml. Additionally, all ELISA data were adjusted for tissue weight and are presented as pg of protein per mg of tissue.

2.4. RNA Extraction

Tissue samples stored in RNAlater were homogenised using rotor-stator homogeniser and then RNA was extracted using RNeasy Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Briefly, samples were homogenised in 600μl of RLT buffer for about 20-40s and then were centrifuged for 3min at ≥10,000rpm for 3 minutes. The pellet was discarded and supernatant was mixed with equal volume of 70% ethanol (Sigma Aldrich, Dorset, UK). Samples were then put on the RNeasy spin column and centrifuged at ≥10,000rpm for 15 seconds. Column was then washed with 350μl of RW1 buffer, followed by on-column DNA digestion for 20 minutes using the RNase-Free DNase Set (Qiagen, Manchester, UK). After DNA digestion, another wash with 350μl of RW1 and 2 washes with 500μl of RPE buffer were performed. After the last wash the column was centrifuged for 2 minutes and an additional 1 minute spin with new collection tube was performed to remove any residual ethanol. The flow-
through was discarded after each of the steps. The RNA was then eluted using 50μl of RNase-free water (supplied with the kit) and stored at -20°C.

2.5. Measuring RNA Concentration

RNA concentration was measured using LVis Plate using POLARstar OMEGA microplate reader (both BMG Labtech, Aylesbury, UK). Blanks (2μl of RNase-free water) were loaded onto the LVis Plate first, followed by 2μl of RNA sample. Absorbance was read at four discrete wavelengths, i.e. 230, 260, 280 and 340nm. RNA concentration was calculated using MARS software with the assumption that 40μg of RNA has an optical density equal to 1.

2.6. Complementary DNA synthesis

To synthesise complementary DNA (cDNA), RNA was diluted in RNase free water to 25ng/μl. cDNA was synthesised using Verso cDNA Synthesis Kit (Fisher Scientific, Loughborough, UK). Exact volumes of different kit components for a typical reaction are presented in Table 2-2.

Each cDNA synthesis reaction also included two controls: no template control where instead of RNA template 2μl of water was added and no enzyme (or –RT) reaction where Verso enzyme was substituted with 1μl of water.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master Mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5x cDNA synthesis buffer</td>
<td>4μl</td>
<td>1x</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2μl</td>
<td>500μM each</td>
</tr>
<tr>
<td>Random hexamers</td>
<td>1μl</td>
<td>400ng</td>
</tr>
<tr>
<td>RT enhancer</td>
<td>1μl</td>
<td></td>
</tr>
<tr>
<td>Verso enzyme</td>
<td>1μl</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>9μl</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Template</td>
<td>2μl</td>
<td>50ng</td>
</tr>
<tr>
<td>Total</td>
<td>20μl</td>
<td></td>
</tr>
</tbody>
</table>

PCR reaction was performed using S-96 Quanta Biotech Thermal Cycler. The programme included hot start phase at 42°C for 2min, cDNA annealing phase at 42°C for 30min and cDNA
denaturation phase at 95°C for 2min. The programme was performed with heated lid at 110°C. After completion, cDNA samples were stored at -20°C for subsequent qRT-PCR.

2.7. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

To measure the levels of mRNA of several genes of interest quantitative real-time polymerase chain reaction (qPCR) was performed using Absolute Blue qPCR Mix (Fisher Scientific, Loughborough, UK) and specific gene probes and primers (Taqman) (Life Technologies, Paisley, UK) (see Table 2-3 for list of primers used). PCR was performed using Stratagene Mx3005P qPCR system and gene expression was normalised to GAPDH using $2^{-\Delta\Delta CT}$ method. The programme consisted of 15min enzyme activation phase at 95°C, and 40 cycles of 15s denaturation at 95°C and 1min annealing phase at 60°C.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe product number</th>
<th>Volume per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>4352934E</td>
<td>0.75μl</td>
</tr>
<tr>
<td>TNFα</td>
<td>Hs01113624_g1</td>
<td>0.875μl</td>
</tr>
<tr>
<td>CCL5</td>
<td>Hs00174575_m1</td>
<td>0.75μl</td>
</tr>
<tr>
<td>IL-6</td>
<td>Hs00985639_m1</td>
<td>0.5μl</td>
</tr>
<tr>
<td>IFNα1</td>
<td>Hs00256882_s1</td>
<td>0.25μl</td>
</tr>
<tr>
<td>IFNβ1</td>
<td>Hs01077958_s1</td>
<td>0.25μl</td>
</tr>
<tr>
<td>IFNλ1</td>
<td>Hs00601677_g1</td>
<td>0.25μl</td>
</tr>
</tbody>
</table>

IFNs are the classical anti-viral molecules measured when conducting experiments using viruses. The other approach here could be to measure the expression of a panel of anti-viral molecules, including interferon-stimulated genes, by luminex or gene array to compare whether there is a difference in response to viruses between subject groups.
2.8. **Statistics**

All data were analysed and checked for normality using Kolmogorov-Smirnov test and were plotted in GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA).

2.8.1. **Time Course**

Absolute levels of cytokine release or relative gene expression in response to a TLR ligand or rhinovirus at each time point were compared with time-matched unstimulated controls using paired t-tests (or Wilcoxon for nonparametric data). Unpaired t-tests (Mann-Whitney) between subject groups (COPD and Smokers) at each time point were utilised to verify if any significant differences in cytokine release or gene expression were present.

2.8.2. **Dose response**

Absolute levels of cytokine release in response to TLR ligands were analysed by one-way ANOVA with Dunnett post-test. CCL5 dataset was analysed using Friedman test with Dunn post-test comparing all ligand doses versus unstimulated control. Unpaired t-tests (Mann-Whitney) between subject groups (COPD and Smokers) at each time point were utilised to verify if any significant differences in cytokine release were present.

2.8.3. **Combination of TLR Ligands**

The levels of TNFα and IL-6 released in response to simultaneous use of TLR3 and TLR7/8 ligands were compared to the levels induced by the two ligands on their own using paired t-test (or Wilcoxon test for CCL5). The combination effect was also compared to hypothetical sum (where absolute levels of cytokines released by both ligands were added together) with the use of paired t-test (or Wilcoxon test for CCL5).

2.8.4. **Effect of Smoking on Cytokine and IFN Protein and Gene Expression**
In addition to tests described above for Time course, Dose response and Combination (sections 2.8.1. to 2.8.3.), unpaired t-tests (or Mann-Whitney tests) were utilised to compare Current Smokers with Ex-Smokers in order to establish whether smoking status influenced these datasets.

2.8.5. **TNFα Neutralisation**

Each set of data (unstimulated, poly(I:C) and R848) was analysed using multiple repeats ANOVA with Dunnett’s post-test and Friedman with Dunn’s post-test for nonparametric data.

2.8.6. **Inhibitors**

The effect of inhibitor on cytokine production and mRNA levels were compared with time-matched vehicle (DMSO) control using paired t-tests (normally distributed data) or using Wilcoxon test for non-parametric data.

2.8.7. **Rhinovirus Stimulation of BEAS-2B Cells**

Multiple repeats ANOVA with Dunnett’s post-test was used to analyse dose-response data. Where only two columns were analysed, i.e. unstimulated control with positive control (poly(I:C)), a paired t-test was used.

2.8.8. **Comparison Between RV Additional Control Samples**

Control samples included: (1) unstimulated control (U), (2) RV, (3) UV-RV, and (4) HeLa extract (H). Repeated measures ANOVA with Bonferroni’s post-test (or Friedman with Dunn’s post-test for nonparametric data) were used to analyse these datasets to compare the following columns: (a) U vs RV, (b) U vs UV-RV, (c) U vs H, (d) RV vs UV-RV, (e) RV vs H and (f) UV-RV vs H.
3.1. Introduction

Microorganisms, especially viruses, are proposed to be one of the most important triggers of COPD exacerbations [57, 340, 345]. Acute exacerbations of COPD lead to a rapid and life-threatening decline in lung function and a significant increase in respiratory symptoms, such as cough or mucus production, that also increase the rate of patient hospitalisation [234, 317]. Although the triggers and events that lead to COPD exacerbations are actively investigated, little is known about any defective roles of different airway cell types and any misdirected cooperation and signalling between them.

A few cell types have been frequently used to study functional and phenotypic changes that occur in vivo and in vitro models of COPD exacerbations. These cells include: airway epithelial cells, alveolar macrophages and neutrophils, and changes in all of them have been implicated in the progression of COPD. Smoking was shown to instigate changes in the composition of epithelial layer in the lungs (i.e. decrease in number of ciliated cells which are replaced by squamous cells) that can diminish the rate of pathogen clearance and therefore cause airway obstruction [352, 389]. The number of alveolar macrophages in the lungs of COPD patients was also shown to be increased [310], however their phagocytic capabilities are decreased [35]. This leads to the presence of a large number of cells capable of secreting factors involved in inflammation and tissue remodelling pathways which at the same time are ineffective in pathogen clearance and thus enhance the local inflammation. Additionally, there is an increased number of neutrophils present in COPD airways [192] resulting in increased ROS production and neutrophil elastase release that further damage already inflamed airways. In summary, the presence of increased number of macrophages and neutrophils in COPD airways leads to an enhanced airway inflammation initially caused by noxious gases.

Although epithelial cells, macrophages and neutrophils can be studied on their own to further elucidate their response to common pathogens or cigarette smoke, it is also imperative to study the tissue as a whole as the immune response of whole tissue could be different from
the one produced by single cell type cultures. This has been attempted by using human lung tissue in culture [80, 144]. However, there are limited data available about how human lung tissue responds to viruses or bacteria and only bacterial components (lipopolysaccharide – LPS) [144] and synthetic analogues of viral dsRNA (poly(I:C)) [80] have been used thus far to study TLR responses in human tissue \textit{ex vivo}. These initial attempts to characterise and explain the complex cooperation between human lung cells are the first steps towards our understanding of the innate immune responses in the lung.

Numerous studies have examined the response of airway cells to a single TLR ligand, such as LPS and poly(I:C). However, if the aim is to mimic bacterial or viral infection, it is important to remember that bacteria and viruses will not just activate one receptor but their different components may activate a plethora of innate immune receptors (including NLRs and RLRs for bacterial and viral infections, respectively). It is therefore essential to study the effects of not only activation of one TLR ligand, but also a combination of ligands activating multiple PRRs at the same time. Such a model would be more relevant to mimic viral/bacterial infection when using appropriate pathogen is not possible.

The main aim of this chapter was to establish whether human lung tissue explants are susceptible to TLR stimulation as a synthetic virus infection model. The experiments had the following objectives:

1. To characterise innate immune response to TLR3 and TLR7/8 ligands, by:
   a. quantifying pro-inflammatory cytokines release from the supernatant by ELISA;
   b. measuring gene expression of pro-inflammatory cytokines in tissue by qRT-PCR;
   c. measuring gene expression of type I and III IFN in tissue by qRT-PCR.
2. To assess whether activation of multiple TLRs induces synergistic release of pro-inflammatory cytokines, by measuring the release of TNFα, CCL5 and IL-6 in response to combined TLR ligands.

3. To compare the TLR-induced innate immune response of lung tissue from smokers and COPD patients.
3.2. Materials and Methods

Poly(I:C) and R848 were purchased from Invivogen (Source Bioscience, Nottingham, UK). Stock solutions of poly(I:C) and R848 were diluted in supplemented RPMI medium to 10x or 50x final concentrations (depending on the experiment). The concentrations of poly(I:C) used ranged from 0.01μg/ml to 1000μg/ml, whereas the R848 concentrations varied from 0.0001μg/ml to 10μg/ml.

All supernatants and tissue samples were collected after appropriate time of incubation at 37°C, 5% CO₂ and stored at -20°C and -80°C, respectively. First tissue sample of each condition was immersed in 200μl of RNAlater (Invitrogen, Paisley, UK) and kept overnight at 4°C, then stored at -80°C. Protein levels of TNFα, CCL5 and IL-6 in supernatants were measured by ELISA (see section 2.3) and mRNA fold-induction of IFNα, IFNβ and IFNλ were analysed by qRT-PCR (see sections 2.6 and 2.7).

3.2.1. Time and Dose-Response of Lung Tissue to TLR ligands

Following overnight incubation of WTE (see section 2.2), old medium was discarded and fresh supplemented RPMI medium was added. Only medium (800μl) was put into control wells whereas 720μl of fresh medium were added to the experimental wells. Then 80μl of 10x TLR agonist were added to the appropriate wells to obtain a total volume of 800μl. There were three wells used per condition for each patient.

For the time course experiment four time points were chosen: 1, 6, 24 and 48 hours post-stimulation. Based on pilot dose-response experiment the following concentrations were used: 100μg/ml of poly(I:C) and 10μg/ml of R848. For dose-response, all samples were collected after 24 hours and the dose of TLR ligands ranged from 0.1-1000μg/ml and 0.01-10μg/ml for poly(I:C) and R848, respectively. Demographics of subjects used in these studies are summarised in Table 3-1.
Table 3-1 Demographics of subjects used in poly(I:C) and R848 time course and dose response experiments.

<table>
<thead>
<tr>
<th></th>
<th>Time course</th>
<th>Dose response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COPD (n=7)</td>
<td>Smokers (n=6)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>4/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Age</td>
<td>73.9±6.7</td>
<td>69.8±6.7</td>
</tr>
<tr>
<td>FEV1</td>
<td>1.45±0.59</td>
<td>2.73±0.73</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>62±13.2</td>
<td>99.0±18.2</td>
</tr>
<tr>
<td>FEV1/FVC % ratio</td>
<td>52.9±12.2</td>
<td>74.8±6.5</td>
</tr>
<tr>
<td>Smoking status (Current/Ex)</td>
<td>2/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Smoking history (pack-years)</td>
<td>46 (13-88)</td>
<td>37 (17-54)</td>
</tr>
<tr>
<td>ICS use? (Y/N)</td>
<td>2/5</td>
<td>0/6</td>
</tr>
</tbody>
</table>

FVC data for one Smoker was not available.

3.2.2. Simultaneous stimulation of TLR3 and TLR7/8

As in the previous experiment, old medium was discarded and 800μl of fresh medium were added to control wells, 720μl of fresh medium were added to single-ligand wells, and 640μl of medium were added to combination wells. Then 80μl of 10x TLR ligands (poly(I:C) and/or R848) were added to the appropriate wells to obtain total volume of 800μl. The ligand concentrations used were the same as in time course experiment above, i.e. 100μg/ml of poly(I:C) and 10μg/ml of R848. All supernatants and tissue samples were collected after 24 hour incubation. Demographics of patients used in this experiment is summarised in Table 3-2.

Table 3-2 Demographics of subjects used in the combination experiment.

<table>
<thead>
<tr>
<th></th>
<th>COPD (n=12)</th>
<th>Smokers (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>8/4</td>
<td>6/4</td>
</tr>
<tr>
<td>Age</td>
<td>68.1±6.5</td>
<td>66.4±9.8</td>
</tr>
<tr>
<td>FEV1</td>
<td>2.07±0.67</td>
<td>2.45±0.54</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>76.8±18.1</td>
<td>102.5±23.0</td>
</tr>
<tr>
<td>FEV1/FVC % ratio</td>
<td>58.9±10.2</td>
<td>75.9±8.3</td>
</tr>
<tr>
<td>Smoking status (Current/Ex)</td>
<td>6/6</td>
<td>5/5</td>
</tr>
<tr>
<td>Smoking history (pack-years)</td>
<td>53 (13-126)</td>
<td>33 (2-66)</td>
</tr>
<tr>
<td>ICS use? (Y/N)</td>
<td>7/5</td>
<td>0/10</td>
</tr>
</tbody>
</table>

FVC data for one Smoker was not available.

3.2.3. Statistics

All data were checked for normality using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as mean with SEM where the datasets passed the normality tests, and as median with range where they did not.
3.2.3.1. **Time course**

Absolute levels of cytokine release or relative gene expression in response to a TLR ligand at each time point were compared with matching unstimulated controls using paired t-tests (or Wilcoxon for nonparametric data). Unpaired t-tests (Mann-Whitney) between COPD and Smokers group at each time point were utilised to verify if any significant differences in cytokine release or gene expression were present.

3.2.3.2. **Dose response**

Absolute levels of cytokine release in response to TLR ligands were analysed by one-way ANOVA with Dunnett post-test. CCL5 dataset was analysed using Friedman test with Dunn post-test comparing all ligand doses versus unstimulated control.

3.2.3.3. **Combination of TLR ligands**

The levels of TNFα and IL-6 released in response to simultaneous use of TLR3 and TLR7/8 ligands were compared to the levels induced by the two ligands on their own using paired t-test (or Wilcoxon test for CCL5). The combination effect was also compared to hypothetical sum (where absolute levels of cytokines released by both ligands were added together) with the use of paired t-test (or Wilcoxon test for CCL5).

3.2.3.4. **Effect of smoking status on cytokine and IFN expression**

Unpaired t-tests (or Mann-Whitney tests for non-parametric data) were utilised to compare Current Smokers with Ex-Smokers in order to establish whether smoking status had an effect on cytokine and IFN expression.
3.3. Results

3.3.1. Secretion of Pro-Inflammatory Cytokines

3.3.1.1. Time course

Basal levels of TNFα and CCL5 were almost undetectable, however the release of IL-6 from unstimulated tissue was elevated (app. 11000pg/mg tissue after 48 hours). Analysis of the effects of poly(I:C) and R848 on human lung tissue over time revealed that it significantly induced the release of TNFα, CCL5 and IL-6 compared to time-matched controls (Figure 3-1).

In response to poly(I:C), secretion of TNFα and CCL5 (Figure 3-1, A and C) peaked at 24 hours after which their levels were decreasing. Poly(I:C)-induced IL-6 levels (Figure 3-1, E), however, significantly increased after 24h (p<0.01 and 0.05; Smokers and COPD, respectively) and continued to increase up to 48h.

R848 induced TNFα release (p<0.001) after just 6 hours (Figure 3-1 B) after which cytokine levels decreased. Levels of CCL5 were also significantly increased after 24h in smokers (p<0.05) and after 6, 24 and 48h in tissue from COPD patients (p<0.05) when compared to unstimulated control (Figure 3-1, D). The release of IL-6 was increased in the smokers group after just 6h (p<0.001), reached significance in both groups after 24h (p<0.01) and then continued to increase up to 48h (p<0.01) (Figure 3-1, F).

The data show that R848 is a much more potent inducer of TNFα than poly(I:C). R848 caused a release of approximately 300pg/mg tissue of TNFα after 24h, when compared to about 40pg/mg tissue in response to poly(I:C). On the other hand, poly(I:C) was able to induce higher levels of CCL5 when compared to R848 (20pg/mg tissue vs 5 and 12pg/mg tissue for smokers and COPD, respectively; after 24h). IL-6 levels, however, were similar in response to both ligands throughout the time course.
Comparison between smokers and COPD at this stage revealed that tissue from COPD patients released significantly higher amounts of TNFα (323.04 vs 457.22 pg/mg tissue, p=0.0382, Figure 3-1 B) in response to R848 stimulation after 6 hours, and higher amounts of CCL5 in response to poly(I:C) after 1 hour (0.34 vs 0.79 pg/mg tissue, p=0.0047, Figure 3-1 C) and in response to R848 after 1 hour (0.37 vs 0.84 pg/mg tissue, p=0.0082) and 48 hours (2.05 vs 4.39 pg/mg, p=0.0221) (Figure 3-1 D).

In summary, human lung tissue in response to poly(I:C) and R848 released significantly elevated levels of pro-inflammatory cytokines compared to unstimulated control and peak release was time-dependent. At several time points, tissue derived from COPD patients secreted significantly higher amounts of TNFα and CCL5.
Figure 3-1 Production of pro-inflammatory cytokines in TLR-stimulated human lung tissue over time.

The production of TNFα (A,B), CCL5 (C,D) and IL-6 (E,F) in poly(I:C) (100μg/mL) (A,C,E) and R848 (10μg/mL) (B,D,F) stimulated tissue from smokers (n=6) and COPD patients (n=7). Data are presented as scatter plots with grand mean (TNFα and IL-6) or grand median (CCL5). Technical replicates from the same donor are shown using the same symbol (n=3). #, ##, ### = p<0.05, 0.01, 0.001 significantly over unstimulated control, respectively. *, ** = p<0.05, 0.01 significant difference between subject groups, respectively.
3.3.1.2. **Dose Response**

To examine how human tissue responds to varying doses of both ligands, a range of poly(I:C) (0.1-1000µg/ml) and R848 (0.01-10µg/ml) doses were used and the levels of TNFα, CCL5 and IL-6 released after 24h were measured by ELISA (Figure 3-2).

Poly(I:C) significantly induced TNFα in COPD group at a concentration of 100µg/ml (p<0.01) and in both groups at the highest dose (p<0.001) (Figure 3-2 A), CCL5 levels were significantly higher in both groups than unstimulated controls at concentrations of 10 (p<0.05), 100 and 1000µg/ml (p<0.001) (Figure 3-2 C). IL-6 protein level was also significantly higher in both groups (p<0.001) than unstimulated control at 100 and 1000µg/ml of poly(I:C) (Figure 3-2 E).

R848 in doses of 1 and 10µg/ml significantly induced the release of TNFα, CCL5 and IL-6 (p<0.001) in both patient groups (Figure 3-2 B and D). In addition, the levels of IL-6 were significantly higher than time-matched unstimulated control (p<0.01) in the COPD group at 0.1µg/ml of R848 (Figure 3-2 F).

A comparison between the two patient groups was also carried out. COPD patients released significantly higher amounts of TNFα and CCL5 compared to Smokers (Figure 3-2 A-D) in response to: 0.1 (CCL5: 0.68 vs 1.89pg/mg tissue, p=0.014), 1 (CCL5: 0.65 vs 1.85pg/mg tissue, p=0.0098), 10 (TNFα: 1.01 vs 10.19pg/mg tissue, p=0.022; CCL5: 1.52 vs 4.21pg/mg tissue, p=0.0015) and 1000µg/ml (CCL5: 46.18 vs 105.29pg/mg tissue, p=0.040) of poly(I:C) and 0.01 (CCL5: 0.57 vs 1.69pg/mg tissue, p=0.0098), 0.1 (TNFα: 5.74 vs 12.79pg/mg tissue, p=0.0042; CCL5: 1.14 vs 4.87pg/mg tissue, p=0.016), 1 (CCL5: 4.11 vs 11.33pg/mg tissue, p=0.041) and 10µg/ml of R848 (5.28 vs 13.66pg/mg tissue, p=0.041).
Figure 3-2 Production of pro-inflammatory cytokines from human lung tissue in response to increasing doses of TLR ligands.

The production of TNFα (A,B), CCL5 (C,D) and IL-6 (E,F) in poly(I:C) (A,C,E) and R848 (B,D,F) stimulated tissue from smokers (n=13) and COPD patients (n=13) after 24 hours. Data are presented as scatter plots with grand mean (TNFα and IL-6) or grand median (CCL5). Technical replicates from the same donor are shown using the same symbol (n=3). *, **, *** = p<0.05, 0.01, 0.001 significantly over unstimulated control, respectively. *, ** = p<0.05, 0.01 significant difference between subject groups, respectively.
In summary, these findings show, for the first time, that human lung tissue is susceptible to both poly(I:C) and R848 stimulation. Out of the three cytokines measured, IL-6 was most ubiquitously released. R848 was a more potent inducer of TNFα compared to poly(I:C) but the reverse was true for CCL5. However, tissue derived from COPD patients released higher amounts of both TNFα and CCL5 compared to tissue from smokers.

3.3.2. Gene Expression of Pro-inflammatory Cytokines

RNA was extracted from tissue used in the time course experiment and gene expression of TNFα, CCL5 and IL-6 was measured by qRT-PCR. The data are presented as fold-increase over unstimulated control and were normalised to GAPDH expression (Figure 3-3).

Poly(I:C)-induced gene expression of TNFα was significantly increased after 6, 24 and 48 hours (p<0.05). TNFα expression peaked after 6 hours in response to poly(I:C) (approximately 4-fold increase, Figure 3-3, A) and then started to decrease. CCL5 mRNA expression was significantly increased throughout the time course (p<0.05) but peaked after 24 hours in response to poly(I:C) (19- and 9-fold increase for smokers and COPD, respectively; Figure 3-3, C). Poly(I:C)-induced IL-6 mRNA levels were significantly increased in smokers after 1h (p<0.05) and in the COPD group throughout the time course (p<0.05) (cf. Figure 3-3, E).

R848 caused a significant increase of TNFα gene expression at all time points (p<0.05). TNFα mRNA levels; peaked after just 1 hour (61- and 39-fold increase for smokers and COPD, respectively; Figure 3-3 B) after which the levels started to decrease. R848-induced CCL5 gene expression was significantly increased at all time points (p<0.05) however its levels reached a peak after 6 hours (approximately 3-fold increase for both groups) but remained constant up to 48 hours (Figure 3-3, D). IL-6 expression, however, was significantly induced in response to R848 only in the COPD group (app. 2-fold increase after 6, 24 and 28h; p<0.05; Figure 3-3, F).

A comparison between the two patient groups revealed that there was a decreased CCL5 expression in smokers as compared with COPD after 1 hour stimulation with 100μg/ml of
poly(I:C) (0.9- and 1.5-fold increase, respectively, p=0.018); however, after 6 hour stimulation it was the Smokers group that expressed significantly higher levels of CCL5 than COPD (10.6- vs 4.5-fold increase, p=0.0082) (Figure 3-3 C).

In summary, gene expression of pro-inflammatory cytokines was significantly induced in poly(I:C)- and R848-stimulated tissue. Similarly to protein expression, TNFα mRNA levels were higher in response to R848 as compared to poly(I:C), and, again, the reverse was true for CCL5 mRNA levels. IL-6 expression, however, was not significantly induced in the Smokers group but significantly increased levels were detected in the COPD group.
Figure 3-3 Expression of pro-inflammatory cytokines in TLR-stimulated human lung tissue over time.

The expression of TNFα (A,B), CCL5 (C,D) and IL-6 (E,F) mRNA in poly(I:C) (A,C,E) and R848 (B,D,F) stimulated tissue from smokers (n=6) and COPD patients (n=7). Data are presented as scatter plots with grand median. Technical replicates from the same donor are shown using the same symbol (n=1) and correspond to the protein data shown in Figure 3-1. Horizontal line at y=1 represents the unstimulated control. # = p<0.05 significantly over unstimulated control. *, ** = p<0.05, 0.01 significant difference between subject groups, respectively.
3.3.3. Gene Expression of Interferons

Previous sections examined protein and gene expression of pro-inflammatory cytokines in human lung tissue in response to TLR3 (poly(I:C)) and TLR7/8 (R848) ligands. The next step was to verify that this model system was also able to express key antiviral genes: interferons (IFNs). The gene expression of type I (IFNα1 and IFNβ1) and type III IFNs (IFNλ1) in response to 100μg/ml poly(I:C) and 10μg/ml R848 were measured by qRT-PCR. The results are presented as fold-change and were normalised to GAPDH (Figure 3-4).

In response to poly(I:C), IFNα1 mRNA was significantly induced only in the smokers group after 6-hour stimulation compared with unstimulated control (2-fold change, p=0.012, Figure 3-4, A). Poly(I:C)-induced IFNβ1 mRNA expression peaked after 6h and then started to gradually decrease. IFNβ1 mRNA was significantly increased in smokers after 6 hours (44-fold increase, p=0.0053) and in COPD group after 6, 24 and 48 hours (19-, 11- and 4-fold increase; p=0.0029, 0.012, 0.043, respectively) (Figure 3-4, C). Lastly, IFNλ1 mRNA levels also peaked after 6h stimulation with poly(I:C) and then decreased. IFNλ1 gene expression was significantly increased after 6, 24 and 48 hours (Smokers: 399-, 223- and 72-fold increase, respectively, p=0.032; COPD: 148-, 73- and 22-fold increase, respectively, p=0.016; Figure 3-4, E).

R848 did not cause any significant changes in the expression of IFNα1, although mRNA levels after 1h in the COPD group was numerically increased (Figure 3-4, B). R848-induced IFNβ1 gene expression peaked after just 1h and then decreased to just above the basal levels. mRNA levels of IFNβ1 were significantly higher in stimulated than in unstimulated control after 1, 6 and 24 hours in Smokers (7.5-, 2- and 3-fold change, p=0.0001, 0.046, 0.011, respectively), and 1 and 24 hours in the COPD group (17- and 2.5-fold increase, p=0.016, 0.037, respectively) (Figure 3-4, D). In response to R848, IFNλ1 gene expression was not significantly increased until 24h in both groups (1.7 and 3.3-fold increase for smokers and COPD, p=0.032) and then slightly decreased in the smokers group (1.5-fold increase above unstimulated control,
p=0.032) but further increased in the COPD group (5.0-fold increase above unstimulated control, p=0.016) (cf. Figure 3-4, F).

A comparison between Smokers and COPD was also carried out. Smokers expressed significantly higher levels of IFNβ1 in response to poly(I:C) after 6 hours compared to COPD patients (44- vs 19-fold increase, respectively, p=0.0216, Figure 3-4 C). On the other hand, IFNλ1 mRNA levels were lower in smokers compared to COPD in response to R848 after 48 hours (1.5- vs 5.0-fold increase, respectively, p=0.0221, Figure 3-4 F).
Figure 3-4 Expression of type I and III IFN in TLR-stimulated human lung tissue over time.

The expression of IFNα1 (A,B), IFNβ (C,D) and IFNλ1 (E,F) mRNA in poly(I:C) (A,C,E) and R848 (B,D,F) stimulated tissue from smokers (n=6) and COPD patients (n=7). Data are presented as scatter plots with grand mean (IFNα1 and IFNβ) or with grand median (IFNλ1). Technical replicates from the same donor are shown using the same symbol (n=1) and correspond to the protein and gene expression data shown in Figures 3-1 and 3-3. Horizontal line at y=1 represents the unstimulated control. #, ##, ### = p<0.05, 0.01, 0.001 significantly over unstimulated control, respectively. * = p<0.05 significant difference between subject groups.

In summary, IFNα1 mRNA levels were generally not significantly induced in response to either ligand. IFNβ1 gene expression peaked at 6 hours and was higher in the Smokers group. Poly(I:C) was a potent inducer of IFNλ1 gene expression, however COPD had higher levels of IFNλ1 mRNA compared to smokers in response to R848.
3.3.4. Simultaneous stimulation of TLR3 and TLR7/8

To further investigate how TLR ligands stimulate the innate immune system in human lung tissue, poly(I:C) and R848 were used in combination. Simultaneous activation of TLR3 and TLR7/8 was used to make this artificial model of virus infection more relevant, as rhinovirus is known to activate more than one TLR [380].

Poly(I:C) (100μg/ml) and R848 (10μg/ml) were used on their own and in combination to examine whether activation of multiple TLRs produces additive or synergistic response. The concentrations of TLR ligands were the same as the ones used in the time-course presented above. Absolute levels of TNFα, CCL5 and IL-6 proteins were measured by ELISA. Cytokine levels in response to each of the ligands were compared to the levels produced in response to the combination. In addition, the protein levels secreted by the tissue in response to combination were compared to hypothetical result, i.e. sum of absolute levels of cytokines produced by the two TLR ligands.

Statistical analysis revealed that poly(I:C) and R848 used in combination led to a significantly higher release of TNFα from tissue (both Smokers and COPD) when compared to either ligand alone (Figure 3-5, A and B). Simultaneous activation of TLR3 and TLR7/8 led to synergistic release of TNFα which was significantly higher than the amount produced in response to poly(I:C) and R848 alone (Figure 3-5 A-B). Combination of ligands also induced significantly higher levels of TNFα than the hypothetical sum (411 vs 274pg/mg tissue, p=0.024 (Smokers); 523 vs 371pg/mg tissue, p=0.010 (COPD)).

Similar results were observed for CCL5. Absolute levels of CCL5 secreted in response to combination were significantly higher than any ligand alone (Figure 3-5, C and D). CCL5 levels in response to combination were also significantly higher than the hypothetical sum in both patient groups (37 vs 19pg/mg tissue, p=0.0059 (Smokers); 68 vs 56pg/mg tissue, p=0.016 (COPD)).
IL-6 levels in response to combination were significantly higher than those produced in response to poly(I:C) (29246 vs 24951pg/mg tissue, p=0.0057 (COPD only)) and R848 (25229 vs 15934pg/mg tissue, p=0.0007 (Smokers); 29246 vs 20771pg/mg tissue, p<0.0001 (COPD)). Combination effect, however, was not higher than the hypothetical sum.

In summary, human lung tissue in response to simultaneous activation of TLR3 and TLR7/8 produced significantly higher levels of TNFα and CCL5 compared to the levels induced by any of the ligands alone or hypothetical sum. IL-6 levels in response to ligand combination were also higher compared to poly(I:C) or R848 alone, however, they were not higher than the hypothetical sum.
Figure 3-5 Effects of simultaneous activation of TLR3 and TLR7/8 on pro-inflammatory cytokine production in human lung tissue.

The effects of simultaneous TLR3 and TLR7/8 activation on the production of TNFα (A,B), CCL5 (C,D) and IL-6 (E,F) in WTE from smokers (n=10) and COPD patients (n=12) after 24 hours. Data are presented as scatter plots with grand mean (TNFα and IL-6) or grand median (CCL5). Technical replicates from the same donor are shown using the same symbol (n=3). Hypothetical result (“Sum”) was calculated by adding the averages of poly(I:C) and R848 data for each patient. *, **, *** = p<0.025, 0.005, 0.0005 significant difference between conditions, respectively. #, ## = p<0.05, 0.01 significantly above hypothetical additive result, respectively.
3.3.5. The Effects of Smoking Status

Additional statistical analyses were performed in order to establish whether smoking status of the patients had an effect on cytokine expression in this tissue model. In these analyses, samples were split into two groups: current and ex-smokers. For the dose-response and combination experiments, because of the larger sample size, it was possible to also compare current and ex-smokers within the COPD and Smokers groups. In general, there were no significant differences in the release and gene expression of pro-inflammatory cytokines and the expression of IFNs. However, a sub-analysis on the Smokers group suggested that the release of TNFα is attenuated in samples derived from current smokers, especially in response to R848 (see Table 3-3). Other cytokines were not smoking-dependent and there were no significant differences in the COPD group. The remaining tables showing these analyses are presented in Appendix II.
Table 3-3 The effect of smoking status on cytokine release in response to increasing doses of poly(I:C) and R848 from samples derived from Smokers group only.

<table>
<thead>
<tr>
<th>Dose response</th>
<th>Protein</th>
<th>Smokers only</th>
<th></th>
<th></th>
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<tr>
<td></td>
<td>PolyI:C</td>
<td>EX (n=7)</td>
<td>CURR (n=6)</td>
<td>P value</td>
<td>R848</td>
<td>EX (n=7)</td>
<td>CURR (n=6)</td>
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<tr>
<td>TNFα</td>
<td>0.1</td>
<td>0.76 ±0.11</td>
<td>0.60 ±0.13</td>
<td>NS</td>
<td>0.01</td>
<td>0.80 ±0.17</td>
<td>0.52 ±0.11</td>
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<td></td>
<td>1</td>
<td>0.89 ±0.15</td>
<td>0.77 ±0.17</td>
<td>NS</td>
<td>0.1</td>
<td>8.99 ±1.22</td>
<td>1.95 ±0.19</td>
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<td></td>
<td>10</td>
<td>1.22 ±0.13</td>
<td>0.77 ±0.17</td>
<td>NS</td>
<td>1</td>
<td>272.9 ±38.87</td>
<td>77.42 ±9.94</td>
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<td></td>
<td>100</td>
<td>19.68 ±4.47</td>
<td>10.13 ±5.11</td>
<td>NS</td>
<td>10</td>
<td>318.62 ±48.67</td>
<td>151.31 ±40.03</td>
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<td></td>
<td>1000</td>
<td>69.38 ±15.65</td>
<td>14.11 ±2.50</td>
<td>0.0082</td>
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<td>CCL5</td>
<td>0.1</td>
<td>0.86 ±0.28</td>
<td>0.87 ±0.29</td>
<td>NS</td>
<td>0.01</td>
<td>1.18 ±0.32</td>
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<td>8.97 ±3.18</td>
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<td>9.20 ±3.36</td>
<td>5.31 ±2.26</td>
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<tr>
<td></td>
<td>1000</td>
<td>72.35 ±20.08</td>
<td>42.94 ±9.58</td>
<td>NS</td>
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<td>IL-6</td>
<td>0.1</td>
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<td>7034 ±1245</td>
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<td>8937 ±2156</td>
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<td>17706 ±4293</td>
<td>NS</td>
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</table>

Data are expressed as pg/mg of tissue and presented by mean ± SEM. There were 3 technical replicates for each patient sample. Current and Ex-smokers were compared using Unpaired t-tests for each condition. EX – ex-smokers, CURR – current smokers.
3.4. Discussion

Human lung tissue produced a pro-inflammatory and anti-viral response to TLR ligands *ex vivo* by increasing the levels of cytokines and interferons. These experiments have confirmed that whole tissue explants are responsive to TLR3 and/or TLR7/8 activation and are therefore a good model to study human innate immune response *in vitro*. Significant differences in cytokine release and gene expression of tissue from Smokers and COPD also suggest that this model could be used to compare the innate immune response of unobstructed and obstructed lungs and could further elucidate the mechanisms that lead to the development and progression of COPD.

3.4.1. Pro-inflammatory Cytokine Secretion

Analysis of pro-inflammatory cytokine induction in response to poly(I:C) and R848 revealed that TNFα, CCL5 and IL-6 were all significantly induced in a time-dependent and dose-dependent manner. Little is known about human lung tissue response to the activation of TLR3 and TLR7/8. Data published so far are limited to the effects after 24 hour stimulation with poly(I:C): precision cut lung slices released approximately 100pg/ml TNFα, 1000pg/ml CCL5 and 100000pg/ml IL-6 in response to 100µg/ml poly(I:C) [80]. The results in that study were not adjusted for tissue weight and it is therefore difficult to compare the two models. However, studies conducted by Cooper et al. and the results presented in this chapter show that human lung tissue responds to TLR3 activation when prepared by two different methods and the response is similar, at least after 24 hours [80]. Cooper et al. also observed no differences in cytokine levels in response to either 10 or 100µg/ml poly(I:C) [80]. In this current study, cytokine levels released in response to different doses of ligands have not been compared, but there was a notable increase in TNFα, CCL5 and IL-6 levels after stimulation with 100µg/ml of poly(I:C) compared to 10µg/ml. This could be explained by the different preparation method. Precision cut lung slices are very thin and flat whereas whole tissue explants are cubes of tissue and thus the surface to volume ratio is higher in the lung slices. Ligands such as poly(I:C) are
therefore more readily available to the lung cells in precision cut lung slices when compared to whole tissue explants hence the concentration of ligand required to induce maximal response is lower.

There are no published studies that assess the response of human lung tissue to TLR7/8 stimulation, however the effects of both poly(I:C) and R848 in many different primary cell types and cell lines have been examined (see summary in Table 3-4) [133, 143, 218, 252, 286, 363, 403]. For example, stimulation with 0.1 and 1µg/ml poly(I:C) resulted in only modest increase in TNFα and IL-6 in PBMCs from asthmatic children, whereas these cells in response to 1 and 10µg/ml of R848 secreted significantly higher amounts of both TNFα and IL-6 compared to unstimulated control [218]. Guilliot et al. investigated the release of poly(I:C)-induced CCL5 and IL-6 over time in BEAS2B cells and showed that the levels of these proteins gradually increased over time and did not reach their peak point after 24h (the end point of the experiment), which is in accordance with the data presented in the current study [143]. CCL5 was also up-regulated following poly(I:C) exposure in BEAS2B cells in a different study [133] and following both poly(I:C) and R848 stimulation in bone marrow-derived dendritic cells from BALB/c mice [252].

There are scarce data describing the effects of R848 in human respiratory cells therefore no direct comparisons can be made with findings from current study. R848 induced significant release of TNFα and IL-6 from monocyte-derived dendritic cells [286, 403], RAW264.7 macrophage cell line and bone marrow-derived macrophages from BALB/c mice [363]. The levels of CCL5 in other studies were generally higher than in this whole tissue explant model. However, increased CCL5 production in BEAS2B cells [133], mouse dendritic cells [252] and in the precision cut lung slices [80] (where epithelial cells are exposed to the ligand) implies that CCL5 is secreted by specific cell types rather than all the cells present in the lungs.
In other studies, Butchar et al. showed that TNFα levels increase in peripheral blood monocytes following overnight R848 stimulation [58]. Moreover, Yanagisawa et al. investigated the response to R848 in peripheral blood neutrophils and found that TNFα was released in a dose-dependent manner following 24h R848 stimulation [417]. The data presented in current study showed that the production of TNFα following R848 stimulation peaked at 6h (at least 400-fold increase) whereas at 24h cytokine levels decreased to only 200pg/mg of tissue (compared to controls) when exposed to 1 and 10μg/ml of R848. Data from the current study are therefore in accordance with findings of others, i.e. TNFα is released in a dose-dependent manner. However other groups did not examine the responses at earlier time points and may have therefore missed the maximal cytokine release.

In this study CCL5 induction was significant but low compared to the other two cytokines. Weak induction of CCL5 in response to R848 stimulation was also observed by Jensen and Gad [173] who reported that CCL5 induction (in monocyte-derived dendritic cells) following 1μg/ml R848 exposure did not exceed 10-fold increase in protein level. Due to the relatively small number of studies that investigated the effects of R848 in human cells it is impossible to make any sound comparisons. However, data obtained by independent groups confirmed a potent induction of TNFα and IL-6 in response to R848, at least in the inflammatory cells [58, 286].

<table>
<thead>
<tr>
<th>Ligands used</th>
<th>Cell type</th>
<th>Mediators measured</th>
<th>Main findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(I:C)</td>
<td>Precision cut lung slices</td>
<td>TNFα, CCL5, IL-6</td>
<td>Poly(I:C) (100µg/ml) induced the release of TNFα (100pg/ml), CCL5 (1ng/ml) and IL-6 (100ng/ml) after 24h. No difference in the levels of cytokines in response to either 10 or 100pg/ml of poly(I:C).</td>
<td>Cooper et al. [80]</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>BEAS-2B</td>
<td>CCL5</td>
<td>Dose-dependent secretion of CCL5 after poly(I:C) stimulation. Levels of CCL5 increased to app. 7ng/ml after 24h.</td>
<td>Gern et al. [133]</td>
</tr>
<tr>
<td>Ligands used</td>
<td>Cell type</td>
<td>Mediators measured</td>
<td>Main findings</td>
<td>Authors</td>
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<tr>
<td>Poly(I:C)</td>
<td>BEAS-2B</td>
<td>CCL5, IL-6</td>
<td>Time and dose-dependent secretion of both cytokines following poly(I:C) stimulation. Both cytokine levels increased gradually until reaching a peak (2 and 30ng/ml for CCL5 and IL-6, respectively) after 24h (end of experiment).</td>
<td>Guillot et al. [143]</td>
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<td>Poly(I:C); R848</td>
<td>PBMCs (asthmatic children)</td>
<td>TNFα, IL-6</td>
<td>Low TNFα levels released after poly(I:C) stimulation. Levels of TNFα and IL-6 were lower in cells from children at exacerbation when compared to convalescence stage. Increased levels of both cytokines after R848 stimulation. TNFα and IL-6 after R848 stimulation were higher in cells collected at exacerbation compared to convalescence stage.</td>
<td>Lee et al. [218]</td>
</tr>
<tr>
<td>Poly(I:C); R848</td>
<td>BMDMs (BALB/c mice)</td>
<td>CCL5</td>
<td>Increased CCL5 levels after poly(I:C) and R848 stimulation (app. 18 and 10ng/ml, respectively)</td>
<td>Mitchell et al. [252]</td>
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<tr>
<td>Poly(I:C); R848</td>
<td>RAW264.7; BMDMs (BALB/c mice)</td>
<td>TNFα, IL-6</td>
<td>Poly(I:C) caused a higher increase in TNFα levels than R848 in RAW264.7 cells – opposite was found in BM-DMs. IL-6 was only released in response to R848 in BM-DMs.</td>
<td>Suet Ting Tan et al. [363]</td>
</tr>
<tr>
<td>Poly(I:C); R848</td>
<td>moDCs</td>
<td>TNFα, IL-6</td>
<td>Limited induction of TNFα in response to 25µg/ml poly(I:C) - app.300pg/ml after 24h. Increased levels of TNFα (8ng/ml) and IL-6 (10ng/ml) after R848 (2µg/ml) stimulation.</td>
<td>Wenink et al. [403]</td>
</tr>
<tr>
<td>R848</td>
<td>PBMCs</td>
<td>TNFα</td>
<td>TNFα levels increase to 7ng/ml after overnight incubation with 1µM of R848.</td>
<td>Butchar et al. [58]</td>
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<td>R848</td>
<td>PBMCs</td>
<td>TNFα, IL-6</td>
<td>R848 caused an increase in the levels of TNFα and IL-6 (200 and 20ng/ml, respectively, after 24h).</td>
<td>Paustian et al. [286]</td>
</tr>
<tr>
<td>R848</td>
<td>PBMCs</td>
<td>TNFα, IL-6</td>
<td>Both cytokine levels were released in dose-dependent manner. R848 caused a significant increase in TNFα and IL-6 levels compared to controls.</td>
<td>Yanagisawa et al. [417]</td>
</tr>
<tr>
<td>R848</td>
<td>moDCs</td>
<td>TNFα, CCL5, IL-6</td>
<td>Following 24h exposure to R848, MD-DCs secreted high levels of TNFα and IL-6 (&gt;1000-fold increase) and only limited levels of CCL5 (1-10-fold increase).</td>
<td>Jensen and Gad [173]</td>
</tr>
</tbody>
</table>

BMDM – bone marrow-derived macrophage; moDC – monocyte-derived dendritic cell; PBMC – peripheral blood mononuclear cell
Basal levels of IL-6 in both whole tissue explants and precision cult lung slices were elevated compared to other cytokines studied, which confirms that this is a common phenomenon when human lung tissue is in culture [80, 144]. On the other hand, it has been shown that airway epithelial cells (e.g. BEAS2B cell line) also release high basal levels of IL-6 [211], suggesting that high IL-6 secretion is normal for airway cells studied in vitro. There is evidence that aging contributes to high IL-6 secretion in humans [248], however this does not explain the increase in IL-6 in BEAS2B cells. Culture medium supplements could also play a role and cells can react to the components of bovine serum that is commonly added. Hackett et al. do not mention any serum additions to their whole tissue explant culture medium and the basal IL-6 levels presented in their study are lower than those presented in the current study (where medium supplemented with 10% (v/v) foetal bovine serum was used) [144]. There are published data that suggest the addition of serum changes the level of secreted cytokines in response to bacterial stimuli [118]. The actual influence of serum on human lung tissue has not been studied. In the context of the findings of the present study, however, high basal IL-6 levels do not influence the fact that tissue from both smokers and COPD release increased levels of various pro-inflammatory cytokines in response to TLR activation and that there are significant differences between the groups.

In summary, these findings show that cell cultures required lower concentrations of poly(I:C) and R848 than lung tissue models to produce similar levels of pro-inflammatory cytokines. However, single cell type models use a monolayer of cells and, as in the case of precision cut lung slices, stimuli are readily available for the uptake by the cells. Although at first it seems that the cytokine levels produced by other models were higher than in this study, it must be underlined that the results presented here were adjusted for tissue weight effectively decreasing the values per ml approximately 30 times (as values per ml were divided by mg tissue weight). It is therefore difficult to compare the data obtained from different models as the number of cells and/or tissue weight used in other studies is probably different from the tissue
weight used here. However, similar findings made by other groups suggest that the model presented in this study is a reliable representation of the human lung tissue response to TLR3 and TLR7/8 activation.

3.4.2. Pro-inflammatory Cytokine Gene Expression

The analysis of gene expression pattern of pro-inflammatory cytokines revealed that TNFα and CCL5 mRNA levels were significantly increased in time-dependent manner. TNFα expression peaked at 6 hours in response to poly(I:C) and after just 1 hour after R848 stimulation, which reflects the release of TNFα protein. The gene expression of CCL5, however, did not predict the levels of CCL5 protein release to the supernatant as CCL5 protein release peaked after 24h but CCL5 mRNA remained at similar level throughout the time course (especially for R848). Previous studies suggest that CCL5 is constitutively transcribed in airway cells [37] which could explain relatively constant levels of CCL5 mRNA observed in the present study. Nevertheless, both poly(I:C) and R848 induced a small but significant increase in CCL5 mRNA. The expression of IL-6, however, was significantly induced only in the COPD group. Limited increase in mRNA levels of IL-6 when compared to large quantities of IL-6 protein released into the supernatant suggest that IL-6, as a marker of general inflammation, is already actively transcribed. This could be due to lung injury caused by tissue processing (high basal levels of IL-6 in all samples), or perhaps due to nonspecific induction of IL-6 by the components of culture medium.

Limited research has been done to analyse TNFα, CCL5 and IL-6 mRNA levels in airway cells in response to poly(I:C) and/or R848. Others used poly(I:C) and R848 to study TLRs in different cells, including human monocyte-derived macrophages and dendritic cells (see Table 3-5 for summary) [224, 231]. TNFα mRNA levels were significantly higher after 9h incubation with 10µM R848 in both macrophages and dendritic cells, although the fold-change was greater in macrophages compared to dendritic cells (200 vs 150 fold, respectively) [232]. The change in TNFα mRNA was less pronounced in response to 30µg/ml poly(I:C) (compared to R848) and
was higher at 9h compared to 24h. The more rapid response of a monolayer of cells vs explant tissue model could be again explained by the culture method. In a different study, Lombardi et al. observed modest 10 fold increase in TNFα mRNA levels in monocyte-derived dendritic cells after 24h incubation with 1µg/ml R848 [224]. Taking into account this and others’ findings, by 24h TNFα gene expression fell dramatically compared to earlier time points, suggesting that Lombardi et al. may have missed a crucial, earlier time point. TNFα, CCL5 and IL-6 mRNA levels were also increased in bone marrow-derived macrophages from mice in response to 20µg/ml poly(I:C) (approximately 150, 100 and 250 fold-change, respectively) and 40nM R848 (150, 50 and 250 fold-change, respectively) [421]. Others demonstrated a pronounced fold-increase in IL-6 mRNA following poly(I:C) and R848 stimulation (in this case, levels in macrophages were lower than in dendritic cells) [224, 232]. This relatively high IL-6 gene induction differs from the novel tissue model presented in this chapter. As explained above, by cutting tissue with a scalpel, a direct injury to the prepared tissue is sustained, and this could explain high basal mRNA and protein levels of IL-6.

In summary, the kinetics of pro-inflammatory cytokine gene expression are not well elucidated in human lung tissue. The data presented above are the first to show the pattern of TNFα, CCL5 and IL-6 gene expression in response to poly(I:C) and R848 at multiple time points. Comparison to other studies conducted either in human or murine immune cells indicates that TNFα and CCL5 patterns are comparable to this human lung tissue model. IL-6 expression in tissue is already high and therefore additional induction by TLR ligands is minimal (at the gene expression level), as opposed to other studies conducted in single cell type cultures. Despite the differences in the IL-6 gene expression pattern, this model is a reliable one to study gene expression in human lung tissue.
Table 3-5 A summary of studies examining poly(I:C)- and R848-induced pro-inflammatory cytokine gene expression.

<table>
<thead>
<tr>
<th>Ligands used</th>
<th>Cell type</th>
<th>Mediators measured</th>
<th>Main findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(I:C); R848</td>
<td>MDMs, moDCs</td>
<td>TNFα, IL-6</td>
<td><strong>In MDMs:</strong> 200-fold and 50-fold increase of TNFα mRNA after 9 and 24h stimulation with 10µM R848, respectively. Limited TNFα expression after exposure to 30µg/ml poly(I:C). IL-6 expression increased about 25000 times after 24h. Again, limited expression of IL-6 following poly(I:C) stimulation. <strong>In moDCs:</strong> Quick induction of TNFα gene expression after 9h in response to both ligands (app. 50-fold) – decreased after 24h. Quick increase in IL-6 mRNA levels after 9h (about 10000-fold) in response to both ligands which also decreased after 24h. Mäkelä et al. [231]</td>
<td></td>
</tr>
<tr>
<td>Poly(I:C); R848</td>
<td>moDCs</td>
<td>IL-6</td>
<td>Limited IL-6 mRNA levels after poly(I:C) exposure. Quick increase in IL-6 gene expression following R848 stimulation – 5000-fold after 4 and 8h. Limited expression after 24h. Mäkelä et al. [232]</td>
<td></td>
</tr>
<tr>
<td>Poly(I:C); R848</td>
<td>BMDMs (mouse)</td>
<td>TNFα, CCL5, IL-6</td>
<td>Increased TNFα mRNA levels (&gt;100-fold) following 3h exposure to poly(I:C) (25µg/ml) and R848 (40nM). CCL5 gene expression increased app. 150-fold after poly(I:C) stimulation and about 30-fold in response to R848. Levels of IL-6 mRNA also increased in response to both ligands (app. 250-fold increase). Yang et al. [421]</td>
<td></td>
</tr>
<tr>
<td>R848</td>
<td>moDCs</td>
<td>TNFα, IL-6</td>
<td>10-fold increase of TNFα and 100-fold increase of IL-6 gene expression after 24h stimulation with 1µg/ml R848. Lombardi et al. [224]</td>
<td></td>
</tr>
</tbody>
</table>

BMDM – bone marrow-derived macrophage; MDM – monocyte-derived macrophage; moDC – monocyte-derived dendritic cell
3.4.3. Interferon Gene Expression

Gene expression pattern of interferons showed a limited increase of IFNα1 in response to both poly(I:C) and R848, however, poly(I:C) induced a potent increase of both IFNβ1 and IFNλ1 mRNA, whereas the response to R848 was more modest. As there are 13 IFNα variants present in humans [155], the reasons for low levels of IFNα1 induction in this model could be that this particular IFNα variant is not an appropriate one to study TLR3 and TLR7/8 activation in human lung tissue. IFNα1 mRNA expression was examined because others have assessed that particular variant in various studies examining TLR responses [155, 167], including responses to 25μg/ml poly(I:C) and 10μM imiquimod (TLR7 ligand) and found that IFNα1 is the most prominent variant expressed in myeloid dendritic cells (see Table 3-6 for summary) [155]. R848 was demonstrated to be a potent IFNα inducer [369], and the IFNα1 mRNA expression levels after 1 hour suggest that R848 indeed generated higher IFNα1 mRNA levels in human lung tissue when compared to poly(I:C); although the fold-change did not reach statistical significance. On the other hand, R848 was shown to decrease IFNα protein levels when used in combination with TLR9 ligand [36, 239], suggesting differential activation of genes in response to a different combination of signals. Human innate immune response is emerging to be minutely tailored to specific pathogen- and danger-associated signals and evidence suggest that the expression of IFNα variants is signal- and cell type-specific [155]. It is therefore possible that IFNα1 plays a lesser role under these circumstances and thus is not transcribed.

The levels of IFNβ1 mRNA were significantly up-regulated in response to both poly(I:C) and R848 in the tissue explants. In many studies poly(I:C) triggered IFNβ expression in human lung cells (cf. Table 3-6); for example BEAS2B and A549 cell lines and small airway primary epithelial cells [143, 312]. R848, however, is not a good inducer of IFNβ1 gene expression, which has been demonstrated before in monocyte-derived dendritic cells [126, 232]. Poly(I:C) was also more potent at inducing IFNλ1 mRNA levels in the tissue compared to R848, which is in accordance with results obtained from monocyte-derived dendritic cells [126]. However,
in a population of plasmocytoid dendritic cells it was imiquimod (similar to R848) that induced higher levels of IFNλ1 protein than poly(I:C) [246].

To date, there are no data describing type I and type III IFN responses to TLR activation in human lung tissue as a whole. Analysis of poly(I:C) stimulation on human bronchial smooth muscle cells revealed that both IFNβ1 and IFNλ1 gene expression was significantly up-regulated after 3 and 24h [63], which also reflects the pattern in this model. In this model, it was established that whole tissue explants show characteristics that are similar to single cell type systems, e.g. limited IFNα1 response and more potent IFNβ1 and IFNλ1 gene expression to stimulation with poly(I:C) when compared to R848. These data again suggest that whole lung tissue model is appropriate to study innate immune response in the human lung.
<table>
<thead>
<tr>
<th>Ligands used</th>
<th>Cell type</th>
<th>Mediators measured</th>
<th>Main findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(I:C)</td>
<td>BEAS-2B</td>
<td>IFNβ</td>
<td>IFNβ levels significantly increased peaking 6h post-stimulation</td>
<td>Guillot et al. [143]</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Primary lung epithelial cells</td>
<td>IFNβ</td>
<td>IFNβ protein increased after 6 and 24h post-infection. Peak levels after 6h.</td>
<td>Ritter et al. [312]</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>BSMCs</td>
<td>IFNβ, IFNλ1</td>
<td>Gene expression of both IFN significantly increased in dose-dependent manner after 3 and 24h. Highest IFNβ expression after 3h (&gt;5000 fold-increase), but IFNλ1 peaked after 24h (app. 150 fold-increase).</td>
<td>Calvén et al. [63]</td>
</tr>
<tr>
<td>Poly(I:C); R848</td>
<td>HBECs, PBMCs</td>
<td>IFNα, IFNβ, IFNλ1</td>
<td>IFNβ and IFNλ1 mRNA levels increased in HBECs from healthy and asthmatics donors after 8h stimulation with 10mg/ml poly(I:C). IFNβ gene expression after 24h R848 (1µM) stimulation increased in HBECs from healthy donors but not asthmatics, however IFNλ1 increased in both groups. Potent IFNα protein expression in response to R848 in PBMCs.</td>
<td>Sykes et al. [369]</td>
</tr>
<tr>
<td>Poly(I:C); R848</td>
<td>DCs (mouse and human)</td>
<td>IFNα, IFNβ, IFNλ1</td>
<td>Quantitative induction of IFNα and IFNβ mRNA by poly(I:C) and R848. IFNλ1 in response to R848 most prominent IFN expressed.</td>
<td>Gautier et al. [126]</td>
</tr>
<tr>
<td>Poly(I:C); R848</td>
<td>moDCs</td>
<td>IFNα, IFNβ, IFNλ1</td>
<td>In response to poly(I:C) cells expressed limited IFNα and prominent IFNβ and IFNλ1 (peak after 3h). None of IFN increased after R848 stimulation.</td>
<td>Mäkelä et al. [232]</td>
</tr>
<tr>
<td>Poly(I:C); Imiquimod</td>
<td>pDCs</td>
<td>IFNλ1</td>
<td>Production of IFNλ1 in pDCs after 24h was increased in response to imiquimod (3000pg/ml vs &lt;1000pg/ml for controls). Poly(I:C) did not induce IFNλ1 levels above time-matched control.</td>
<td>Megjugo-rac et al. [246]</td>
</tr>
<tr>
<td>R848</td>
<td>PBLs, PBMCs</td>
<td>IFNα</td>
<td>R848 causes modest IFNα release from PBL, however R848 decreases IFNα levels when used in co-stimulation with TLR9 ligand when compared to levels induced by TLR9 ligand alone.</td>
<td>Berghöfer et al. [36] and Marshall et al. [239]</td>
</tr>
</tbody>
</table>

BSMC – bronchial smooth muscle cell; HBEC – human bronchial epithelial cell; mDC – myeloid dendritic cell; moDC – monocyte-derived dendritic cell; PBL – peripheral blood leukocyte; PBMC – peripheral blood mononuclear cell; pDC – plasmacytoid dendritic cell
3.4.4. Simultaneous activation of TLR3 and TLR7/8

Both TLR3 and TLR7/8 ligands were used in combination to assess any synergistic effects that could be produced in response to the two stimuli. As rhinoviruses were shown to stimulate several receptors [380], such a combination would better represent what is happening during viral infection. Results confirmed that activation of TLR3 and TLR7/8 at the same time caused a synergistic release of TNFα and CCL5. There was no synergy observed for IL-6 protein, most likely due to the fact that levels of IL-6 in response to any of the ligand alone were high and the tissue was not able to produce any more of the cytokine as it was maximally stimulated. Perhaps using lower (sub-optimal) concentrations of ligands would have been better for this type of experiment. There are several studies that examined the effects of simultaneous activation of different TLRs in different cell types, e.g. monocyte-derived dendritic cells and macrophages (cf. Table 3-7) [45, 231, 232, 258, 316, 363]. Using poly(I:C) (10 µg/ml) and R848 (25 ng/ml) in combination produced synergistic release of both TNFα and IL-6 from RAW264.7 macrophage cell line and bone marrow-derived macrophages from BALB/c mice [363]. In monocyte-derived dendritic cells, synergy in response to 30 µg/ml of poly(I:C) and 5 µM of R848 was observed in the release of a plethora of cytokines, including TNFα and IL-6 [231], and, in a separate study TNFα production was also synergistically up-regulated in response to TLR3 and TLR7/8 activation [316]. However this effect was not repeated in monocyte-derived macrophages [231]. Taken together, synergy in response to multiple TLR activation was observed in many different cell types as well as in this model. Lack of synergy observed in monocyte-derived macrophages underscores the view that components of the innate immune system are minutely tailored depending on the signal received and activate cells that are most likely to help to clear the infection. Nevertheless, human lung cells are always in contact with different cell types present in the tissue. They will therefore confer immune signals following any infection and may activate cells that normally would not respond directly to the pathogen.
Table 3-7 A summary of studies examining combination effect of poly(I:C) and R848 on pro-inflammatory cytokine release.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mediators measured</th>
<th>Main findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>moDCs</td>
<td>TNFα</td>
<td>Combination-induced &gt;3000pg/ml of cytokine but additive effect of both ligands was &lt;1000pg/ml.</td>
<td>Roelofs et al. [316]</td>
</tr>
<tr>
<td>moDCs; MDMs</td>
<td>TNFα, IL-6</td>
<td>In moDCs: Combination-induced TNFα protein levels were over 5000pg/ml compared to &lt;2500pg/ml for each ligand alone (24h). IL-6 protein levels in response to combination increased to app. 7500pg/ml compared to about 2000pg/ml for ligands alone (24h). Synergistic gene expression caused by ligand combination also observed in this study (only after 9h but not 24h). In MDMs: No synergy observed in release of TNFα and IL-6 when compared to sum of both ligands. Gene expression of IL-6 was synergistically increased only after 24h and not 9h. No such observations for TNFα mRNA.</td>
<td>Mäkelä et al. [231]</td>
</tr>
<tr>
<td>RAW264.7; BMDM (mouse)</td>
<td>TNFα, IL-6</td>
<td>TNFα and IL-6 protein levels induced by combination of the two ligands was synergistically increased when compared to any of the ligands alone (after 24h). Interestingly, when ligands were administered in sequence, i.e. (1) first poly(I:C) then R848 or (2) first R848 then poly(I:C), the first combination resulted in significantly higher TNFα and IL-6 gene expression when compared to the second combination.</td>
<td>Suet Ting Tan et al. [363]</td>
</tr>
<tr>
<td>moDCs</td>
<td>IL-6</td>
<td>Synergistic IL-6 mRNA increase in response to combination after 4, 8 and 24h. For example, after 8h combination-induced cytokine levels were increased &gt;15000-fold, compared to &lt;1000-fold and &lt;5000-fold increase in response to poly(I:C) and R848, respectively.</td>
<td>Mäkelä et al. [232]</td>
</tr>
</tbody>
</table>

BMDM – bone marrow-derived macrophage; moDC – monocyte-derived dendritic cell; MDM – monocyte-derived macrophage

3.4.5. Differences between Smokers and COPD

The last point to be discussed is the difference in pro-inflammatory response between the two study groups: smokers and COPD. COPD exacerbations lead to hospitalisations and deaths and it became evident that patients suffering from COPD have increased respiratory symptoms and require specialised treatment to common pathogens such as rhinoviruses [234]. In the present study, it was shown that indeed the tissue from the COPD group released significantly
higher amounts of TNFα and CCL5 than the tissue from smokers. This observation is in accordance with other studies that used whole lung tissue to examine TLR responses, e.g. activation of TLR4 by LPS [144]. On the other hand, there are studies that examined the response of alveolar macrophages to LPS and found that cells from COPD patients had attenuated inflammatory response compared to non-smoking controls [11]. As mentioned in the introduction, the number of macrophages and neutrophils in COPD lungs is increased which could be the reason why COPD whole tissue explants respond with greater pro-inflammatory cytokine release compared to Smokers.

The analysis of gene expression of pro-inflammatory cytokines revealed that smokers had increased gene expression levels of CCL5 compared to COPD after 1 and 6 hours post-stimulation with poly(I:C). This is a surprising result that is in contrast with the protein data and further investigation with an increase in the number of patients could confirm whether this is a true outcome. A study carried out by Di Stefano et al. showed that CCL5 expression in bronchial mucosa is higher in the COPD group compared to smokers with normal lung function and these results are also in conflict with those presented here [99]. However, these two studies were conducted in different tissues and Di Stefano et al. measured only basal cytokine gene expression.

In addition, after 6h Smokers had significantly higher IFNβ1 gene expression compared to COPD in response to poly(I:C). This is in accordance with other studies suggesting that patients with COPD have impaired IFNβ production in response to viral infections [234]. On the other hand, R848 triggered higher IFNβ mRNA levels in the COPD group compared to Smokers, although the difference was not statistically significant. Interestingly, it was the COPD group which responded with higher IFNλ1 expression to R848 after 48 hours. There are no other studies that investigated differences in IFN gene expression in tissue from Smokers with normal lung function and COPD, especially in response to R848. These novel findings are therefore first to show differential IFN expression in response to TLR ligands in Smokers and COPD.
Further investigations are required to reveal the mechanisms that selectively activate pathways leading to IFN expression.

Interestingly, however, smoking status seems to be an important factor for the release of TNFα from the tissue in response to poly(I:C), R848 and combination but only when Smokers were divided into current and ex-smokers. When the same subanalysis was done in the COPD group, there was no evidence that current smokers have attenuated levels of TNFα. Suppression of the pro-inflammatory response to TLR ligands in smokers has been observed by Chen et al. in alveolar macrophages, however their study suggested that smoking status does not affect TLR3 responses [75]. In this tissue model, only at the highest concentration of poly(I:C) TNFα levels were significantly reduced in current smokers, suggesting that TLR3 signalling is not as affected by smoking as the pathway of other TLRs. Additionally, a study conducted solely on primary bronchial epithelial cells showed that the cells from COPD and not smokers had attenuated release of IL-6 and IL-8 following LPS stimulation [76]. However, this attenuation was observed after exposure to cigarette smoke performed in vitro rather than stimulating cells that were derived from current or ex-smokers with and without COPD. The data presented in this thesis suggest that smoking status does not influence the outcome of how the COPD tissue responds to TLR3 and TLR7/8 ligands. It is possible that if these experiments were performed only on selected cell types present in the tissue, e.g. only epithelial cells or alveolar macrophages, the results would be different. These data therefore suggest that the whole tissue may respond differently to the similar stimulus when compared to single cell type cultures.

3.4.6. Data Variability

It must be noted that the data has a high degree of variability, especially in the CCL5 set. The variations of CCL5 release and gene expression are most probably due to differences in the cell numbers within each sample and technical replicate. Since CCL5 is known to be produced by epithelial cells and lymphocytes, the levels of CCL5 probably reflect the number of these cells present in each sample. Another reason for data variability in the experiments presented
above can be the smoking status of the patients from whom the samples were derived. This was statistically demonstrated for the TNFα release in the Smokers group. In addition, when samples were divided into current and ex-smokers, more datasets passed the normality test suggesting that smoking status does influence the variability of the presented data. Therefore, the data presented in this chapter is highly reflective of the model used and more caution is needed when evaluating differences between Smokers and COPD patients.

3.4.7. Conclusions

In this chapter, human whole lung tissue explant model was used to characterise innate immune response to TLR3 and TLR7/8 activation. In sum, it was hypothesised that the human whole lung tissue explant model would produce an inflammatory response to poly(I:C) and R848 stimulation and this has been demonstrated. Furthermore, studies conducted by other independent groups also showed that the three cytokines measured were up-regulated following poly(I:C) and R848 stimulation in other models and cell types thereby confirming the authenticity of these results. These data suggest that whole lung tissue model is more relevant to study innate immune response in the human lung than single cell type models. The increased clinical symptoms observed during viral/bacterial exacerbations are mirrored in this model by an increase in pro-inflammatory cytokine release in COPD lung tissue. Furthermore, this model also showed the impaired IFNβ response observed by different groups in other COPD exacerbations models. The importance of studying human lung tissue as a whole is further underscored by conflicting results obtained from culturing one type of cells and the whole tissue. The novel model described in this chapter is more relevant than others as it incorporates all observations from different single cell studies as well as other tissue based models, including synergy in response to multiple TLR activation and impaired IFNβ response in COPD. In addition, whole tissue explants require less processing than precision cut lung slices but produce very similar results and also allow for cooperation between different cell types.
Chapter 4. The effects of TNFα, dexamethasone, p38 MAPK, IKK-2 and IRAK1/4 inhibition on innate immune response in human lung tissue explants.
4.1. Introduction

In the previous chapter it was established that human whole tissue explants respond to TLR3 and TLR7/8 stimulation. The experiments described in this chapter were carried out to ascertain that this model of acute viral exacerbations can be manipulated to investigate drug effects.

Several cytokines play a role in an increased pro-inflammatory response in COPD, e.g. TNFα and IL-6 [287, 388, 422]. Evidence suggests that in COPD, as well as in other diseases with deregulated immune responses, levels of TNFα are elevated [113, 192]. TNFα is a potent cytokine released by various cell types, including epithelial cells and macrophages, and TNF receptors are present on the majority of cells in the human body [11, 331, 397]. It has therefore been hypothesised that blocking TNFα could benefit patients with COPD, as previously observed with rheumatic arthritis or psoriasis [195, 271]. There are also data suggesting that the levels of TNFα in serum of stable COPD patients are not elevated when compared to healthy controls, however TNFα levels were increased at exacerbation [62]. The unclear role of TNFα in COPD may be the reason why, to date, systemic treatments with monoclonal antibodies against TNFα (infliximab) have not been successful in COPD [309, 387].

Anti-inflammatory targets of interest are kinases such as p38 MAPK, IKK-2 or the family of IRAKs that are important in downstream signalling of TLRs [25]. Inhibitors of p38 are often used in COPD research and show potential in decreasing the inflammation in studies using models of acute exacerbations [47, 194]. p38 MAPK inhibitors are also tested in clinical trials for stable COPD management [226]. IKK-2 and IRAKs have received more attention recently as steroid treatment becomes ineffective in patients with COPD [22]. NF-κB is central for pro-inflammatory gene expression and inhibition of IKK-2 prevents the translocation of NF-κB to the nucleus [371]. IRAKs are central kinases in the signalling of the majority of TLRs and could therefore have the potential of being effective in multiple pathogen-induced exacerbations [204, 366].
The main aim of this chapter was to assess the role of whole tissue explant model as a potential platform to test novel anti-inflammatory drugs for acute exacerbations of COPD. The experiments had the following objectives:

1. To use the model to investigate the role of TLR-induced TNFα on subsequent pro-inflammatory response in patients with COPD by quantifying CCL5 and IL-6 release by ELISA.

2. To assess whether this model is a possible platform to test novel drugs for TLR-induced inflammation in COPD tissue. This will be done by:
   a. pre-incubating tissue with anti-inflammatory drugs and subsequent stimulation with TLR3, TLR7/8 or combination of the two ligands;
   b. quantifying the release of pro-inflammatory cytokines into supernatant by ELISA and measuring type I and III IFN response in tissue by qRT-PCR.
4.2. Materials and Methods

Tissue was prepared as described in section 2.2. All supernatants and tissue samples were collected after appropriate time of incubation at 37°C, 5% CO₂ and stored at -20°C and -80°C, respectively. First tissue sample of each condition was immersed in 200μl of RNaLater (Invitrogen, Paisley, UK) and kept overnight at 4°C, then stored at -80°C. Protein levels of TNFα, CCL5 and IL-6 were measured by ELISA (see section 2.3) and mRNA fold-induction of IFNα, IFNβ and IFNλ were analysed by qRT-PCR (see sections 2.6 and 2.7).

4.2.1. Neutralisation of TNFα

Human TNFα Antibody (Clone #28401) and Mouse IgG1 Isotype (Clone #11711) were purchased from R&D Systems Europe (Abingdon, UK) and stock solutions were prepared according to manufacturer’s instructions. Antibodies were diluted in supplemented RPMI medium to 20x final concentration (1μg/ml).

In this experiment, the old medium was also discarded and 744μl of fresh supplemented RPMI medium were added to all wells. Then 40μl of medium or 20x antibody solutions were added to the appropriate wells and incubated for 1 hour at 37°C, 5% (v/v) CO₂. Subsequently, 16μl fresh medium were added to the control wells and 16μl of 50x TLR ligand were added to experimental wells and incubated for 24 hours. Demographics of patients used in this experiment are shown in Table 4-1.

Table 4-1 Demographics of subjects used in TNFα neutralisation assay.

<table>
<thead>
<tr>
<th>COPD (n=6)</th>
<th>COPD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>3/3</td>
</tr>
<tr>
<td>Age</td>
<td>67.8±6.9</td>
</tr>
<tr>
<td>FEV1</td>
<td>2.01±0.36</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>77.7±17.1</td>
</tr>
<tr>
<td>FEV1/FVC % ratio</td>
<td>67.7±11.6</td>
</tr>
<tr>
<td>Smoking status (Current/Ex)</td>
<td>4/2</td>
</tr>
<tr>
<td>Smoking history (pack-years)</td>
<td>46 (27-65)</td>
</tr>
<tr>
<td>History of ICS use? (Y/N)</td>
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</tr>
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</table>

FEV1 data for one subject was not available.
4.2.2. Inhibition of Inflammatory Response to TLR ligands

Three inhibitors, against p38 MAP kinase (BIRB 796, CAS 285983-48-4, Calbiochem), IKK-2 (BMS-345541, CAS 445430-58-0, Calbiochem) and IRAK1/4 (CAS 509093-47-4, Calbiochem) were bought from Millipore (Watford, United Kingdom) and a glucocorticoid steroid, dexamethasone, was bought from Sigma-Aldrich (Dorset, UK). All inhibitors were diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Dorset, UK) to stock concentrations of 10mM (for preliminary experiments showing p38 and p65 activation in tissue see Appendix II).

This experiment was designed in the similar manner to the TNFα neutralisation above; old medium was discarded and 744μl of fresh medium were added to all wells. Then 40μl of 20x inhibitor dilution were added to the experimental wells to reach final concentration of 1μM and an equivalent volume of DMSO was added to the control wells. Final DMSO concentration present in all conditions was 0.01% (v/v). After 1 hour incubation at 37°C, 5% (v/v) CO₂, 16μl of 50x of poly(I:C), R848 or combination was added to the tissue and then incubated for 6 and 24 hours. Demographics of patients used in this experiment are shown in Table 4-2.

<table>
<thead>
<tr>
<th>COPD (n=6)</th>
<th>Sex (M/F)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Age</td>
<td>67.3±9.5</td>
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<tr>
<td></td>
<td>FEV1</td>
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<td></td>
<td>FEV1 % predicted</td>
<td>71.1±14.6</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>Smoking status (Current/Ex)</td>
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</tr>
<tr>
<td></td>
<td>Smoking history (pack-years)</td>
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</tr>
<tr>
<td></td>
<td>History of ICS use? (Y/N)</td>
<td>1/5</td>
</tr>
</tbody>
</table>

4.2.3. Statistics

4.2.3.1. TNF Neutralisation

Each set of data (unstimulated, poly(I:C) and R848) was analysed using multiple repeats ANOVA with Dunnett’s post-test.
4.2.3.2. **Inhibitors**

The effect of inhibitor on cytokine production and mRNA levels were compared with time-matched vehicle (DMSO) control using paired t-tests (normally distributed data) or using Wilcoxon test for non-parametric data.
4.3. Results

4.3.1. TNFα Neutralisation

Pre-exposure of whole lung tissue explants to neutralising TNFα antibody resulted in significant decrease of CCL5 (p<0.05) in response to poly(I:C) stimulation and IL-6 (p<0.05) following R848 stimulation (Figure 4-1). There was also a decrease in IL-6 protein levels after poly(I:C) exposure, however it was not statistically significant. CCL5 and IL-6 levels remained unchanged when treated with control IgG antibody. In addition, neither TNFα nor IgG isotype antibody had any effect on basal cytokine release (in Figure 4-1). These findings show that the release of CCL5 following poly(I:C) stimulation and IL-6 after R848 exposure is, at least in part, dependent on TNFα.

![Figure 4-1](image)

**Figure 4-1** Effects of TNFα neutralisation on pro-inflammatory cytokine production from human lung tissue.

The effect of pre-exposure to TNFα neutralising antibody and IgG control antibody on CCL5 (left) and IL-6 (right) secretion in whole tissue explants from COPD patients (n=6) that were either unstimulated or stimulated with poly(I:C) (100μg/ml) or R848 (10μg/ml). Data are presented as scatter plots with grand median (CCL5) or grand mean (IL-6). Technical replicates from the same donor are shown using the same symbol (n=3). * = p<0.05 significantly below untreated levels.
4.3.2. Anti-Inflammatory Drugs

A set of experiments was designed to examine the effects of glucocorticosteroid (dexamethasone) and three inhibitors targeting TLR pathways (p38 MAPK, IKK-2 and IRAK1/4). Here, samples were used to assess the effect of these anti-inflammatory drugs on poly(I:C)-, R848- and combination-stimulated tissue (due to limited sample availability only one drug concentration was used (1µM) and the effect of drug on unstimulated tissue was not examined).

4.3.2.1. Effect of Anti-Inflammatory Drugs on Pro-Inflammatory Cytokine Release

Dexamethasone

Pre-incubation of lung tissue with 1µM of dexamethasone caused a significant inhibition of all three pro-inflammatory cytokines measured (Figure 4-2). Dexamethasone inhibited TNFα release (6h; p=0.036 poly(I:C); p=0.042 R848; p=0.0069 combination) but this effect was prolonged until after 24h only with the use of R848 as stimulus (with other two stimuli numerically but not significantly lower). Optimal production of CCL5 and IL-6 was at 24h, and at that time the use of dexamethasone significantly decreased CCL5 and IL-6 levels in all conditions compared to time-matched vehicle control. These data show that dexamethasone is efficient in inhibiting pro-inflammatory cytokine release from human lung tissue and is able to significantly reduce their levels in their optimal production times.
Figure 4-2 Effects of dexamethasone on the release of pro-inflammatory cytokines from TLR-stimulated human lung tissue.

The effect of dexamethasone (1µM) on poly(I:C) (100µg/ml), R848 (10µg/ml), and combination-induced protein release of TNFα, CCL5 and IL-6 after 6h (top) and 24h (bottom) in whole tissue explants from donors with COPD (n=6). Data are presented as scatter plots with grand mean (TNFα and IL-6) or grand median (CCL5). Technical replicates from the same donor are shown using the same symbol (n=1 (6h) and n=3 (24h)). *, **, *** = p<0.05, 0.01, 0.001 significantly below vehicle control.
p38 MAPK Inhibitor

Exposure to 1µM of p38 MAPK inhibitor resulted in significant decrease of all three cytokines released from the lung tissue (Figure 4-3). Interestingly, p38 MAPK inhibitor significantly decreased R848- and combination-induced TNFα levels after 6h (p=0.018 and 0.0084, respectively), and only poly(I:C)-induced levels after 24h when compared to vehicle controls (p=0.042). Poly(I:C), in general, was slower at inducing TNFα production in comparison to R848 and the data show that p38 MAPK inhibitor successfully decreased cytokine levels at their optimal times. CCL5 levels were decreased after 6 and 24h following poly(I:C) stimulation (p=0.032) suggesting that its TLR3-induced production is dependent on p38 MAPK. CCL5 was also inhibited after 24h in response to stimulation with the ligand combination, but the combination-induced CCL5 is clearly driven by poly(I:C). p38 MAPK inhibitor was efficient at decreasing IL-6 levels in response to all conditions and at both time points (p<0.05). Inhibition of IL-6 was, however, more pronounced after 24h (p<0.01).
The effect of p38 MAPK inhibitor (BIRB 796) (1µM) on poly(I:C) (100µg/ml), R848 (10µg/ml), and combination-induced protein release of TNFα, CCL5 and IL-6 after 6h (top) and 24h (bottom) in whole tissue explants from donors with COPD (n=6). Data are presented as scatter plots with grand mean (TNFα and IL-6) or grand median (CCL5). Technical replicates from the same donor are shown using the same symbol (n=1 (6h) and n=3 (24h)). *, **, *** = p<0.05, 0.01, 0.001 significantly below vehicle control.
IKK-2 Inhibitor

The effect of IKK-2 inhibitor on the pro-inflammatory cytokine release in human lung tissue was limited (Figure 4-4). TNFα levels were significantly reduced after 6h in combination-stimulated tissue (p=0.0021) and after 24h in response to R848 (p=0.019) (other conditions also showed a numerical decrease but it was not statistically significant). The levels of CCL5 were reduced by this inhibitor after 6h when tissue was exposed to poly(I:C) and after 24h in response to combination (p=0.032 for both). As in the case of TNFα, CCL5 levels secreted in response to other conditions were also numerically, but not statistically, lower. IKK-2 inhibitor, however, had no effect on IL-6 secretion in response to all of the conditions after 6h; after 24h poly(I:C)-induced IL-6 was significantly decreased (p=0.015) and combination-induced IL-6 was also numerically lower (p=0.059). These data suggest that IKK-2 (and therefore NF-κB) plays only a partial role in both TLR3 and TLR7/8 signalling resulting in the release of pro-inflammatory cytokines.
Figure 4-4 Effects of IKK-2 inhibitor on the release of pro-inflammatory cytokines from TLR-stimulated human lung tissue.

The effect of IKK-2 inhibitor (BMS-345541) (1µM) on poly(I:C)- (100µg/ml), R848- (10µg/ml), and combination-induced protein release of TNFα, CCL5 and IL-6 after 6h (top) and 24h (bottom) in whole tissue explants from donors with COPD (n=6). Data are presented as scatter plots with grand mean (TNFα and IL-6) or grand median (CCL5). Technical replicates from the same donor are shown using the same symbol (n=1 (6h) and n=3 (24h)). *, ** = p<0.05, 0.01 significantly below vehicle control.
IRAK1/4 Inhibitor

Pre-treatment of tissue with IRAK1/4 inhibitor had a limited effect on the release of pro-inflammatory cytokines (Figure 4-5). TNFα levels were numerically lower in supernatants from tissue exposed to all ligands for 6h, however, that change was not significant. After 24h, only R848-induced secretion of TNFα was significantly decreased by the inhibitor (p=0.049), and for the other conditions lower levels were also observed but, again, were not significant. IRAK1/4 did not have any effect on either CCL5 or IL-6 secretions following exposure to any of the conditions used. These data are surprising since IRAK1 and IRAK4 have been shown to be central in TLR7/8 signalling. More studies are therefore required to fully assess the role of IRAKs in pro-inflammatory cytokine induction in human lung tissue.
The effect of IRAK1/4 inhibitor (CAS 509093-47-4) (1µM) on poly(I:C) (100µg/ml), R848 (10µg/ml), and combination-induced protein release of TNFα, CCL5 and IL-6 after 6h (top) and 24h (bottom) in whole tissue explants from donors with COPD (n=6). Data are presented as scatter plots with grand mean (TNFα and IL-6) or grand median (CCL5). Technical replicates from the same donor are shown using the same symbol (n=1 (6h) and n=3 (24h)). * = p<0.05 significantly below vehicle control.
4.3.2.2. **Effects of Anti-Inflammatory Drugs on Type I and III IFN Gene Expression**

**Dexamethasone**

Examination of IFNα, IFNβ and IFNλ gene expression in TLR-stimulated tissue following pre-treatment with dexamethasone revealed only R848-induced IFNα1 mRNA levels were decreased compared to vehicle control \((p=0.014)\) (Figure 4-6). Interestingly, poly(I:C)-induced IFNλ1 was increased by dexamethasone 2.0-fold. The remaining conditions revealed little influence of dexamethasone treatment on IFN gene expression. These data show that after 6h dexamethasone did not diminish an already attenuated IFN response in the COPD lung tissue.

**Figure 4-6** Effects of dexamethasone on the expression of type I and III IFN in TLR-stimulated human lung tissue.

The effect of dexamethasone \((1\mu M)\) on poly(I:C)\(-\) \((100\mu g/ml)\), R848\(-\) \((10\mu g/ml)\), and combination-induced gene expression of IFNα1, IFNβ1 and IFNλ1 after 6h in whole tissue explants from donors with COPD \((n=6)\). Data are presented as scatter plots with grand mean. Technical replicates from the same donor are shown using the same symbol \((n=1)\) and correspond to protein data as shown in Figure 4-2. Horizontal line at \(y=1\) represents vehicle control. \# = \(p<0.05\) significantly below vehicle control.
p38 MAPK Inhibitor

Pre-treatment of tissue with p38 MAPK inhibitor resulted in lower levels of IFN mRNA in response to some, but not all, conditions when compared to time-matched vehicle controls. The inhibitor attenuated gene expression of R848-induced IFNx1 (p=0.044), poly(I:C)-induced expression of IFNβ1 (p=0.0008), and R848- and combination-induced mRNA levels of IFNλ1 (p=0.029 and 0.0056, respectively). These data demonstrate that inhibition of p38 MAPK can further decrease IFN expression in already IFN-diminished COPD lungs.

Effect of p38 inhibitor (p38i) on TLR-induced type I and III IFN gene expression

Figure 4-7 Effects of p38 MAPK inhibitor on the expression of type I and III IFN in TLR-stimulated human lung tissue.

The effect of p38 MAPK inhibitor (BIRB 796) (1μM) on poly(I:C)- (100μg/ml), R848- (10μg/ml) and combination-induced gene expression of IFNα1, IFNβ1 and IFNλ1 after 6h in whole tissue explants from donors with COPD (n=6). Data are presented as scatter plots with grand mean. Technical replicates from the same donor are shown using the same symbol (n=1) and correspond to protein data as shown in Figure 4-3. Horizontal line at y=1 represents vehicle control. #, ##, ### = p<0.05, 0.01, 0.001 significantly below vehicle control.
IKK-2 Inhibitor

IKK-2 inhibitor did not have any effect on type I IFN gene expression. It has, however, significantly decreased R848-induced IFNλ1 mRNA levels (p=0.0082). On the other hand, poly(I:C)- and combination-induced IFNλ levels were numerically higher (3.6- and 1.5-fold, respectively). These data show that in this human tissue model, only type III IFN expression is dependent on p65 NF-κB. However, the effect of the inhibitor could vary depending on the TLR activated. IKK-2 inhibition therefore seems to play a limited role in type I IFN gene expression.

Figure 4-8 Effects of IKK-2 inhibitor on the expression of type I and III IFN in TLR-stimulated human lung tissue.

The effect of IKK-2 inhibitor (BMS-345541) (1µM) on poly(I:C)- (100µg/ml), R848- (10µg/ml), and combination-induced gene expression of IFNα1, IFNβ1 and IFNλ1 after 6h in whole tissue explants from donors with COPD (n=6). Data are presented as scatter plots with grand mean. Technical replicates from the same donor are shown using the same symbol (n=1) and correspond to protein data as shown in Figure 4-4. Horizontal line at y=1 represents vehicle control. ## = p<0.01 significantly below vehicle control.
IRAK1/4 Inhibitor

Exposure to IRAK1/4 inhibitor did not decrease IFN mRNA levels following TLR stimulation. Surprisingly, inhibition of IRAK resulted in a numerical increase of all three IFN genes when compared to vehicle control. The small increase, however, was not significant for IFNα1 and IFNλ1. Only combination-induced expression of IFNβ1 was significantly increased (p=0.0086) and R848-induced IFNβ1 mRNA levels also had an increasing but not statistically significant trend (1.4-fold increase). These data, therefore, reveal a potential influence of IRAK kinases on type I and III IFN gene expression in the tissue.

Figure 4-9 Effects of IRAK1/4 inhibitor (IRAK1/4i) on TLR-induced type I and III IFN gene expression

The effect of IRAK1/4 inhibitor (CAS 509093-47-4) (1µM) on poly(I:C)- (100µg/ml), R848- (10µg/ml), and combination-induced gene expression of IFNα1, IFNβ1 and IFNλ1 after 6h in whole tissue explants from donors with COPD (n=6). Data are presented as scatter plots with grand mean. Technical replicates from the same donor are shown using the same symbol (n=1) and correspond to protein data as shown in Figure 4-5. Horizontal line at y=1 represents vehicle control. ## = p<0.01 significantly above vehicle control.
4.4. Discussion

4.4.1. Effect of TNFα Neutralisation on Pro-Inflammatory Cytokine Production

Exposure of human lung tissue to TNFα neutralising antibody resulted in the attenuation of the subsequent release of CCL5 and IL-6. These observations confirm the view that TNFα is, at least partially, required for maximal induction of other mediators. In another study, Hackett et al. have also observed attenuation of IL-6 in response to TNFα neutralisation and following LPS stimulation [144]. The role of TNFα in inflammatory diseases is well-established. However anti-TNFα treatment (infliximab) in patients with stable COPD was not successful in reducing clinical markers of disease (e.g. FEV1) [387]. The model presented here is a viral exacerbation model and, to-date, infliximab has not been tried to relieve increased symptoms at exacerbation. In addition, the safety of anti-TNFα therapy has been questioned as it may lead to an increased cancer risk [309]. Nevertheless, TNFα plays a role in the induction of other pro-inflammatory cytokines and therefore other ways that limit TNFα expression should be investigated. Since induction of TLRs also triggers an increase in TNFα protein levels, targeting other proteins that play a role in TLR signalling should also limit the amount of TNFα (and other cytokines) secreted in response to TLR ligands.

4.4.2. Effect of Anti-Inflammatory Drugs on Pro-Inflammatory Cytokine Production

Dexamethasone, as a member of corticosteroid family, was used in these experiments to compare its action on TLR-stimulated tissue to other inhibitors that target intracellular kinases. Inhibitors against p38 MAPK are already in development for COPD, while other molecules (IRAKs and NF-κB) play a central role in TLR signalling and are therefore interesting anti-inflammatory targets for COPD. The inhibitors targeting kinases in the TLR pathway were also
used to try to improve our understanding of how TLR ligands increase inflammation in human lung tissue.

Dexamethasone attenuated the release of all three pro-inflammatory cytokines measured at all of the time points. These observations suggest that, at exacerbation, human lung tissue is partially sensitive to corticosteroid treatment (app. 40-80% inhibition of cytokine release). Studies conducted by others also show that during acute exacerbation of COPD corticosteroid treatment can improve symptoms and decrease chances of a relapse [91, 115, 267, 356, 378]. Dexamethasone has also been used in other in vitro studies and was shown to inhibit pro-inflammatory cytokine release from various cell types, including alveolar macrophages, monocyte-derived macrophages and primary bronchial epithelial cells [11, 151, 194, 216]. There is, however, evidence suggesting that some of the pro-inflammatory genes are less sensitive to corticosteroid treatment. For example, Kaur et al. showed that IFNγ and IL-2 released by activated T lymphocytes are not significantly reduced by dexamethasone pre-treatment and Perry et al. demonstrated that IL-8 released from airway smooth muscle cells from COPD exhibit steroid insensitivity [189, 289]. In another study Culpitt et al. showed that alveolar macrophages from COPD patients are less sensitive to dexamethasone treatment when compared to macrophages from smokers [86].

Another actively investigated target for COPD treatment is p38 MAP kinase [226, 399]. In this study, a p38 MAPK inhibitor has been used in human tissue and it has significantly reduced the levels of TNFα, CCL5 and IL-6. The data show that p38 plays a role in both TLR3 and TLR7/8 signalling in the tissue. This is not surprising as p38 MAPK is known to play a role in the TLR pathway. In previous studies, it was observed that inhibition of p38 MAPK in alveolar macrophages significantly inhibited the release of LPS-induced TNFα, IL-6 and IL-8 from alveolar macrophages [11]. Poly(I:C)-, R848- and combination-induced TNFα and IL-6 levels were also decreased in response to p38 MAPK inhibition in monocyte-derived dendritic cells [231]. Since cytokine abrogation in this and other models is not complete when using p38
MAPK inhibitor it is worth considering the use of multiple drugs to limit the inflammation. For example, exposure to p38 MAPK inhibitor in combination with dexamethasone was more effective than any of the drugs alone [11]. These data show that inhibition of p38 MAPK reduces the overall airway inflammation caused by either viral or bacterial ligands. COPD patients have higher levels of active p38 MAPK in their lungs [123] therefore the use of p38 MAPK inhibitor is also being evaluated as a treatment in stable COPD to stop the decline in lung function [226]. The use of oral p38 MAPK inhibitors however may be controversial as trials in patients with rheumatoid arthritis resulted in numerous common and several serious adverse events [127]. Inhibition of p38 MAPK therefore shows promise to limit the underlying inflammation in stable COPD patients and as a treatment for exacerbation and thus help to stop the progression of lung function decline.

Similarly to p38 MAPK, NF-κB also plays a central role in TLR signalling and is therefore a potential target for exacerbation treatment. In this study, inhibition of IKK-2 did not result in a pronounced inhibition of pro-inflammatory cytokines. Interestingly, in a recent study conducted by Lamb et al. knock-out IKK-2 (lung epithelium only) mice were protected from LPS-induced BALF neutrophilia and released smaller levels of pro-inflammatory cytokines in response to LPS when compared to wild type mice [212]. This effect was not repeated when mice were exposed to cigarette smoke extract suggesting that the epithelium plays a bigger role in producing inflammatory responses to pathogens rather than environmental factors. These observations further underscore the use of IKK-2 inhibitors to treat COPD exacerbations. In this model, IKK-2 had a limited effect on the release of pro-inflammatory cytokines suggesting that cytokine secretion may be independent of IKK-2. It is also possible, however, that the IKK-2 inhibitor used was not potent enough to exert a significant inhibitory effect in the tissue.

IRAK inhibitors are also in development for inflammatory diseases, including COPD [319]. Here, IRAK1/4 inhibitor significantly reduced R848-induced TNFα release after 24h with little effect on all other conditions. Due to IRAKs central role in TLR signalling it was expected for
IRAK inhibitor to play a role in, at least, R848-induced inflammation in the tissue [334]. The lack of effect suggests that (1) TLR7/8 pathway can circumvent IRAK and directly activate NF-κB, (2) the dose used for this experiment was inefficient or (3) the drug was not potent enough to inhibit the release of cytokines to the stimuli. Others have suggested that intracellular TLRs such as TLR7 and TLR8 are able to activate NF-κB without the aid of IRAK1/4: Irak1 knock-out mice produced normal levels of pro-inflammatory cytokines in response to TLR ligands [383]. One study observed that IRAK-4 was required for cytokine release in poly(I:C)-stimulated macrophages which is in contrast with other data suggesting that TLR3 signalling is not IRAK-dependent [174, 366]. The data therefore suggest that also in the human lung tissue IRAK1 and IRAK4 may redundant for pro-inflammatory response to poly(I:C) and R848.

In summary, dexamethasone and p38 MAPK inhibitor were the most effective at reducing the levels of pro-inflammatory cytokines in response to both poly(I:C) and R848. IKK-2 and IRAK1/4 inhibitors significantly reduced cytokine release only at some of the conditions.

### 4.4.3. Effect of Anti-Inflammatory Drugs on Type I and III IFN Gene Expression

In COPD exacerbations it is desirable to control the associated inflammation and influx of inflammatory cells. At the same time, the ideal treatment should not interfere with antiviral responses that will limit the rate of viral replication and thus resolve the infection earlier. Gene expression of type I and III IFN was therefore measured in this study to evaluate the actions of anti-inflammatory drugs on antiviral responses.

Dexamethasone had very little influence on IFN gene expression and only R848-induced IFNα was markedly lower than in vehicle control. This limited effect is not surprising since others demonstrated that treating viral infection in primary bronchial epithelial cells with dexamethasone did not influence the levels of secreted IFNβ [49, 396]. On the other hand, studies conducted in bronchial smooth muscle cells showed that dsRNA-induced IFNβ and
IFNλ1 was abrogated when cells were pre-treated with dexamethasone [63]. These data suggest that some cell types, such as epithelial cells, do not have impaired IFN production after steroid treatment. Similarly, in the whole tissue dexamethasone had a minimal effect on IFN gene expression.

The inhibition of p38 MAPK in the whole tissue also resulted in significant decrease of IFN gene expression. This is an undesirable effect since tissue with COPD already has an impaired IFN expression in response to TLR activation. Makela et al. also observed that inhibition of p38 MAPK in monocyte-derived dendritic cells abrogates IFNβ and IFNλ1 gene expression [232]. In macrophages, however, IFNβ was inhibited after stimulation with vesicular stomatitis virus (VSV) and not after stimulation with poly(I:C) [307]. Interestingly, Börgeling et al. conducted a study on mice infected with influenza virus and found that inhibition of p38 MAPK also reduces IFNβ gene expression [47]. It has also been suggested that inhibition of p38 may be a good antiviral strategy since it has protected mice from lethal dose of virus (80% vs 20% survival). These data show that IFNs may not be as important in viral clearance as initially thought and limiting the release of pro-inflammatory cytokines should have priority. Since the effects of p38 MAPK inhibition on IFN gene expression seems to be stimulus and cell specific, more studies in human cells and tissues should be conducted to fully investigate the role of IFNs in virus-induced COPD exacerbations.

The inhibition of IKK-2 in this model did not significantly influence the expression of type I and III IFNs. Only IFNλ1 was significantly inhibited in R848-stimulated tissue. Interestingly, poly(I:C) and combination-stimulated tissue had enhanced IFN gene expression. It could be due to the fact that blocking one pathway, in this case p65, increases the efficiency of another – TBK1 activation of IRFs and subsequent enhanced IFN gene expression. Since this observation was only made in response to poly(I:C) and not R848 – another receptor, MDA-5, could play an important role here. Poly(I:C) is known to activate MDA-5 and signalling downstream of MDA-5 (including adapter MAVS or IPS-1) has been shown to independently
activate NF-κB and IRFs. Peritoneal macrophages but not bone marrow derived macrophages from MAVS-deficient mice stimulated with poly(I:C) showed diminished IFN responses [205, 364]. This phenomenon could also be dependent on TRIF since TLR3 uses TRIF and not MyD88 as its sole adaptor molecule [330]. TRIF-deficient mice also have deficient IFN production following dsRNA exposure, and recently TRIF has been shown to interact with MAVS [156, 413, 430]. These observations again suggest that IFN expression is signal- and cell-specific, e.g. IFN induction to rotavirus (dsRNA virus) is solely dependent on MDA-5/MAVS and not TLR3/TRIF [50]. There are scarce data examining the effects of IKK-2 or p65 inhibition on IFN gene expression. In bronchial epithelial cells, direct inhibition of p65 (by siRNA) did not inhibit type I IFN expression after RV infection [28]. Taken together, however, these data imply that p65 is redundant for IFN gene expression during viral infections.

IRAK1/4 inhibitor used in the lung tissue enhanced the expression of all IFNs. This was most pronounced for IFNb in combination-stimulated tissue. This is in accordance with a few novel studies conducted on mice and in cell lines where IRAK1 malfunction or deficiency led to an increased IFN gene and protein expression [6, 53]. On the other hand, however, Kim et al. have demonstrated that R848-induced IFNα and IFNβ secretion in plasmocytoid dendritic cells is abrogated when IRAK4 is deprived of its kinase activity [200]. The exact effects of IRAKs on IFN gene and protein expression still need to be elucidated. The increase of IFN expression after IRAK inhibition could lead to the development of a novel COPD treatment that could potentially have double benefits, i.e. control of pro-inflammatory cytokine release and increase of the attenuated IFN in COPD.

In summary, only inhibition of p38 showed a marked decrease in IFN gene expression, dexamethasone had no effect, and IKK-2 (poly(I:C) and combination only) and IRAK1/4 inhibition resulted in a small increase of IFNs. As in the case of pro-inflammatory cytokines, due to limited tissue availability it was not possible to test multiple doses of inhibitors and therefore different concentrations of IKK-2 and IRAK1/4 inhibitor could have led to a more
pronounced effect. There is evidence from both this study and studies conducted by others that IRAKs are negative regulators of IFN gene expression. Further research is required to specify the role of each IRAK subtype in this phenomenon.

4.4.4. Data Variability

The data presented in this chapter were obtained using tissue from COPD patients only. In addition, only 2 out of 6 samples in the TNFα neutralisation experiment and none in the inhibitors experiments were ex-smokers. Therefore, the majority of datasets were normally distributed which demonstrates reduced variability of data. The only dataset that was again highly variable was the CCL5 protein release which again suggests that CCL5 is produced by selected cell types, most probably epithelial cells or lymphocytes and the WTE model is therefore reflective of that.

In addition, one patient in the inhibitors experiment received inhaled corticosteroid treatment (see inverted light blue triangles on Figure 4-2). It is not possible to make any plausible claims based only on one patient sample, but in this case the pattern of response to dexamethasone did not seem to be different from the remaining five. However, if these experiments were to be repeated in a larger sample size, the effect of use of steroids could also be evaluated, especially in the context of steroid resistance observed in patients with COPD [86].

4.4.5. Conclusions

The data presented in this chapter clearly show that the whole tissue explant model of COPD exacerbation will be useful in multiple ways: it can be used to investigate the mechanisms of pathogen-induced inflammatory pathways as well as to test novel inhibitors in the whole tissue environment. Simplified TLR3/MDA-5 and TLR7/8 pathways and the influence of each inhibitor thereon is depicted in Figure 4-10. The observations made in this model confirmed that dexamethasone is a potent inhibitor of pro-inflammatory response and it does not decrease
IFN production (see Figure 4-10, B). The inhibition of p38 MAPK also resulted in lower cytokine levels but IFN response was significantly affected and that is also in accordance with studies conducted by other groups (Figure 4-10, C). The IKK-2 played a role in cytokine production in response to both poly(I:C) and R848. These observations also confirm the existence of an alternative signalling pathway downstream of TLR3 and/or MDA-5 that is dependent on IKK-2 (see Figure 4-10, D). The most surprising results were observed following IRAK1/4 inhibition (Figure 4-10, E). Scarce reports suggesting IRAKs as negative regulators of IFN production were confirmed in this model. The inefficacy of the inhibitor to limit R848-induced CCL5 and IL-6 suggests that these IRAKs may be redundant for their production. In summary, dexamethasone and p38 are potent inflammation inhibitors also in the lung tissue and more data is required to confirm the role of IRAKs and IKK-2 in IFN production.
The effects of inhibitors on TLR3 and TLR7/8 pathway taking into account MDA-5 as an alternative dsRNA sensor.

(A) Depiction of a simplified TLR3, TLR7/8 and MDA-5 signalling pathway without any interference. TLR3 uses TRIF, TRAF3 and TBK1 to activate IRFs, NF-κB and p38 MAPK. TLR7/8 uses MyD88, IRAKs, TRAF6 and TAK1 to activate NF-κB (via NEMO) and p38 MAPK; TLR7/8 can also activate IRF7 directly. MDA-5 associates with IPS-1 and TRAF3 to activate IRFs, NF-κB and possibly p38. MDA-5 is also thought to be able to associate with TRIF and therefore use the same pathway as TLR3. Adapted from Cell Signaling Technology [71].

(B) The effects of dexamethasone on the pathway. Dexamethasone binds to glucocorticoid receptor (GR) which then translocates to nucleus and starts the transcription of anti-inflammatory genes such as IL-10. Dexamethasone can also bind directly to NF-κB and limit the expression of pro-inflammatory cytokines and chemokines. It does not seem to interfere with IFN gene expression.

(C) The effects of p38 MAPK inhibitor on the pathway. The inhibitor prevents p38 to activate expression of pro-inflammatory cytokines and chemokines as well as decreases IFN gene expression.

(D) The effect of IKK-2 inhibitor on the pathway. Inhibition of IKK-2 results in IκB not dissociating from NF-κB. It should therefore limit the transcription of pro-inflammatory genes without affecting IFN gene expression. Limited cytokine inhibition could be due to inadequate dose in the tissue system.

(E) The effect of IRAK1/4 inhibitor on the pathway. Inhibiting IRAK1/4 should result in pro-inflammatory cytokine abrogation. Until recently IRAK1/4 were not thought to play a role in IFN production. Recent data however show that IRAK1 and IRAK4 can act as negative regulators of IFN gene expression and therefore inhibition of IRAKs leads to an enhanced IFN gene and protein expression. In whole tissue explants, R848-induced TNFα was inhibited suggesting that CCL5 and IL-6 are produced by activation of other pathways, i.e. p38, in an alternative way (e.g. type I IFN).

CCL5 – chemokine (C-C motif) ligand 5; IFN – interferon; IKK – IkappaB kinase; IL – interleukin; IPS-1 – interferonβ promoter stimulator-1 (also known as MAVS/VISA/Cardif); IRAK – IL-1 receptor associated kinase; IRF – IFN regulatory factor; MDA-5 – melanoma differentiation associated protein-5; MyD88 – myeloid differentiation primary response 88; NEMO – NF-κB essential modulator; NF-κB – nuclear factor-kappaB; TAK1 – TGFβ-activated kinase 1; TBK1 – TANK binding kinase 1; TLR – Toll-like receptor; TRAF – TNF receptor associated factor; TRIF – Toll/IL-1 receptor-domain-containing adapter-inducing IFNβ.
5.1. Introduction

Human rhinoviruses are the most important viral trigger of COPD exacerbations. It is therefore essential to study the effect of RV infections in COPD patients in an attempt to find effective preventative and/or exacerbation management treatments. Studies that examine RV-induced immune response in COPD patients are scarce. RV infections of single cell type models can be misleading as there are conflicting data that suggest differential roles for various types of cells in antiviral immunity, i.e. dominance of PBMCs in IFNα production. The roles of diverse cell types will overlay in vivo and thus may produce a different final outcome. Although several human in vivo challenges have been completed, it is difficult to obtain tissue samples from these studies to conduct a detailed investigation of molecular pathways involved in RV infections. Such challenges, however, will also be of benefit in the final stages of clinical trials to assess the effects of new treatments. The tissue model presented in this thesis could be used, in addition to other human cell models, to enhance the understanding of the inflammatory pathways activated by RV infection in subjects with COPD.

In the previous chapters, it was demonstrated that human lung tissue can be employed to study innate immune responses and compare them between subject groups. It was also possible to manipulate such a model with the range of experimental approaches, including the use of targeted inhibitors. However, the above studies were conducted with the use of synthetic TLR ligands (or combination thereof). Although these are frequently used to mimic viral infections (where it is not possible to use intact virus), they do not fully represent the events occurring in humans during virus infections.
The main aim of this chapter was to characterise innate immune response to two RV serotypes: RV-16 (belonging to major group) and RV-1B (minor group). The experiments had the following objectives:

1. To infect whole tissue explants with major (RV-16) and minor (RV-1B) group rhinovirus and quantify subsequent inflammatory response by:
   a. Measuring pro-inflammatory cytokines in the supernatants by ELISA;
   b. Measuring type I and III IFN gene expression in the tissue by qRT-PCR.
2. To compare the RV-induced immune response in smokers to COPD.
5.2. Materials and Methods

5.2.1. Cell culture

BEAS2B and HeLa (H1 and Ohio strains) were a gift from Dr Nathan Bartlett (Imperial College, London). BEAS2B cells were cultured in RPMI-1640 medium (Sigma-Aldrich, Dorset, UK) supplemented with 10% (v/v) foetal bovine serum, 2mM L-glutamine, 37.5mg sodium bicarbonate and 12.5mM HEPES (Invitrogen, Paisley, UK). HeLa cells were cultured in DMEM (high glucose) medium supplemented with 10% (v/v) foetal bovine serum, 37.5mg sodium bicarbonate and 10mM HEPES (Invitrogen, Paisley, UK). Cells were incubated at 37°C, 5% (v/v) CO₂ and split when they reached 80% confluence.

5.2.2. Human Rhinovirus Preparation

Human rhinoviruses RV-16 and RV-1B were obtained from Dr Nathan Bartlett (Imperial College, London).

5.2.2.1. Propagation

HRV-16 and HRV-1B were propagated in HeLa H1 cells according to the protocol from as described before [281]. Briefly, a 3ml aliquot of previously propagated rhinovirus was diluted in 100ml of HeLa infection media (DMEM supplemented with 2% (v/v) foetal bovine serum (Invitrogen, Paisley, UK)) and was used to infect 5 confluent 175cm² cell culture flasks. The cells with virus were put on a shaker for 1 hour following which they were transferred to an incubator (37°C, 5% (v/v) CO₂). The next day, infected cells with virus were harvested and frozen and thawed twice. Then, cell debris was removed by centrifugation and the supernatant was filtered through 0.2μm syringe filter (Pall Life Sciences, Portsmouth, UK) and was diluted in 500ml of infection media and used immediately to infect a further 25 confluent 175cm² cell culture flasks of HeLa H1 cells. Cells with virus were again put on a shaker for 1h, incubated overnight and the freeze-thaw cycle repeated and supernatant collected and filtered the
following day. The filtered supernatant from 25 flasks was then aliquoted and stored at -80°C for future use.

5.2.2.2. Titration Assay

HRV titration assay was performed in HeLa cells (Ohio strain). Rhinovirus was diluted in HeLa infection medium to obtain a series of dilutions ranging from neat to 10\(^{-9}\). In a 96-well flat-bottom plate, 50μl of each virus dilution were added to 8 wells (50 μL of HeLa infection medium were added to control wells). Subsequently, 1x10\(^5\) cells were added to each well (total volume 200μl) and the plate was incubated at 37°C, 5% (v/v) CO\(_2\). The cells were checked after 24, 48, 72 and 96h for cytopathic effect and virus titre (TCID\(_{50}\)) was calculated using Spearman-Kärber method.

5.2.2.3. Neutralisation Assay

To verify that the virus aliquots obtained as described above are the expected rhinovirus serotype a neutralisation assay was performed. Antisera specific against HRV-16 and HRV-1B were bought from American Type Culture Collection (ATCC) (LGC Standards, Middlesex, UK). Stocks of antisera were prepared according to manufacturer’s instructions.

HRV neutralisation assay was performed on a 96-well flat-bottom plate. Firstly, 20μl of serum was diluted in 180μl of 1x Dulbecco’s phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK). Then 1800μl of neutralisation medium (DMEM, high glucose supplemented with 4% (v/v) foetal bovine serum (Invitrogen, Paisley, UK)) was added to the antibody dilution. Virus stocks were diluted in neutralisation medium to have 2x10\(^3\) and 5x10\(^3\) TCID\(_{50}\)/ml for both serotypes. A suspension of HeLa cells (Ohio strain) was prepared to have 1x10\(^8\) cells/ml. Initially, a serial dilution (1:2 in neutralisation media) of antiserum was prepared in rows B-H (row H containing highest antiserum concentration). Row A was used for negative and positive controls and only 50μl of neutralisation media was added to these wells. Then, 50μl of virus dilution (or 50μl of medium to negative control wells) were added to the appropriate wells. The
plate with virus and antiserum was put on a shaker for 1 h after which 100 μl of cell suspension was added to each well. Neutralisation plate was then incubated at 37°C, 5% (v/v) CO₂ and cells were checked for the presence of cytopathic effect after 96 h.

5.2.3. **Stimulation of BEAS2B Cells with Human Rhinovirus**

BEAS2B cells were grown to confluence, harvested, counted and diluted in culture medium to obtain a suspension of 1.7x10⁵ cells/ml. Then 1 ml of cell suspension was added to each well in 12-well plates pre-coated with collagen for 30 min (from calf skin, Sigma-Aldrich, Dorset, UK) and transferred to an incubator (37°C, 5% (v/v) CO₂). After 24 h culture medium was replaced with 500 μl of infection medium (RPMI-1640 (Sigma-Aldrich, Dorset, UK) supplemented with 2% (v/v) foetal bovine serum, 2 mM L-glutamine, 37.5 mg sodium bicarbonate and 12.5 mM HEPES (Invitrogen, Paisley, UK)) and cells were incubated for another 24 h. On the day of infection, old infection medium was removed and replaced with 200 μl of virus dilution for infection wells, 0.1 μg/ml poly(I:C) for positive control wells and infection medium for negative control wells. The plates were then put on a shaker for an hour, after which the virus/poly(I:C)/medium from each well were removed and replaced with 500 μl of infection media (to poly(I:C) wells 500 μl of 0.1 μg/ml poly(I:C) solution was added). The plates were then incubated for the appropriate time (6 and 24 h). In one experiment, 500 μl of virus/poly(I:C)/medium were used and were not replaced after 1 hour on a shaker. The supernatants were collected after 24 h. All experiments were performed in triplicate.

5.2.4. **Stimulation of Lung Tissue with Human Rhinovirus**

Whole tissue explants were prepared as before (see section 2.2). Pilot experiments to optimise correct virus dose and time points were performed first. Viral stocks were diluted in RPMI-1640 medium (Sigma-Aldrich, Dorset, UK) supplemented with 10% (v/v) foetal bovine serum (Invitrogen, Paisley, UK), 100 U penicillin/0.1 mg streptomycin (Sigma-Aldrich, Dorset, UK) and 2 mM L-glutamine (Invitrogen, Paisley, UK) to obtain final TCID₅₀/ml values ranging
from $1 \times 10^4$ to $1 \times 10^7$. Following overnight incubation of whole tissue explants, old medium was discarded and 800μl of medium, RV16, RV1B or poly(I:C) were added to the correct wells. Plates were then put on a shaker for 1h and then incubated at $37^\circ C$, 5% (v/v) CO$_2$. Supernatants were initially collected after: 1, 6, 24 and 48h. Negative control at 0h was also included. Levels of TNFα, CCL5 and IL-6 were analysed by ELISA. In these pilot experiments, each condition was performed in duplicate.

Pilot experiment allowed for one dose to be chosen which was used for the subsequent experiment. The optimal dose of $1 \times 10^7$ TCID$_{50}$/ml could not be used because the new viral stock prepared for this time course experiment was weaker than the one used in the pilot experiments. It was therefore decided to use the closest possible concentration, i.e. $1 \times 10^{6.5}$ TCID$_{50}$/ml. Virus stocks were diluted to obtain final value of $1 \times 10^{6.5}$ TCID$_{50}$/ml. Additional controls were also prepared: UV-irradiated virus and HeLa extract. Virus was irradiated with UV light in Envair Biosafety Cabinet (Rosendale, UK) equipped with 30 Watt UV light bulb (G30W T8, Sylvania, Newhaven, UK) for 30min. HeLa extract was prepared as described in section 5.2.2.1 but without virus infection. HeLa extract was diluted with the same volume required to obtain final RV-16 dilution. As before, 800μl of medium, virus preparation, UV-RV and HeLa extract were added to the appropriate wells. Supernatants and tissue were collected after 6, 24, 48 and 72h. Due to limited tissue availability, all UV-RV and HeLa controls were only collected after 24h. The additional controls were included to show whether non replicating virus is able to induce cytokine production and IFN expression (UV-RV) and to demonstrate that there is no contamination in the viral preparation from HeLa cells that could interfere with virus infection (HeLa). All conditions were performed in triplicate. Demographics of subjects used in this experiment are presented in Table 5-1.
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<td><strong>History of steroid use (Y/N)</strong></td>
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5.2.5. **Statistics**

All data were checked for normality using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as mean with SEM where the datasets passes the normality tests, and as median with range where they did not.

5.2.5.1. **Rhinovirus Stimulation of BEAS-2B Cells**

Multiple repeats ANOVA with Dunnett’s post-test was used to analyse dose-response data. Where only two columns were analysed, i.e. unstimulated control with positive control (poly(I:C)), a paired t-test was used.

5.2.5.2. **Rhinovirus Stimulation of Whole Tissue Explants**

Absolute levels of cytokine release or relative gene expression in response to RV at each time point were compared with matching unstimulated controls using paired t-tests (or Wilcoxon for gene expression data). Any differences in cytokine release or IFN gene expression between subject groups were tested using unpaired t-tests (Mann-Whitney) at each time point.

5.2.5.3. **Comparison Between Control Samples**

Control samples included: (1) unstimulated control (U), (2) RV, (3) UV-RV, and (4) HeLa extract (H). Repeated measures ANOVA with Bonferroni post-test (or Friedman with Dunn post-test for nonparametric data) were used to analyse these datasets to compare the following columns: (a) U vs RV, (b) U vs UV-RV, (c), U vs H, (d) RV vs UV-RV, (e) RV vs H and (f) UV-RV vs H.
5.3. **Results**

5.3.1. **Stimulation of BEAS-2B cells with RV-16 and RV-1B**

To establish a successful RV infection in the tissue, initial experiments with virus were carried out on a BEAS-2B cell line. This was done because these cells have been previously successfully infected with both RV-16 and RV-1B and in order to confirm that the rhinovirus propagated in the new laboratory was inducing similar levels of pro-inflammatory cytokines and chemokines as previously described.

First attempt to infect BEAS-2B cells involved using three RV concentrations (TCID$_{50}$/ml), i.e. 1x10$^2$, 1x10$^3$ and 1x10$^4$. After 24h post-infection, BEAS-2B released approximately 400pg/ml of CCL5 in response to poly(I:C) (positive control). Only in response to RV-16 at 1x10$^3$ did the cells release noticeable increase of CCL5 over the basal levels (control) (see Figure 5-1, A).

In the next experiment, the virus concentrations were increased to 1x10$^4$, 1x10$^5$ and 1x10$^7$ TCID$_{50}$/ml. Here, cells released 40pg/ml of CCL5 and about 600pg/ml of IL-6 in response to positive control poly(I:C) (see Figure 5-1, B and C, respectively). The levels of both cytokines after rhinovirus infection was not increased after 24h at any of the concentrations. The same experiment using all the virus concentrations was repeated and 6h time point was chosen to ensure that any changes in the cytokine levels were not missed. In this case, poly(I:C) after 6h induced a release of 60pg/ml of CCL5, but all of the rhinovirus concentrations and unstimulated controls did not released detectable levels of CCL5 (see Figure 5-1, D).
Figure 5-1 Release of pro-inflammatory cytokines from BEAS-2B cells in response to poly(I:C) and a range of RV-16 and RV-1B concentrations.

The release of CCL5 (A,B,D) and IL-6 (C) in response to poly(I:C) (0.1µg/ml) and various doses of RV-16 and RV-1B in BEAS-2B cells after 1h virus pre-incubation and subsequent incubation after 6 (D) and 24h (A-C). Data are presented as mean with SEM (n=3).

As the levels of CCL5 following the same concentration of poly(I:C) varied 10-fold (400 vs 40pg/ml), it was decided to try the all of the previously used viral concentrations and measure cytokine release again after 24h. This time, however, after 1h on a shaker media was not changed.
to allow virus more time to infect the cells, as no effect was seen when the virus was removed after 1h. Under these circumstances, cells released 15pg/ml of CCL5 (p<0.05 above unstimulated control) and 300pg/ml of IL-6 (not significant) in response to positive control – poly(I:C) (see Figure 5-2, A and B, respectively). This time, the highest concentration of RV-1B induced significant increase (p<0.001) of CCL5 over unstimulated control (RV-1B dose response repeated measures ANOVA p<0.0001, Figure 5-2, A). However, it was the highest concentration of RV-16 that induced significant increase (p<0.01) of IL-6 (ANOVA p=0.0146) (see Figure 5-2, B).

![Figure 5-2](image-url)

**Figure 5-2** Release of pro-inflammatory cytokines from BEAS-2B cells in response to poly(I:C) and extended range of RV-16 and RV-1B concentrations.

The release of CCL5 (A) and IL-6 (B) following stimulation with poly(I:C) [0.1µg/ml] and various doses of RV-16 and RV-1B. The levels of cytokines after 24h were measured after 1h on a shaker and then after 24 incubation (without media change). Data are presented as mean with SEM (n=3).

All these data taken together show that the levels of CCL5 and IL-6 released from BEAS-2B cells in response to either poly(I:C) or rhinovirus were not repeatable in these experiments. The variation in the release of CCL5 in response to the same dose of poly(I:C) suggest that it was not a successful attempt, however limited increase of cytokine levels in response to highest
concentrations of rhinovirus was a positive indicator that the virus preparation was viable. It was therefore decided to proceed to infect whole tissue explants.

5.3.2. Preliminary Time and Dose Response

Following the attempt to infect BEAS-2B cells with limited success with either of the RV serotype, it was decided to infect whole tissue explants with both, RV-16 and RV-1B. Pilot dose-response and time-course experiments were carried out to assess the cytokine release from tissue following RV infection (n=3).

5.3.2.1. Effects of HRV Stimulation on Proinflammatory Cytokine Secretion

Stimulation of tissue with rhinovirus resulted in time-dependent release of TNFα (Figure 5-3), CCL5 (Figure 5-4) and IL-6 (Figure 5-5). Tissue from all three donors released pro-inflammatory cytokines in a similar pattern. Poly(I:C) was used as a positive control.

In response to RV-16, levels of TNFα peaked after 24h (20-fold increase) when the highest concentration of virus was used. The lowest and middle virus concentrations, however, increased the levels of TNFα gradually reaching a peak after 48h (Figure 5-3, B). In response to RV-1B, TNFα levels increased 2-fold only after stimulation with the highest concentration of the virus, with the two other concentrations not increasing cytokine levels above the unstimulated control (Figure 5-3, C).

In terms of CCL5, RV-16 caused 10 to 20-fold increase after 48h at the highest concentrations for two out of three subjects. The two lower RV concentrations did not increase levels of CCL5 above unstimulated controls. The tissue from the third donor did not release CCL5 in response to any of the RV-16 concentrations used (Figure 5-4, B). In response to RV-1B there was at least a 5-fold increase in CCL5 levels in response to highest virus concentrations, with tissue from one donor reaching over 10-fold increase after 48h. Again, lower concentrations did not induce the release of CCL5 (Figure 5-4, C).
IL-6 levels in response to RV-16 were gradually increasing up to 48h for all three virus concentrations, with one donor increasing 8-fold over unstimulated control after 48h stimulation with middle virus concentration (Figure 5-5, B). Similar pattern was observed with RV-1B with IL-6 levels increasing up to 48h in response to all concentrations. Again, tissue from one donor released higher IL-6 levels after 48h (in response to the lowest RV-1B concentration) compared to the remaining samples (Figure 5-5, C).

Taken together, these data suggest that in order to obtain an increase in pro-inflammatory cytokine release from whole tissue explants the highest concentration should be used. Due to the increasing levels of CCL5 and IL-6 after 48h it was also decided to extend the experiment to 72h and abandon the 1h time point. The next step was to characterise the pattern of cytokine release and IFN gene expression in a larger sample of tissue donors.
Figure 5-3 Release of TNFα from human lung tissue in response to poly(I:C) and a range of RV-16 and RV-1B concentrations.

The effect of poly(I:C) [100μg/ml], RV-16 and RV-1B on TNFα release from human lung tissue explants (n=3). Each line represents time course from a separate tissue donor. Data are presented as mean of 2 technical replicates per each condition.
Figure 5-4 Release of CCL5 from human lung tissue in response to poly(I:C) and a range of RV-16 and RV-1B concentrations.

The effect of poly(I:C) [100μg/ml], RV-16 and RV-1B on CCL5 release from human lung tissue explants (n=3). Each line represents time course from a separate tissue donor. Data are presented as mean of 2 technical replicates per each condition.
Figure 5-5. Release of IL-6 from human lung tissue in response to poly(I:C) and a range of RV-16 and RV-1B concentrations.

The effect of poly(I:C) [100μg/ml], RV-16 and RV-1B on IL-6 release from human lung tissue explants (n=3). Each line represents time course from a separate tissue donor. Data are presented as mean of 2 technical replicates per each condition.
5.1.1. RV Stimulation of Lung Tissue over Time

Tissue from smokers with normal lung function (n=6) and donors with COPD (n=6) were stimulated with RV-16 and RV-1B. To characterise the innate immune response in tissue over time the levels of pro-inflammatory cytokines and type I and III IFN gene expression were measured and compared between the two subject groups.

5.1.1.1. Effects on Proinflammatory Cytokine Secretion

Stimulation of whole tissue explants with RV-16 resulted in significant increase of TNFα and CCL5, whereas IL-6 was not significantly induced except for a few time points (Figure 5-6).
Figure 5-6 Release of pro-inflammatory cytokines in response to RV-16 from human lung tissue over time.

The effects of RV-16 stimulation on the release of TNFα, CCL5 and IL-6 from human lung tissue explants from smokers (left, \( n=6 \)) and COPD patients (right, \( n=6 \)) over time. Data are presented as scatter plots with grand mean. Technical replicates from the same donor are shown using the same symbol (\( n=3 \)). *, **, *** = \( p<0.05, 0.01, 0.001 \) significantly over unstimulated control, respectively.

TNFα levels were significantly increased after 6, 24 and 48h post-stimulation in both subject groups (approximately 10-fold increase, \( p<0.01 \)), decreasing after 72h (Figure 5-6, A). CCL5 protein levels were also significantly increased (3-4-fold) in smokers (\( p<0.001 \) and COPD...
patients (p<0.05) after 6 and 24h, and additionally in smokers after 48h (Figure 5-6, B). Levels of IL-6 were continuously increasing throughout the time course (5-fold increase over unstimulated control), with significant increase in smokers after 6 and 24h and in COPD patients only after 72h (p<0.05). Although there was a trend in the smokers group, the IL-6 levels did not reach significance after either 48 or 72h (p=0.0688 and 0.0761, respectively; Figure 5-6, C).

Further comparisons were carried out to investigate any differences in cytokine release between smokers and COPD. There were no differences between subject groups at any of the time points of all three cytokines analysed.

Next, additional controls collected after 24h (UV-RV-16, and HeLa extract) were compared with RV-16 stimulated samples and time-matched unstimulated controls (Figure 5-7). For TNFα and CCL5, RV-16 induced significantly higher levels of these cytokines compared to unstimulated control, UV inactivated virus and HeLa extract which confirms that replicating RV-16 is required for maximal production of the two cytokines. UV-RV-16, however, also significantly increased cytokine levels compared to unstimulated control (Figure 5-7, A and B). This shows that even UV-inactivated virus is able to induce TNFα and CCL5 protein release, although such induction is significantly lower when compared to live virus (p<0.05). For IL-6, RV-16-induced stimulation of whole tissue explants resulted in significant increase of cytokine levels compared to unstimulated control or HeLa extract. However, there was no significant induction of IL-6 by UV-RV-16 compared to unstimulated control (Figure 5-7, C) which suggests replicating RV-16 virus is required for any significant changes in IL-6 production in this model. For all three cytokines, HeLa extract did not increase cytokine levels significantly above unstimulated control. This shows that none of the components of HeLa cells in virus preparation is responsible for the increase in the pro-inflammatory response in the lung tissue.
Figure 5-7 Effects of using UV-RV-16 and HeLa extract on pro-inflammatory cytokine release from human lung tissue.

The effects of RV-16, UV irradiated-(UV)-RV-16 and HeLa extract stimulation on the release of TNFα, CCL5 and IL-6 from whole tissue explants from smokers and COPD patients (combined, n=12) after 24 hours. Data are presented as scatter plots with grand mean. Technical replicates from the same donor are shown using the same symbol (n=3). *, **, *** = p<0.05, 0.01, 0.001 significantly over unstimulated control, respectively.

A similar experiment was conducted using RV-1B (Figure 5-8). In this case, TNFα levels were significantly increased in the smokers group after 24 and 48h, and in the COPD group after 48h (p<0.05; Figure 5-8, A). There was no significant increase in the levels of CCL5 throughout the time course for any of the subject groups (Figure 5-8, B). The level of IL-6, however, was significantly up-regulated only after 72h in the COPD group (Figure 5-8, C). Again, there was a trend in the smokers group after 24, 48 and 72h (p=0.082, 0.057, 0.077) and in COPD after 48h (p=0.097), but the levels did not reach statistical significance.

Again, a comparison of cytokine levels released by the two groups was carried out. As in the case of RV-16, there were no differences between smokers and COPD.
Figure 5-8 Release of pro-inflammatory cytokines in response to RV-1B from human lung tissue over time.

The effects of RV-1B stimulation on the release of TNFα, CCL5 and IL-6 from human lung tissue explants from smokers (left, n=6) and COPD patients (right, n=6) over time. Data are presented as scatter plots with grand mean. Technical replicates from the same donor are shown using the same symbol (n=3). *, **, *** = p<0.05, 0.01, 0.001 significantly over unstimulated control, respectively.
The additional control samples, i.e. UV-RV-1B and HeLa extract, were also collected after 24h (Figure 5-9). The analysis revealed that RV-1B and UV-RV-1B induced significantly higher levels of TNFα than unstimulated control (p<0.01 and 0.05, respectively), suggesting that replicating virus is not required to increase TNFα levels. HeLa extract, however, did not significantly increase TNFα protein levels over unstimulated controls, again confirming that none of the components from HeLa cells are involved in the cytokine production. For CCL5, neither intact nor inactivated virus (RV-1B and UV-RV-1B) increased cytokine levels. Statistically, however, HeLa extract induced levels significantly higher than unstimulated control (p<0.01) and RV-1B (p<0.05) (Figure 5-9, B). These data could suggest that there is a small influence of HeLa cell extract on CCL5 production in the tissue. In terms of IL-6, RV-1B caused a significant increase of cytokine levels compared to unstimulated control (p<0.05), but not compared to UV-RV-1B or HeLa extract. There were also no differences when HeLa extract was compared with either unstimulated control or UV-RV-1B. These data show that increased IL-6 production is solely dependent on the virus and not HeLa cell components that may be present in the viral preparation used.
The effects of RV-1B, UV irradiated (UV)-RV-1B and HeLa extract stimulation on the release of TNFα, CCL5 and IL-6 from whole tissue explants from smokers and COPD patients (combined, n=12) after 24 hours. Data are presented as scatter plots with grand mean. Technical replicates from the same donor are shown using the same symbol (n=3). *, ** = p<0.05, 0.01 significantly over unstimulated control, respectively.

In summary, RV-16 caused an increase in the levels of TNFα, CCL5 and, to some extent, IL-6. Intact RV-16 was required to obtain maximal release of pro-inflammatory cytokines, at least after 24h. UV-RV-16 also increased levels of TNFα and CCL5 suggesting a role of its structural components in the induction of the innate immune response in human lung tissue. RV-1B, however, was weaker at inducing pro-inflammatory cytokine release, with limited increase of TNFα and IL-6 levels. RV-1B induced significantly higher levels of TNFα and IL-6 than unstimulated controls after 24h, but these were not dependent on replicating RV-1B.
5.3.2.2. Effects on Type I and III Interferon Gene Expression

The second step to characterise innate immune response in human lung tissue to rhinovirus was to examine type I and III IFN gene expression. mRNA levels of IFNα1, IFNβ1 and IFNλ1 were measured from the tissue.

RV-16 induced a significant induction of IFNα1 (peak at 24h) in smokers after 6 and 24h (2- and 8-fold increase), and in the COPD group after 24h (7-fold). In smokers, after 48 and 72h there was a considerable increase in IFNα1 mRNA, however it did not reach statistical significance (p=0.063) (Figure 5-10, A). IFNβ1 gene expression was significantly up-regulated in smokers throughout the time course (p=0.032), and in the COPD group only after 6 and 24h (p=0.032). In COPD group, levels of IFNβ1 mRNA were also increased, but not statistically significant, after 48 and 72h (p=0.063) (Figure 5-10, B). Gene expression of IFNλ1 was significantly elevated in smokers after 24, 48 and 72h (p=0.032) and in COPD after 24 and 72h (p=0.032) with levels after 48h almost reaching statistical significance (p=0.063) (Figure 5-10, C).

Further analysis to compare the levels of mRNA in smokers vs COPD was also carried out. There were no differences in the levels of IFNα1 gene expression between the two subject groups. For IFNβ1, COPD group had significantly lower mRNA levels after 48h (p=0.026) and numerically lower levels after 72h (p=0.065) (Figure 5-10, B). Similarly, COPD group expressed less of IFNλ1 in response to RV-16 when compared to smokers, also after 48h (p=0.041) (Figure 5-10, C).
Figure 5-10 Expression of type I and III IFN in response to RV-16 in human lung tissue over time.

The expression of IFNα1, IFNβ and IFNλ1 mRNA in RV-16-stimulated tissue from smokers (n=6) and COPD patients (n=6). Data are presented as scatter plots with grand median. Technical replicates from the same donor are shown using the same symbol (n=1). Horizontal line at y=1 represents the unstimulated control. # = p<0.05 significantly over unstimulated control. * = p<0.05 significant difference between subject groups.

Additional controls, i.e. UV-RV-1B and HeLa extract, were also examined after 24h to verify whether intact virus was needed for maximal IFN gene expression. For all three IFN genes analysed, RV-16 induced significantly higher mRNA levels compared to unstimulated control and HeLa extract. There were no differences in gene expression in RV-16 and UV-RV-16 stimulated tissue, indicating that replicating virus was not required for maximal IFN gene expression. Additionally, HeLa extract did not increase gene expression compared to unstimulated control confirming that increased IFN mRNA levels were solely virus-dependent (Figure 5-11).
The effects of RV-16, UV irradiated-(UV)-RV-16 and HeLa extract stimulation on the gene expression of IFNα1, IFNβ1 and IFNλ1 from whole tissue explants from smokers and COPD patients (combined, n=12) after 24 hours. Data are presented as scatter plots with grand median. Technical replicates from the same donor are shown using the same symbol (n=1). *, **, *** = p<0.05, 0.01, 0.001 significant difference between designated groups, respectively.

Again, the same experiments were carried out with RV-1B (Figure 5-12). RV-1B induced significant increase in IFNα1 mRNA levels after 24h in COPD group (p=0.032) with smokers almost reaching statistical significance after 24h (p=0.063) (Figure 5-12, A). IFNβ1 gene expression, however, was only significantly increased in smokers after 24, 48 and 72h (p=0.032) (Figure 5-12, B). Finally, mRNA levels of IFNλ1 were significantly increased in smokers after 24, 48 and 72h (p=0.0313) and in COPD group after 6 and 48h (p=0.032) with levels after 24h almost reaching statistical significance (p=0.063) (Figure 5-12, C).
A comparison between smokers and COPD groups was also carried out at each time point for all three IFN genes. The statistical analysis revealed that COPD patients had impaired IFNλ1 gene expression after 48h when compared to smokers (p=0.026) (Figure 5-12, C).

**RV-1B**

![Graph showing expression of IFNα1, IFNβ1, and IFNλ1 mRNA](image)

**Figure 5-12 Expression of type I and III IFN in response to RV-1B in human lung tissue over time.**

The expression of IFNα1, IFNβ1 and IFNλ1 mRNA in RV-1B stimulated tissue from smokers (n=6) and COPD patients (n=6). Data are presented as scatter plots with grand median. Technical replicates from the same donor are shown using the same symbol (n=1). Horizontal line at y=1 represents the unstimulated control. # = p<0.05 significantly over unstimulated control. * = p<0.05 significant difference between subject groups.

The analysis carried out to compare additional control samples after 24h showed that RV-1B induced an increase in expression of all three IFN genes measured over unstimulated control and HeLa extract. This shows that the rise of IFN mRNA levels was solely virus-dependent. There were also no differences between the levels of IFN gene induction between intact RV-1B and UV-RV-1B (Figure 5-13) suggesting that replicating virus is not required for IFN gene expression.
The effects of RV-1B, UV irradiated (UV)-RV-1B and HeLa extract stimulation on the gene expression of IFNα1, IFNβ1 and IFNλ1 from whole tissue explants from smokers and COPD patients (combined, n=12) after 24 hours. Data are presented as scatter plots with grand median. Technical replicates from the same donor are shown using the same symbol (n=1). *, **, *** = p<0.05, 0.01, 0.001 significant difference between designated groups, respectively.

In summary, there was a significant increase in type I and III IFN gene expression in response to both serotypes of RV used compared to unstimulated time-matched controls. Tissue from COPD donors had attenuated expression of IFNβ1 (RV-16 only) and IFNλ1 (both serotypes) after 48h when compared to smokers. Intact virus was not required for maximal gene expression.
5.4. Discussion

5.4.1. Rhinovirus Optimisation Experiments

The reason to infect BEAS-2B cells before proceeding to whole tissue explants was that such an attempt will confirm that the newly propagated virus is causing similar effects on the cells as previously shown by others. There was, however, a limited success with obtaining measurable and repeatable cytokine levels in rhinovirus-stimulated BEAS-2B cells. Only the highest virus concentration used (1x10^7 TCID_{50}/ml) was able to significantly increase CCL5 (RV-1B) and IL-6 (RV-16) after 24h incubation. The cytokine levels released by cells after 1h pre-incubation with RV and subsequent medium change did not result in any increase above unstimulated control. The results are surprising since BEAS-2B cells are commonly used to study RV-induced immune response [108, 109, 140, 279, 362, 393, 426]. This perhaps could be explained by the unrepeatable results using the positive control – the levels of cytokines released from poly(I:C)-stimulated BEAS-2B cells varied 10-fold. It is difficult to explain such a difference in cell line culture as the same protocol had been followed. It was, however, an indication that any experiments carried out on these cells were to be interpreted with caution.

There are reports by other groups indicating that RV-16 infection in BEAS-2B cells is limited [303], which is in accordance with the results obtained using this tissue model. On the other hand, others that report successful RV infections have used different RV serotypes, e.g. RV-2, RV-39 or RV-14, to measure TNFα, CCL5 or IL-6 cytokine levels [140, 362], or used a different medium [108, 140, 279, 362, 426] which could alter the extent of innate immune response. Some groups also purify virus preparations against a sucrose gradient before stimulating BEAS-2B cells [108, 140, 426]. All of these factors contribute to the high discrepancy of data obtained from RV-infection studies in various cell types and models and therefore it is hard to make any comparisons of results reported by different groups.
Infection of BEAS-2B cells with RV was not successful, however highest virus concentration used on the cells suggested that virus is present and triggers an increase in cytokine levels. Moreover, HeLa cells exhibited cytopathic effect when exposed to virus preparations and specific RV antisera prevented the RV-induced morphological changes of the cells. These further suggested that the correct virus serotypes were present in the prepared stocks. It was therefore decided to proceed to infect whole tissue explants.

Pilot experiments carried out using tissue from three donors confirmed that RV causes the increase of all three cytokines measured. Since this was a first attempt to infect human lung tissue, it was decided to use higher concentrations than those utilised by others in cell line and primary cell cultures (1x10^{2.5} – 1x10^{5.5}). This is due to the observations made beforehand – higher doses of synthetic TLR ligands were required to induce similar cytokine response in tissue when compared to cell studies (cf. Chapter 3). Based on these observations and on the pilot infections of human whole lung tissue explants a concentration of 1x10^{6.5} was chosen. The optimal virus concentration of 1x10^{7} was not available due to virus batch to batch variation and therefore the highest closest concentration was utilised. The results of that experiment will be discussed below.

5.4.2. Effect of RV Stimulation on Pro-inflammatory Cytokine Production in Human Lung Tissue

The results show that both RV serotypes caused an increase in cytokine release, which is in accordance with previous studies. These include studies conducted in cell lines such as BEAS-2B and A549, primary bronchial epithelial cells and in bronchoalveolar lavage samples collected from patients participating in RV in vivo challenges.

These particular two viral strains were chosen because they represent two classes of RVs, RV-16 belongs to major group (and uses ICAM-1 as attachment receptor) whereas RV-1B belongs to minor group (and uses LDLR). These strains have also been widely used by others.
in different cell types and models and thus using them allows for some degree of comparison between different studies. Due to the fact that the response from two different RV serotypes varied, they will be discussed separately.

5.4.2.1. RV-16

TNFα is one of the first to be released following RV-16 infection. Rhinovirus therefore resembles the way R848 is inducing TNFα with its peak after just 6h, and it is quicker at inducing TNFα when compared to poly(I:C) (cf. Chapter 3). TNFα levels have also been measured from RV-16-stimulated macrophage cells (THP-1-derived), monocyte-derived macrophages (MDM), fibrocytes, bronchoalveolar macrophages and primary bronchial epithelial cells (see Table 5-2) [131, 164, 215, 397]. The levels and kinetics of TNFα release was different in different cell types, with MDM releasing highest concentrations of the cytokine after just 24h (2400pg/ml), whereas levels of TNFα from THP-1-derived cells increased even up to 72h (150ng/ml) [215]. Bronchoalveolar macrophages collected from patients undergoing bronchoalveolar lavage were very sensitive to RV-16 stimulation and released approximately 10ng/ml of TNFα; however UV-RV-16 also induced similar levels of the cytokine suggesting that, at least in these cells, TNFα secretion is not replication-dependent [131]. Wark et al. observed limited TNFα release from primary bronchial epithelial cells from healthy and asthmatic donors in response to RV-16 (5 and 15pg/ml after 48h; with UV-RV-16 also releasing measurable TNFα levels) [397] which is in accordance with the amount of the cytokine released in this model.

CCL5 has been frequently measured in response to RV-16 exposure in different cell types, including primary human bronchial epithelial cells and alveolar epithelial cells (A549) (cf. Table 5-2) [202, 282, 336]. Schroth et al. demonstrated that 250pg/ml of CCL5 is released from primary bronchial epithelial cells after 24h incubation with $10^7$ TCID$_{50}$/ml of RV-16 and that CCL5 induction is replication dependent [336]. Wark et al. also used primary bronchial epithelial cells which released about 600pg/ml of CCL5 in response to RV-16 after 48h [397]. Konno et al., however, observed that primary bronchial epithelial cells released approximately 200pg/ml
of CCL5 after 48h \((10^7 \text{ TCID}_{50}/\text{ml})\), however, in A549 cells, RV-16 did not induce a significant increase of CCL5 levels [202]. On the other hand, Papi et al. also infected A549 cells with RV-16 and observed a significant induction of CCL5 protein \((200\text{pg/ml})\) [282]. Taken together, these data suggest that the release of CCL5 in response to RV-16 varies in different cell types. Significant induction of CCL5 in human lung tissue (as presented in this chapter) is therefore in accordance with the data obtained using primary airway epithelial cells.

IL-6 concentration has also been frequently measured when studying rhinovirus infections (summary in Table 5-2 below) [164, 339, 397, 433]. In the tissue model presented here, IL-6 levels were high when compared to other cytokines, however, as basal cytokine levels were also elevated, only after a few time points RV-induced IL-6 concentrations were significantly above control values. This is in accordance with the in vivo challenge conducted by Mallia et al. where there was high variation of IL-6 secretion, and although there was an increase in IL-6 levels – it was not statistically significant [234]. In other studies, Wark et al. observed IL-6 concentrations of about 250\text{pg/ml} after 48h (significantly above controls) from human primary bronchial epithelial cells [397]. IL-6 was also significantly up-regulated in induced sputum after RV infection, although particular serotypes were not differentiated [339]. There was also an increase in IL-6 concentrations following human in vivo RV-14 (another major RV serotype) challenge, with IL-6 levels in nasal washings peaking after 48h post-infection \((250\text{pg/ml})\) [433], whereas Mallia et al. observed that in response to RV-16, IL-6 concentrations in nasal lavage from COPD patients peaked after 72h reaching about 500\text{pg/ml} [234]. These data again suggest that whole lung tissue explants respond to rhinovirus in a very similar way as respiratory system of patients infected with RV in vivo.

### 5.4.2.2. RV-1B

In the model presented here, RV-1B was less potent than RV-16. It did not cause a significant increase of CCL5 at any of the time points and IL-6 was increased only in COPD group, however TNF\(\alpha\) was significantly increased although the level of induction was moderate.
These observations together with the control data after 24h (cf. Figure 5-9) demonstrate a very limited infection of the human lung tissue.

There are scarce data investigating RV-1B-induced release of cytokines. RV-1B has been used in murine models (BALB/c) and significant cytokine release in BAL has been demonstrated (CCL5 increased from undetectable to 250pg/ml after 24h; IL-6 increased from 5 to 35pg/ml after 48h) [27]. On the other hand, Kim et al. showed that RV-1B on its own does not increase pro-inflammatory cytokine production, only in combination with an allergen a significant increase in cytokine levels is observed [198]. However, primary human bronchial epithelial cells were also infected with RV-1B and IL-6 levels increased 400-fold when compared to unstimulated controls [284]. Similar results were observed in human primary bronchial fibroblasts where IL-6 release was also significantly increased compared to controls [34]. Studies that examined TNFz, CCL5 and IL-6 release in response to RV-1B are included in Table 5-2.

The unsuccessful infection could be due to the limited availability of LDL receptor which acts as an attachment receptor for minor group rhinoviruses. There are no studies conducted to date that look at the expression patterns of LDL receptor in the lung. Such studies could prove useful when evaluating minor RV group infections both in vivo and in vitro. In addition, it could be that these patients were previously exposed to the RV-1B strain. Since there are lymphocytes present in the lungs [323], it is possible that adaptive immune response was responsible for the largely limited release of pro-inflammatory cytokines. In order to establish whether adaptive immunity have influence on WTE explants, the patients’ blood samples should be tested for the presence of antibodies to particular RV serotypes and positive and negative patients should be subsequently compared. Alternatively, RV-1B, as shown previously by others [28, 265], may be only a trigger in already sensitised or inflamed lungs. However, since there were no differences in cytokine release from smokers and COPD, it could be that another pathogen, such as a colonising bacteria, is required to trigger a more potent inflammatory response. The intricate mechanisms of bacterial/viral co-infections need to be further elucidated.
A summary of studies that examined TNFα, CCL5 and IL-6 protein expression in response to RV infection.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>RV serotype used</th>
<th>Mediators measured</th>
<th>Main findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMCs; BAL cells</td>
<td>RV-16</td>
<td>TNFα</td>
<td>RV-stimulated macrophages released increased levels of TNFα.</td>
<td>Gern et al. [129]</td>
</tr>
<tr>
<td>MDMs; THP-1-derived macrophages</td>
<td>RV-2; RV-9; RV-16</td>
<td>TNFα</td>
<td>TNFα increased in both types of cells following RV infection, but peaks earlier in primary cell-derived macrophages.</td>
<td>Laza-Stanca et al [215]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16</td>
<td>TNFα, CCL5, IL-6</td>
<td>Significant induction of all three cytokines following RV-16 infection. Relatively lower levels of TNFα when compared to CCL5 and IL-6.</td>
<td>Wark et al. [397]</td>
</tr>
<tr>
<td>BAL cells (BALB/c mice)</td>
<td>RV-1B</td>
<td>TNFα, CCL5, IL-6</td>
<td>No significant induction of the cytokines after RV-1B challenge. Synergistic increase of TNFα and CCL5 when RV used in combination with an allergen. Increased IL-6 levels after RV-1B+allergen exposure but not statistically significant.</td>
<td>Kim et al. [198]</td>
</tr>
<tr>
<td>Peripheral blood fibrocytes; MDMs</td>
<td>RV-16</td>
<td>TNFα, IL-6</td>
<td>Levels of both cytokines significantly increased following RV-16 exposure. Fibrocytes released significantly higher levels of pro-inflammatory cytokines compared to MDMs.</td>
<td>Isgro et al. [164]</td>
</tr>
<tr>
<td>Primary tracheal epithelial cells</td>
<td>RV-2; RV-14</td>
<td>TNFα, IL-6</td>
<td>TNFα and IL-6 protein levels significantly up-regulated following RV infection.</td>
<td>Terajima et al. [374]</td>
</tr>
<tr>
<td>Induced sputum (smokers and COPD subjects)</td>
<td>RV-16 in vivo challenge</td>
<td>TNFα, IL-6</td>
<td>Numerical increase of TNFα and IL-6 following RV-16 infection but it was not statistically significant. No differences in cytokine release between two subject groups.</td>
<td>Mallia et al. [234]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16; RV-49</td>
<td>CCL5</td>
<td>CCL5 protein levels up-regulated following exposure to either RV or UV-RV.</td>
<td>Schroth et al. [336]</td>
</tr>
<tr>
<td>HBECs; A549</td>
<td>RV-16; RV-49</td>
<td>CCL5</td>
<td>CCL5 significantly increased in HBECs cells after exposure to both RV-16 and RV-49. CCL5 was not increased in A549 cells after RV-16 infection (10^7 TCID_{50}/ml, 48h).</td>
<td>Konno et al. [202]</td>
</tr>
<tr>
<td>A549</td>
<td>RV-16</td>
<td>CCL5</td>
<td>CCL5 significantly increased in supernatants from RV-exposed A549 cells (MOI 1; 24 and 72h).</td>
<td>Papi et al. [282]</td>
</tr>
<tr>
<td>BAL cells (BALB/c mice)</td>
<td>RV-1B</td>
<td>CCL5, IL-6</td>
<td>CCL5 and IL-6 levels significantly increased after RV-1B challenge in BAL cells.</td>
<td>Bartlett et al. [27]</td>
</tr>
</tbody>
</table>
## 5.4.2.3. Summary

Comparatively low levels of cytokine release in this model are not surprising. Experiments with synthetic TLR ligands already confirmed the requirement for higher ligand concentration to induce similar responses in tissue to those observed in different cell cultures (Chapter 3). Therefore the use of similar virus concentrations here would be expected to yield lower cytokine release. Due to the nature of the virus and the method of its propagation it was not possible to produce more concentrated virus preparation at this time.

It is, however, more difficult to infect primary human cells with RV when compared to established cell lines. TNFα levels released from primary human tracheal cells treated with two other RV serotypes, namely RV-14 and RV-9 (10⁵ TCID₅₀/ml), were also measured [374]. The concentrations of TNFα in supernatant in response to both virus serotypes were undetectable until 24h and then peaked after 72h (6pg/ml). IL-6 levels, however, increased to about 300pg/ml after 24h and then began to decrease again. These cells were infected for 1h after

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<table>
<thead>
<tr>
<th>Cell type</th>
<th>RV serotype used</th>
<th>Mediators measured</th>
<th>Main findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced sputum (COPD patients)</td>
<td>RV present at exacerbation</td>
<td>IL-6</td>
<td>IL-6 significantly increased in induced sputum at exacerbation when RV was present. No change in IL-6 when RV was not present during exacerbation.</td>
<td>Seemungal et al. [339]</td>
</tr>
<tr>
<td>Nasal washings (healthy volunteers); MRC-5 lung fibroblasts; A549 cells</td>
<td>RV-39 in vivo challenge; RV-14</td>
<td>IL-6</td>
<td>Significant increase of IL-6 levels in nasal washing after symptomatic RV-39 infection (peak 48h post-infection). Significant increase of IL-6 after 24, 48 and 72h post-infection with RV-14 in supernatants from MRC-5 and A549 cells.</td>
<td>Zhu et al. [433]</td>
</tr>
<tr>
<td>Bronchial fibroblasts</td>
<td>RV-1B</td>
<td>IL-6</td>
<td>Significant induction of IL-6 following RV-1B exposure (24h).</td>
<td>Bedke et al. [34]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-1B</td>
<td>IL-6</td>
<td>Significant increase of IL-6 levels following RV-1B exposure. HBECs from asthmatic donors had lower IL-6 levels compared to healthy controls.</td>
<td>Parsons et al. [284]</td>
</tr>
</tbody>
</table>

BAL – bronchoalveolar lavage; HBEC – human bronchial epithelial cell; MDM – monocyte-derived macrophage; PBMC – peripheral blood mononuclear cell; WTE – whole tissue explant
which the medium was removed. This might explain even lower cytokine levels observed there when compared to this tissue model. Therefore the approach adopted here (i.e. not removing infection medium) takes this obstacle into account.

Others also used different RV serotypes to measure the release of pro-inflammatory cytokines and showed that all of them induced an inflammatory response, which included a significant increase of TNFα, CCL5 and IL-6 levels (see Table 5-2 above) [140, 278, 326]. Differences in the quantity of each cytokine released in response to different virus serotypes were also observed, even within the same “major” RV group [326, 336] which is in accordance with the data presented in this chapter. The method of virus preparation may also have a role in the subsequent cytokine release. To avoid any batch to batch variations, the same virus preparation was used for all the main rhinovirus experiments presented in this chapter.

Nevertheless, it has been demonstrated that, despite the technical difficulties, it is possible to successfully infect human lung tissue with RV. For the first time, it was shown that, upon RV-16 exposure, human lung tissue released increased levels of pro-inflammatory cytokines. It was also possible to evaluate any differences in cytokine release between samples obtained from smokers and COPD patients.

5.4.3. Effect of RV Stimulation on Type I and III Interferon Gene Expression in Human Lung Tissue

IFNα1 was the least expressed IFN gene following RV-infection. However, both RV serotypes significantly increased IFNα1 expression over unstimulated controls after 24h (response to RV-1B was less prominent with only gene expression levels in COPD group significantly increased). This is not surprising, as others also report IFNα gene and protein expression as limited or non-existent in different cells, including primary human epithelial cells (both RV-16 and RV-1B serotypes) and airway epithelial cell lines (Calu-3) (see Table 5-3 for summary) [303, 370]. It was also least expressed IFN in response to synthetic TLR ligands
There is, however, contrasting evidence as it has been shown that RV-16 induces IFNα gene and protein expression in fibrocytes, monocyte-derived macrophages, human peripheral blood monocytes and BAL fluid [90, 107, 164, 234, 298, 335, 368]. There are also data showing that RV-1B induces IFNα protein and gene expression in BAL fluid and lung tissue from RV-infected mice [314, 333] as well as human PBMCs [119]. High discrepancy in gene and protein expression of IFNα in response to rhinovirus is suggesting that dendritic cells and PBMCs are primary producers of IFNα. These observations are also supported by the limited IFNα expression in this tissue model, suggesting that tissue components do not readily express IFNα and leave its production to more specialised cells.

IFNβ1 mRNA levels in response to RV-16 and RV-1B were higher than levels of IFNα1. Multiple groups report similar findings with IFNβ protein and gene expression significantly up-regulated in various cell types in response to RV-16 and RV-1B (cf. Table 5-3) [63, 110, 164, 370, 410]. Several studies revealed a significant up-regulation of IFNβ expression in response to RV-16 and RV-1B in primary bronchial epithelial cells from both healthy donors and asthmatics [110, 370]. Interestingly also IFNβ gene expression seems to be induced in a cell-specific manner. It has been shown that fibrocytes secrete limited IFNβ following RV-16 infection which is in contrast to MDM cells which release significantly higher amounts of the cytokine [164]. Taken together data gathered from this and other studies suggest that expression of IFNβ is significantly increased in response to both RV serotypes.

In the tissue model, IFNλ expression was the highest of all IFN in response to both RV-16 and RV-1B. These observations were also reported by other groups, however the magnitude of IFN release in different experiments can differ 100-fold (summary in Table 5-3) [43, 273, 370]. These discrepancies could be due to different virus preparations and incubation time. Nevertheless, these studies are in accordance with the experiments conducted here. In addition, it has also been suggested that IFNλ is expressed primarily in epithelial cells and on mucosal surfaces and therefore strengthen the antiviral response in the cells that are first to be infected.
These data again underscore the importance of IFNλ in RV-induced innate immune response.

In this model, tissue samples from patients with COPD had significantly lower IFNβ and IFNλ gene expression when compared to tissue samples without COPD. Most of the studies conducted with RV as a trigger of respiratory disease exacerbation examined cells from healthy donors versus asthmatics (see Table 5-4 for summary). Edwards et al. reported that in primary bronchial epithelial cells asthmatics have impaired IFNβ and IFNλ response following RV infection (IFNβ deficiency confirmed by Wark et al.), however, a subsequent study by Sykes et al. revealed that these differences are non-existent when donors with asthma have well-controlled disease [110, 370, 396]. Contoli et al. reported deficient IFNλ gene expression and Sykes et al. showed deficient IFNβ gene and protein expression in BAL cells from asthmatic donors when compared to healthy controls [78, 368].
Table 5-3 A summary of studies that examined type I and III IFN gene and protein expression in response to RV infection.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>RV serotype used</th>
<th>Mediators measured</th>
<th>Main findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMCs</td>
<td>RV-16</td>
<td>IFNα</td>
<td>IFNα protein significantly up-regulated after 24h in cells from healthy and asthmatic donors.</td>
<td>Davies et al. [90]</td>
</tr>
<tr>
<td>PBMCs</td>
<td>RV-16 RV-1A</td>
<td>IFNα</td>
<td>IFNα protein detectable after stimulation with RV-16 or RV-1A (100 and 50 pg/ml, respectively).</td>
<td>Schreiber et al. [335]</td>
</tr>
<tr>
<td>Fibrocytes; MDMs</td>
<td>RV-16</td>
<td>IFNα, IFNβ</td>
<td>Both IFNs (protein) increased in the two cell types. MDMs released higher levels of IFNα and IFNβ when compared to fibrocytes after 72h.</td>
<td>Isgro et al. [164]</td>
</tr>
<tr>
<td>BAL cells; PBMCs</td>
<td>RV-16</td>
<td>IFNα, IFNβ</td>
<td>IFNα and IFNβ protein levels significantly up-regulated in response to RV after 8 and 24h in BAL cells and PBMCs from healthy donors. PBMCs released both IFNs in larger quantities than BAL cells.</td>
<td>Sykes et al. [368]</td>
</tr>
<tr>
<td>PBMCs</td>
<td>RV-16</td>
<td>IFNα, IFNλ</td>
<td>IFNα protein up-regulated app. 100-fold in response to RV. IFNλ1 mRNA not detectable in these cells in response to RV (as opposed to poly(I:C) where 300-fold increase in mRNA levels observed).</td>
<td>Pritchard et al. [298]</td>
</tr>
<tr>
<td>PBMCs</td>
<td>RV-16</td>
<td>IFNα, IFNλ</td>
<td>IFNα and IFNλ increased in RV-16-stimulated cells (1500 and 200 pg/ml, respectively).</td>
<td>Durrani et al. [107]</td>
</tr>
<tr>
<td>Calu-3; PBMCs</td>
<td>RV-14; RV-16</td>
<td>IFNα, IFNλ</td>
<td>IFNα protein levels were not increased in the epithelial cell line after RV infection. The addition of PBMCs and co-culture with Calu-3 resulted in significant increase of IFNα production in response to both serotypes (RV-14 causing a higher IFNα release when compared to RV-16). IFNλ was released in response to both RV serotypes from Calu-3 cells. This response was enhanced when PBMCs were added to Calu-3 culture.</td>
<td>Rajan et al. [303]</td>
</tr>
<tr>
<td>PBMCs</td>
<td>RV-43; RV-1B</td>
<td>IFNα, IFNλ</td>
<td>Prominent IFNα and IFNλ release from PBMCs from healthy donors. IFNλ more ubiquitous than IFNα. RV-43 induced higher IFN protein levels than RV-1B.</td>
<td>Forbes et al. [119]</td>
</tr>
<tr>
<td>Cell type</td>
<td>RV serotype used</td>
<td>Mediators measured</td>
<td>Main findings</td>
<td>Authors</td>
</tr>
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<tr>
<td>HBECs</td>
<td>RV-16; RV-1B</td>
<td>IFNα, IFNβ, IFNλ</td>
<td>All IFN mRNA levels significantly increased after 8, 24 and 48h (both RV serotypes). IFNα least expressed, IFNλ most ubiquitous. IFNα protein not detectable whereas IFNβ and IFNλ significantly increased after 48h.</td>
<td>Sykes at al. [370]</td>
</tr>
<tr>
<td>BAL cells</td>
<td>RV-16</td>
<td>IFNα, IFNβ, IFNλ</td>
<td>All three IFN proteins up-regulated in RV-stimulated cells (after 48h). IFNα most ubiquitous, IFNβ least expressed.</td>
<td>Mallia et al. [234]</td>
</tr>
<tr>
<td>Lung tissue (BALB/c mice)</td>
<td>RV-1B</td>
<td>IFNα, IFNβ, IFNλ</td>
<td>All three IFN gene expression increased after 24h. Limited IFNα expression; IFNλ most ubiquitously expressed. IFNλ increased gene expression lasted for up to 7 days post-infection.</td>
<td>Schneider et al. [333]</td>
</tr>
<tr>
<td>BSMCs</td>
<td>RV-1B</td>
<td>IFNβ, IFNλ</td>
<td>IFNβ and IFNλ mRNA levels significantly up-regulated in response to various doses of RV-1B after 24 and 48h. Higher IFNβ than IFNλ mRNA levels observed.</td>
<td>Calven et al. [63]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16; RV-1B</td>
<td>IFNβ, IFNλ</td>
<td>IFNβ and IFNλ mRNA increased after RV-stimulation in healthy HBECs. IFNβ and IFNλ protein levels numerically increased, but not significant, following RV infection.</td>
<td>Edwards et al. [110]</td>
</tr>
<tr>
<td>NCI-H292</td>
<td>RV-16</td>
<td>IFNβ, IFNλ</td>
<td>IFNβ and IFNλ mRNA increased after RV-stimulation after 4 and 24h (100-200-fold). Both IFN levels decreased when cells transfected to over-express IRAK-M.</td>
<td>Wu et al. [410]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16</td>
<td>IFNβ, IFNλ</td>
<td>IFNβ and IFNλ mRNA increased 2- and 20-fold (respectively) after 24h post-infection.</td>
<td>Bochkov et al. [43]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16</td>
<td>IFNλ</td>
<td>IFNλ protein levels increased to 1000 pg/ml after 24h post-infection.</td>
<td>Parsons et al. [284]</td>
</tr>
</tbody>
</table>

BAL – bronchoalveolar lavage; BSMC – bronchial smooth muscle cell; HBEC – human bronchial epithelial cell; MDM – monocyte-derived macrophage; PBMC – peripheral blood mononuclear cell; WTE – whole tissue explant
Recently, however, more attention has been focused on IFN gene and protein expression in COPD (cf. Table 5-4). Studies conducted to date revealed that, as in the case of asthmatics, patients with COPD also exhibit decreased type I and III IFN response to RV [234]. Mallia et al. infected 13 subjects with RV-16 and examined subsequent symptom scores and viral titers. They have also measured the number of neutrophils and IL-8 levels in sputum following RV infection. However, to examine IFN response to RV, BAL cells obtained at baseline bronchoscopy were infected *ex vivo* with RV-16 and it was found that cells from COPD donors had deficient production of IFNα, IFNβ and IFNλ [234]. On the other hand, other studies showed that primary bronchial epithelial cells from patients with COPD had increased rather than impaired IFNβ and IFNλ gene and protein expression [16, 332]. The smoking status of control subjects was different in the two studies: in the study conducted by Mallia et al. healthy donors were current or ex-smokers [234], whereas Baines et al. and Schneider et al. recruited only healthy non-smoking donors [16, 332]. Cigarette smoke, however, has been shown to decrease antiviral response in multiple cell culture models, including PBMC, fibroblasts and epithelial cells [31, 108, 161, 249]. It must be underlined that some of the studies examining the effects of cigarette smoke on IFN signalling pathways and IFN expression were conducted on cell lines and therefore may not fully represent the events happening *in vivo*. Consequently, it is unlikely that the difference in smoking status of the control groups in the contradicting studies is the reason for this discrepancy. The type of cells used could play a role in the levels of IFN gene expression. Baines et al. and Schneider et al. used epithelial cells which are known to be efficient IFNβ and IFNλ producers, however, Mallia et al. used BAL cells which, in the state of disease, could have altered antiviral signalling pathways and cell type ratios and therefore could have reduced IFN expression [16, 234, 332]. The human lung tissue study presented here takes all the cell types present in the tissue into account and supports the view that COPD patients have decreased IFN responses to RV. These studies, however, had a small sample size and larger investigation into the effects of COPD on IFN production during viral infections needs to be carried out.
Table 5-4 A summary of studies examining RV-stimulated IFN responses in control vs diseased (asthma or COPD) subjects.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>RV serotype used</th>
<th>Main findings</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASTHMA</strong></td>
<td></td>
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<tr>
<td>BAL cells; PBMCs</td>
<td>RV-16; RV-1B</td>
<td>Deficient IFNα and IFNβ production in response to RV-16 in BAL cells from asthmatic subjects. No differences in IFNα production in response to RV-1B. No IFN deficiency in PBMCs.</td>
<td>Sykes et al. [368]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16; RV-1B</td>
<td>No difference in IFNβ and IFNλ protein and gene expression between control vs asthmatic subjects in response to RV-16 and RV-1B.</td>
<td>Sykes et al. [370]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16; RV-1B</td>
<td>Deficient IFNβ and IFNλ mRNA and protein expression in asthmatic cells after stimulation with either RV-16 or RV-1B.</td>
<td>Edwards et al. [110]</td>
</tr>
<tr>
<td>HBECs; BAL cells</td>
<td>RV-16</td>
<td>IFNλ decreased in asthmatic subjects vs control in response to RV-16 in both HBECs and BAL cells.</td>
<td>Contoli et al. [78]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16</td>
<td>IFNβ gene and protein expression deficient in asthmatic cells.</td>
<td>Wark et al. [396]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-1B</td>
<td>IFNβ protein release decreased in asthmatics (app. 50% of healthy controls)</td>
<td>Wark et al. [398]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-16</td>
<td>IFNβ and IFNλ gene expression significantly lower in cells derived from asthmatic patients.</td>
<td>Bochkov et al. [43]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-1B</td>
<td>RV-1B-induced IFNλ protein expression deficient in cells from asthmatic donors (250pg/ml) when compared to healthy donors (1000pg/ml).</td>
<td>Parsons et al. [284]</td>
</tr>
<tr>
<td><strong>COPD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL cells</td>
<td>RV-16</td>
<td>IFNα, IFNβ and IFNλ gene expression deficient in cells from COPD donors.</td>
<td>Mallia et al. [234]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-1B</td>
<td>IFNβ and IFNλ mRNA increased in COPD cells compared to controls. IFNλ protein increased in COPD group.</td>
<td>Baines et al. [16]</td>
</tr>
<tr>
<td>HBECs</td>
<td>RV-39</td>
<td>IFNλ (1 and 2) increased in COPD after 48h but not after 24h.</td>
<td>Schneider et al. [332]</td>
</tr>
</tbody>
</table>

BAL – bronchoalveolar lavage, PBMC – peripheral blood mononuclear cell, HBEC – human bronchial epithelial cell, WTE – whole tissue explant
5.4.4. The Effect of UV-Inactivated RV and HeLa Extract on Immune Response in Human Lung Tissue

Intact virus was required for maximal pro-inflammatory cytokine production in the human lung tissue. UV light was used to render the virus inactive, i.e. UV-RV should not be able to replicate but still have its structural components intact. The use of UV-RV induced a numerical increase of cytokine levels, however that increase was lower when compared to intact virus. These observations are more pronounced in the RV-16 dataset where RV-16 caused a significantly higher induction of cytokines when compared to UV-RV-16. However, most likely due to the limited RV-1B infection in the tissue, intact RV-1B and UV-RV-1B induced similar levels of the cytokines. These data confirm that replicating virus is required for maximal cytokine production. Interestingly, both intact virus and UV-RV induced a prominent IFN response with UV-RV inducing numerically lower levels. These observations suggest that IFN genes are expressed when the tissue detects virus presence and dsRNA produced during replication are not required.

Other groups have also shown that intact virus induces higher cytokine concentrations when compared to UV-RV [78, 164, 396]. There are, however, conflicting observations for UV-RV-induced IFN gene expression. Some groups report markedly lower IFN mRNA levels for UV-RV when compared to intact virus [78, 396], while in this model UV-RV increased the levels of IFN gene expression. These differences could perhaps be explained by the method of UV-irradiation of the virus including the length of time to UV light exposure as well as the distance of samples from the UV lamp. The other difference between this study and those where UV-RV did not induce any significant IFN expression is that the infection medium was not removed after 1h incubation. This allowed more time for the other structural components of the virus to be more effectively recognised by the appropriate pattern recognition receptors. More studies are therefore required to fully assess the role of virus replication in the induction of type I and III IFNs.
HeLa extract was used to confirm that any changes observed in the RV-stimulated tissue were due to the virus and not caused by any HeLa cell components that may have been present in the virus preparation. Data shows that tissue stimulated with HeLa extract did not produce higher cytokine concentrations or IFN mRNA levels. With the exception of CCL5 in response to RV-1B, unstimulated control tissue and HeLa extract-treated tissue had similar levels of cytokines and IFNs. Increased influence of HeLa extract on CCL5 production when compared to RV-1B can again be attributed to the limited effect of RV-1B on CCL5 levels in general. These data show that in this model HeLa extract does not have a significant effect on either cytokine production or IFN expression. Some groups use “sham” infection (e.g. HeLa extract – depending on the virus propagation method) as their negative control in the experiment. Due to the availability of the tissue it was not possible to have “sham” infection as a control here as it would require two separate sets of controls (one for each serotype) as stock concentrations of the two viruses were different. However, including HeLa extract as one of the additional controls at the 24h time point showed that there are no differences in the tissue response to either medium only (unstimulated control) or HeLa extract. In addition, several other studies have also used similar approach. It can therefore be concluded that it is appropriate to use medium alone as the control when examining the effects of multiple virus serotypes and when tissue availability is limited.

5.4.5. Data Variability

Similarly to the previous two chapters, data presented here are variable. Although general patterns of increased tissue response to RV-16 were observed in most of the samples, only several patients have responded to RV-1B (possible explanations were included in Section 5.4.2.2.). This is one of the reasons for increased variability in RV-1B dataset. The release of CCL5 by the tissue again seem to be most variable, thus confirming that CCL5 is probably produced by selected cell types, and the pattern observed for synthetic TLR ligands was also observed when tissue was stimulated with RV.
5.4.6. Limitations

One concern about this model presented at this stage is the fact that it does not confirm whether this is an infection or a stimulation model. As stated before, components of the virus, such as its capsid or genome, are able to be recognised by the tissue PRRs even without viral replication. In order to establish whether a viral infection has taken place, additional experiments could be carried out. Others have used several methods to test whether there was an increase in number of viral particles [27, 260, 278, 370]. For example, qRT-PCR could be used to measure viral RNA, therefore an increase in the quantity of viral RNA would be indicative that, in fact, new virus particles were generated in the WTE model. Another way of checking whether infection has taken place is to perform a titration assay on the supernatants collected during the experiment. Increase in TCID<sub>50</sub> value, and therefore increase of viral titre, would also be indicative of infection and virus replication. In addition, specific antibodies to RV could be used to visualise the virus in the tissue before and after exposure to RV. Therefore, performing at least one of the abovementioned experiments would verify whether RV replication, and thus infection, has occurred.

5.4.7. Conclusions

Data presented in this chapter further establish the whole tissue explant model as a suitable candidate to examine human innate immune response to RV in vitro. Rhinovirus induced cytokine production and type I and III gene expression in human lung tissue in a time-dependent manner. Limited pro-inflammatory cytokine release and prominent type I and III IFN gene expression in response to both serotypes are in accordance with other studies in various models where similar observations were made. It has also been hypothesised that tissue from COPD donors will evoke higher pro-inflammatory cytokine release and decreased IFN response to RV. Although COPD tissue did not release significantly increased levels of cytokines, IFN gene expression was markedly lower in tissue from COPD donors, thus confirming our hypothesis. In addition, it has been suggested that RV on its own is not able to
induce respiratory disease exacerbation which could explain low levels of cytokines measured [28, 164]. Here, it has been demonstrated that human lung tissue is responding to RV stimulation and with additional developments to this model, e.g. virus/bacterial co-infections or the use of specific inhibitors, it could prove a valuable tool to further investigate the mechanisms of RV infections and to test new drugs in the whole tissue environment.
Chapter 6. Conclusion

COPD exacerbations are the leading cause of patient hospitalisations and deaths. Viruses and bacteria are responsible for the majority of COPD exacerbations. It is therefore imperative to understand how exactly pathogens activate the pro-inflammatory pathways in COPD lungs and to develop specific treatments that target and limit the infection. To help to answer these questions, in this thesis, a model of acute viral exacerbation in human lung tissue was developed. Research groups investigating COPD exacerbations use a plethora of models, including single-cell type cultures (such as epithelial cells, macrophages or neutrophils), mouse models and human in vivo challenges. In this thesis, whole tissue explants were used to combine the effects of all cell types present in the human lung and to establish a model that can be used to test novel drugs.

In the initial stages of this project, WTE were stimulated with synthetic TLR ligands known to activate same pathways as RNA viruses (such as rhinovirus). In Chapter 3, it was shown that human tissue responds to TLR ligands by producing both pro-inflammatory cytokines and expressing type I and III IFNs. In addition, using two different TLR ligands at the same time caused a synergistic release of TNFα and CCL5. This synergy was obtained by using two viral synthetic ligands. Perhaps a similar effect could also be observed when using a combination of viral and bacterial ligands as co-infections frequently occur in COPD.

Tissue from COPD patients was also more sensitive to poly(I:C) and R848 treatment. This is in contrast to the studies conducted on single cell type cultures including alveolar macrophages where COPD cells released lower cytokine levels when compared to controls [11]. These differences, however, can be explained by the fact that research conducted on alveolar macrophages uses the same number of cells for comparison, whereas it is known that more macrophages are present in COPD lungs [310]. In the tissue, the ratios of cells should more adequately represent the ratios of cells in vivo which makes the WTE model more advantageous.
to study COPD exacerbations. On the other hand, there is a question whether alveolar macrophages in COPD are alternatively activated, i.e. whether they are anti-inflammatory rather than pro-inflammatory (classical activation). If they are, the lower levels of cytokines obtained in studies conducted solely on macrophages can therefore be ascribed to the different phenotype of these cells. If macrophages are indeed anti-inflammatory in COPD lungs then the different cell types are responsible for increased cytokine levels. While the importance of different macrophage phenotypes in COPD is still under debate, COPD tissue released higher levels of cytokines following TLR stimulation which can be one of the reasons why infections lead to patient hospitalisations. This increase in pro-inflammatory cytokine production would be perhaps most visible in the current smokers’ population: within the Smokers group TNFα release was significantly attenuated when tissue was derived from current smokers.

Having established that this model is responsive to viral TLR ligands, in Chapter 4 it was investigated whether the model can be manipulated to examine, in more detail, which pathways and specific kinases are activated in response to synthetic viral ligands. Previous studies showed that TNFα plays a role as an inflammation-amplifying cytokine [377]. Since TNFα was ubiquitously released by the tissue, especially in response to R848, it was examined how neutralisation of TNFα by a specific antibody affects the release of other cytokines. The results showed that TNFα plays an important role in both TLR3 and TLR7/8 signalling and inhibition of this cytokine caused a significant decrease of CCL5 and IL-6. TNFα has been a target of interest in COPD research for some time now [192]. Clinical trials have been conducted using systemic anti-TNFα therapy to treat stable disease but without success [309, 387].

There are also other targets that are investigated as potential treatment for COPD, including p38 MAPK and NF-κB [25]. Preliminary results from Western blots showed a degree of activation of both p38 MAPK and p65 (NF-κB) in response to TLR ligands (see Appendix II). Therefore, the set of experiments was carried out to examine the effects of p38 MAPK, IKK-2 and IRAK1/4 inhibition in the whole tissue explant model. The results were also compared
with a glucocorticosteroid dexamethasone. Surprisingly, only dexamethasone and p38 MAPK inhibitor showed a marked decrease in cytokine release. IKK-2 inhibitor had a limited effect and IRAK1/4 inhibition did not cause inhibition. The redundancy of IRAK1/4 in this model, especially in response to R848, could be due to the fact that there exists an alternative pathway that bypasses IRAKs in the lung.

The effects of inhibitors on IFN gene expression were also investigated in Chapter 4. Dexamethasone had almost no effect on IFN mRNA levels. Inhibition of p38 MAPK, however, caused a significant reduction of mRNA of all three IFNs measured. These experiments have been conducted on COPD tissue only which means that p38 MAPK inhibitor reduces already significantly impaired IFNβ levels. IKK-2 inhibition affected only the levels of IFNλ1 after R848 exposure. IRAK-1/4 inhibitor, however, increased the gene expression of IFN suggesting a role for IRAKs as negative regulators of IFN.

Chapters 3 and 4 established a model of acute viral exacerbation using synthetic TLR ligands known to activate the same TLRs as viruses. The model was also shown to be able to respond to various treatments. The next step was to introduce an intact virus into the model and rhinovirus was chosen due to the fact that it is most commonly found at COPD exacerbations [339]. In Chapter 5, two rhinovirus serotypes, major (RV-16) and minor (RV-1B), were used on WTE and the innate immune response was characterised. RV-16 caused a significant up-regulation of cytokines, whereas RV-1B had a limited influence on the tissue. There were no differences in cytokine observed between smokers and COPD patients. On the other hand, both serotypes caused a significant induction of IFNs, especially IFNβ and IFNλ. A comparison between smokers and COPD showed that COPD patients had significantly lower IFNβ levels when compared to smokers which is in accordance with observations made by others [234]. These data confirm that diminished IFN response may increase viral load in COPD patients. It can also prolong the viral clearance and therefore make COPD patients more susceptible to secondary bacterial infections. In addition, both cytokine and IFN response to RV-16 was
dependent on the virus and intact virus was required for maximal response. These data show that rhinovirus is able to induce an innate immune response in human lung tissue and thus the model can be used to study antiviral pathways in the human lung.

Considering the data obtained in Chapters 3 and 5, one must conclude that human lung tissue responds differently to intact virus when compared to synthetic ligands. Poly(I:C) and R848 caused a marked increase of all pro-inflammatory cytokines measured whereas the response to the virus was more limited. Conversely, IFNs were induced more prominently by the virus and not the ligands. These observations suggest that although the ligands and the virus are thought to activate the same pathways it may be that the immune response is specifically tailored to the stimulus. Moreover, it is not possible to establish the required amount of synthetic ligand that would be physiologically relevant in terms of virus infection occurring \textit{in vivo}. At the same time, the viral concentrations used in this model were relatively high (1x10^{6.5} TCID_{50}/ml). To obtain a virus-induced exacerbation in \textit{in vivo} challenges a concentration of 10 TCID_{50}/ml was sufficient [233, 234]. This may be due to the fact that breathing facilitates the viral entry into the respiratory system. In the WTE \textit{in vitro} model, however, RV must enter the cells on its own, which may take longer and require higher viral doses.

6.1. **Limitations**

There are limitations to this model that need to be addressed. Taking these into account in future studies using whole tissue explants could therefore limit the variability of the data observed as well as more clearly show patterns of defective antiviral responses that are reflective of the disease rather than the model itself. These limitations will be discussed in more detail in the subsections below.

6.1.1. **Patients**

Whole tissue explant model relies on the tissue obtained from donors that undergo surgery for confirmed or suspected lung cancer. Therefore, the first issue to be addressed here is the
fact that it is not known whether cancerous tissue proximal to the tissue used in this model has any influence on subsequent responses of the model. One way to check that would be to use samples from healthy lungs, e.g. transplants, and compare these responses with the tissue obtained from patients with cancer. However, since both groups, smokers and COPD, were affected by cancer it seems suitable to compare the groups.

Due to the way the samples were collected, it was also not possible to ensure that the tissue from each patient is derived from the same part of the lung and therefore the samples may have contained different cell types and numbers. However a few samples were stained and it was revealed that they contained different cell types including macrophages, epithelial cells, fibroblasts and smooth muscle cells. The differences in cell types’ numbers could therefore be the reason for the variability of the data observed in this model. If the quantity of various cell types present in the tissue was measured by, for example, flow cytometry, it could show us whether there was a correlation between the quantity of cytokines and the composition of each explant sample.

In addition, tissue has been derived from patients from various backgrounds. Therefore any previous respiratory infections or exposure to smoke or different environmental factors could not be controlled for. Also, the microbiome of the lungs may influence the results obtained in the experiments. However, antibiotics were used in the medium and therefore any resident bacterial species should not have influenced the outcomes of the experiments conducted in this thesis. However, the use of antibiotics may not be wanted in studies examining the way tissue from COPD patients responds to infectious agents. Lungs of patients with COPD are known to be colonised with bacteria and these will \textit{in vivo} influence the way subsequent viral infections are cleared.

Because of scarce sample availability, it was also not possible to evaluate in more detail whether other factors, such as sex, smoking status, bacterial colonisation, use of inhaled steroids,
and severity of COPD were influencing the data in this model and whether these are responsible for the variability of the data. Ideally, in a larger study, all of these should be taken into account.

6.1.2. Technical Limitations

This model uses fragments of human lung tissue. These fragments were cut by the same person, however, it was not possible to ensure that all of the pieces are of the same size and volume. This may cause variability in the data because of surface to volume ratios in each sample are probably not the same. The data were adjusted for tissue weight in each well rather than number of fragments. Tissue weight is perhaps the easiest way to adjust for in this type of model, however, adjusting for different cell types, e.g. the number of epithelial cells, could be very interesting and should be considered in future work.

Because of the nature of the samples, it was not possible to make sure that each well contains the same number of various cell types. This is probably one of the causes of CCL5 release variability in the samples. This could be addressed in a few ways: precision cut lung slices were used before and such samples are always of the same depth and contain similar cell types, as airway is the central structure of such samples. In addition, a small biopsy could be taken starting at the top of the airway which again would allow for the samples to contain at least similar number of cells of different types.

The addition of the virus to the tissue culture and not removing the medium after 1h incubation is not the standard procedure in RV experiments conducted on single cell type cultures. However, this is a 3D system of tissue pieces and it was shown in Chapter 3 that WTE require higher doses of poly(I:C) or R848 when compared to other systems. Because the virus stock concentrations were limited, it was not possible to use higher concentration of the virus. It was therefore decided, as in the case of poly(I:C) and R848, to keep the infection medium for the duration of the experiments. To ensure that the intact virus was required for maximal response, the UV-RV and HeLa controls were used. In addition, UV-RV seemed to be sufficient
to induce a potent IFN response. To verify whether the virus was in fact fully inactivated by UV irradiation another set of PCR experiments should be conducted and the number of viral particles should be measured. UV-irradiation, however, is a standard method and it was expected for virus components on its own (without replication) to activate relevant pathways.

### 6.1.3. Model Limitations

Whole tissue explants model is more complicated than single cell type cultures. It is not a whole organ model, however, and it still does not take into account physiological processes such as breathing. This model does allow for the communication between different cell types that are present in the tissue, but this cooperation is limited as there is also no access to circulation therefore it is not possible to look at the influx of different cell types, e.g. neutrophils or eosinophils, following PRR activation.

In addition, because of the nature of sample preparation, any TLR or RV stimulation does not follow the physiological route; the samples were submerged in media that contained the stimuli and therefore cell types that are not the first to be in contact with those stimuli *in vivo* were also directly exposed to them. It would be very interesting to perform these experiments in a similar manner to Jang et al. where they used biopsy punches to obtain similar tissue samples and then put TLR ligands or RV on the apical surface [171]. This would be a more physiological approach as the stimulus would be initially in contact with the epithelial cells of the airway lumen.

### 6.1.4. Analysis Limitations

Due to the nature of sample collection, it was not possible to have a larger sample size in these studies and the experiments were conducted on a relatively small number of samples. However, clear patterns of increased COPD cytokine release in response to TLR ligands and attenuated IFN response were observed. These results were also confirmed by other groups suggesting that the results obtained here are plausible. It was also decided, due to limited sample
availability, that only one concentration of each inhibitor would be used in the experiments. It may be that a different dose of IKK-2 or IRAK-1/4 inhibitor would be more efficient at reducing the pro-inflammatory response. It is therefore not possible to conclusively say that IKK-2 and IRAK-1/4 in this WTE model do no contribute in the TLR-induced pro-inflammatory response.

In addition, since it was not possible to obtain blood samples from tissue donors, it was therefore not possible to perform assay that would test for the presence of specific antibodies for RV-16 and RV-1B. It may be possible that adaptive immunity is responsible for the limited response in these tissue samples.

All of the above-mentioned limitations should be taken into account when using whole tissue explants as a model for virus-induced exacerbations in the future.

6.2. Future Work

This thesis tried to establish a novel model of viral acute exacerbations in COPD. The model can be used in many ways, for example to further investigate the mechanisms of virus-induced exacerbations in COPD. It has been shown that the tissue from COPD patients releases more cytokines and expresses less IFN than WTE from smokers. Using these samples, we have a unique access to examine the responses of the tissue (as in the model described here) and to compare them to responses produced by various cell types isolated from the tissue. It is thought that macrophages are the primary cells responsible for the release of pro-inflammatory cytokines and epithelial cells are major IFN producers. A project involving isolation of, for example, alveolar macrophages, bronchial epithelial cells, smooth muscle cells and fibroblasts as well as using whole tissue explants from the same donor could confirm which cells are responsible for
which cytokines and therefore next generation therapies could be tailored to reach specific cell
types. Such a project would also show if communication between various cell types alters the
pro-inflammatory response in any way.

Another major area for further investigation are viral-bacterial co-infections. Hackett et al.
demonstrated that the tissue also responds to LPS [144]. The next step would be to use live
bacteria, such as non-typeable Haemophilus influenza, in combination with rhinovirus. Such an
experiment could examine: (1) viral infection followed by bacterial infection and vice versa and
(2) simultaneous infection using both pathogens. There is evidence that bacterial infection
predisposes to subsequent virus infection. Such experiments could verify these observations in
the whole tissue settings.

There are still more questions that remain to be answered about how TLR ligands and
rhinovirus induce innate immune response. In Chapter 3 it was shown that the COPD tissue
releases higher levels of pro-inflammatory cytokines in response to poly(I:C) and R848. It would
be interesting to examine the levels of expression of the appropriate receptors, i.e. TLR3,
TLR7/8 and also MDA-5. Evidence to date suggests that in patients with COPD several cell
types have up-regulated TLR4 whereas other cell types have decreased expression of the
receptor and it is not the same for another bacteria-sensing receptor TLR2 [105, 227, 263, 327].
It is therefore still not known how TLRs regulate the innate immune response in COPD. The
expression of viral TLRs, however, have not yet been examined in COPD tissue.

Another experiment conducted in Chapter 3 showed that using two TLR ligands at the same
time causes a synergistic release of TNFα and CCL5. There was no synergy observed for IL-6.
However, since the levels of IL-6 are much higher than the levels of other cytokines, the use of
sub-optimal doses of ligands could reveal whether activating TLR3 and TLR7/8 at the same
time also causes IL-6 synergy. Alternatively, an earlier time point, e.g. 6h, could be used to verify
this. In addition, others have also shown synergy in pro-inflammatory cytokine release when a
variety of TLR ligands were used in combination [231, 232]. Using this model one can combine bacterial and viral signals (synthetic ligands or live bacteria and virus) and examine whether this combination also results in synergy. In addition, bacterial infections were shown to modulate the expression of TLR3 [324]. Therefore, the influence of poly(I:C), R848, and other ligands on the expression of TLRs should also be measured. TLRs could be targets for novel inhibitors that aim to limit the overwhelming inflammation during COPD exacerbation.

In Chapter 4, several inhibitors were used to examine their effect on TLR3 and TLR7/8-stimulated COPD tissue. Although the data obtained using dexamethasone and p38 MAPK were not surprising, the role of both IKK-2 and IRAK1/4 in this model remains controversial. It would be beneficial to conduct a dose-response experiment which will show if higher (or lower) doses of the inhibitor produce a different effect in the tissue. Also, the influence of IRAK1/4 on IFN gene expression and its role as a potential negative regulator should also be examined in more detail. This could be done by, for example, looking at the effect of IRAK1/4 inhibition on expression and/or activation of IRFs.

The novel model of *in vitro* rhinovirus infection of human lung tissue was established in Chapter 5. As the response of the tissue to virus was different from the one obtained in earlier chapters, the mechanisms behind cytokine and IFN induction should be further evaluated. In the first instance, inhibitors already used in Chapter 4 should be introduced to the virus-tissue model to show whether dexamethasone and inhibition of p38 MAPK, IKK-2 or IRAK1/4 produce similar effects to those obtained using synthetic ligands.

And lastly, to have a more complete view of how tissue responds to various stimuli, the panel of cytokines measured should also be expanded. Other cytokines important in COPD should also be evaluated, e.g. chemokine (C-X-C motif) ligand (CXCL) 8 (also known as IL-8) or tumour growth factor (TGF) β. Such experiments will allow for further comparison of this
model to single cell type cultures and assessment whether communication between different cell
types changes the immune response.

6.3. Final Conclusion

In summary, this thesis presents a useful model of acute viral COPD exacerbation. It was
demonstrated that the tissue responds to TLR ligands and RV ex vivo by releasing pro-
inflammatory cytokines and expressing IFN. In addition, activation of multiple TLRs caused
synergistic release of TNFα and CCL5. These data underscore the need for a reliable co-
infection model in COPD cells. Furthermore, it was confirmed that TNFα will enhance the
release of other pro-inflammatory cytokines. Although anti-TNFα treatments were not
successful in stable COPD patients, TLR-induced TNFα release can be attenuated by inhibiting
p38 MAPK or with the use of corticosteroids; both anti-inflammatory agents significantly
decreased the levels of pro-inflammatory cytokines in the COPD tissue. The diminished IFN
response to TLR ligands and RV was also confirmed in this model, thus new
immunomodulators aiming to restore IFN expression in COPD patients can be tested in the
whole tissue explant model.

6.3.1. Meeting Project Aims and Objectives

The main aim of this project was to establish a novel model of virus infection using human
lung tissue. It has been demonstrated that lung WTE from smokers and COPD respond to both
TLR ligands and intact virus. It has also been shown that the model can be further manipulated
with the use of specific inhibitors or steroids. The data presented in this thesis indicate that the
tissue model is suitable to study virus infection in the tissue however more experiments are
required to fully understand the innate immune response to viruses.
Appendix I. A summary of studies examining the effects of RV infection in airway cells.
### Table I-1 A summary of selected studies that examined the effects of rhinoviruses on (primarily) airway cells.

<table>
<thead>
<tr>
<th>Effect of or response to RV</th>
<th>Effect measured by</th>
<th>RV serotype used</th>
<th>Disease involved, if any</th>
<th>Cell type used</th>
<th>Model</th>
<th>Main findings</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>RV is capable of infecting lower airways</td>
<td><em>In situ</em> hybridisation and immunohistochemistry (IHC) used to detect RV RNA or proteins in bronchial biopsy samples</td>
<td>NA</td>
<td>Asthma</td>
<td>Primary bronchial epithelial cells</td>
<td>Human</td>
<td>RV was detected in samples from asthmatic patients and controls in the absence of cold symptoms; RV was significantly more frequently infected in asthmatic patients</td>
<td>Wos et al. [409]</td>
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<tr>
<td>Replication rate at 37°C and 33°C, i.e. temperatures of lower and upper respiratory tract, respectively.</td>
<td>RV-1B, RV-2, (minor) RV-7, RV-9, RV-14, RV-16, RV-41, RV-70 (major)</td>
<td>NA</td>
<td>Ohio HeLa cells</td>
<td>Human</td>
<td>Replication of all viruses observed at both temperatures although some serotypes preferred one temperature over the other</td>
<td>Papadopoulos et al. [277]</td>
<td></td>
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<tr>
<td>Infection rate (measured by <em>in situ</em> hybridisation, RT-PCR and cytokine release)</td>
<td>RV-2 (minor) RV-7 (major)</td>
<td>(although asthma patients also tested)</td>
<td>Primary bronchial epithelial cells and from bronchoscopy specimens following <em>in vivo</em> RV challenge</td>
<td>Human</td>
<td>RV RNA was detectable in cells after 6, 24 and 48h post-infection in lower airway primary cells; mRNA and protein levels of IL-6, IL-8, IL-16 and CCL5 were significantly increased post-infection</td>
<td>Papadopoulos et al. [278]</td>
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<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by RV</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
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<tr>
<td>RV is capable of infecting lower airways (ctd.)</td>
<td>RV RNA presence in samples measured by RT-PCR</td>
<td>RV-16 (major)</td>
<td>Allergy</td>
<td>Primary bronchial epithelial cells (obtained by bronchoscopy) following in vivo RV challenge</td>
<td>Human</td>
<td>Majority of post-infection samples tested positive for RV-16 (negative pre-infection)</td>
<td>Gern et al. [130]</td>
</tr>
<tr>
<td>Cytopathic effect mediated by RV in cells of upper and lower respiratory tract</td>
<td>RV-16 (major)</td>
<td>NA</td>
<td>Primary bronchial epithelial cells Primary adenoidal epithelial cells</td>
<td>Human</td>
<td>Cytopathic effect observed in both types of cells</td>
<td>Mosser et al. [259]</td>
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<tr>
<td>RV induces pro-inflammatory cytokine release</td>
<td>Protein and mRNA levels of IL-8</td>
<td>RV-14, RV-39 (major)</td>
<td>NA</td>
<td>Nasal epithelial cells (in vivo challenge) A549 – alveolar epithelial cell line MRC-5 human foetal lung fibroblasts Normal human bronchial epithelial cells</td>
<td>Human</td>
<td>IL-8 is present in nasal lavages of healthy individuals and its levels increase following RV infection</td>
<td>Zhu et al. [434]</td>
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<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
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<tr>
<td>RV induces pro-inflammatory cytokine release (ctd.)</td>
<td>CCL5 and IL-8 protein levels by ELISA and CCL5 mRNA by RT-PCR; ICAM-1 levels measured by ELISA</td>
<td>RV-16 (major) RV-49 (minor)</td>
<td>NA</td>
<td>Primary bronchial epithelial cells</td>
<td>Human</td>
<td>ICAM-1 expression was not upregulated 24h post-infection; CCL5 protein levels upregulated following exposure to either RV or UV-RV; Hydrocortisone dose-dependently inhibited RV-induced CCL5 release</td>
<td>Schroth et al. [336]</td>
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<tr>
<td>IL-6, IL-8, TNFα and IL-1β ELISA</td>
<td>RV-2 (minor), RV-14 (major)</td>
<td>NA</td>
<td>Primary tracheal epithelial cells</td>
<td>Human</td>
<td>IL-6, IL-8, TNFα and IL-1β protein levels significantly upregulated following RV infection; RV significantly upregulated ICAM-1; Pre-treatment with IL-1β predisposes to RV infection; Blocking IL-1β reduced the viral titer.</td>
<td>Terajima et al. [374]</td>
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<tr>
<td>Cell count on BAL; ELISA on proinflammatory cytokines</td>
<td>RV-1B (minor)</td>
<td>NA</td>
<td>Lung tissue BAL</td>
<td>Mouse</td>
<td>CXCR2 (one of the IL-8 receptors) is required for neutrophilic influx; Production of proinflammatory cytokines, e.g. TNFα were reduced in CXCR2 deficient mice following RV infection</td>
<td>Nagarkar et al. [264]</td>
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<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
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<tr>
<td>RV induces pro-inflammatory cytokine release (ctd.)</td>
<td>Cytokine and mucin protein levels measured by ELISA; Virus particle number measured by RT-PCR, and visualised with staining of anti-sense strand</td>
<td>Asthma – mice model challenged with OVA</td>
<td>Mouse in vivo challenge BAL Lung tissue</td>
<td>Mouse</td>
<td>Neutrophilic inflammation caused by RV detectable at 8h, peaked at 1-2d; RV caused early production of MIP-2, KC (both neutrophil chemoattractants) and MIP-3α (T cell chemokine); Later (1-2d) other T cell chemokines, i.e. IP-10, CCL5 and I-TAC were produced (BAL); TNFα, IL-6, IL-8 and IL-1β were produced, whereas IL-4, IL-13 and IL-17 were undetectable (BAL); IFNα, IFNβ and IFNλ upregulated at 24h post infection (from BAL); Increased number of viral particles in mice (BAL and lung tissue) treated with live RV (as opposed to those treated with UV-RV); OVA challenge resulted in a more pronounced response to RV-1B, increased number of neutrophils (1d) and eosinophils (7d) compared to PBS treated controls; Mucin production was increased in RV-OVA mice compared to controls</td>
<td>Bartlett et al. [27]</td>
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<td>Effect of or response to RV</td>
<td>Effect measured by</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
<td>Model</td>
<td>Main findings</td>
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<tr>
<td>RV induces type I and III IFN</td>
<td>IFNα, IFNβ, IL-6, TNFα and IL-8 protein release measured by ELISA</td>
<td>RV-16 (major)</td>
<td>Asthma</td>
<td>Peripheral blood fibrocytes and MDMs</td>
<td>Human</td>
<td>Significant release of IFNs after 72h; MDMs released higher amounts of IFN; Fibrocytes produced more of IL-6, IL-8 and TNFα; UV virus caused cytokine release, but did not cause IFN production; Addition of house dust mite allergen enhanced pro-inflammatory response.</td>
<td>Isgro et al. [164]</td>
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<tr>
<td>mRNA expression of IFNβ and IFNλ measured by RT-PCR</td>
<td>RV-16 (major) RV-1B (minor)</td>
<td>Asthma</td>
<td>Primary bronchial epithelial cells</td>
<td>Human</td>
<td>Type I and III IFN response to both RV serotypes was increased after 8 and 24h; Cells from asthmatic donors exhibited reduced IFN response when compared to cells from healthy subjects.</td>
<td>Edwards et al. [110]</td>
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<tr>
<td>IFNα, IFNα2 and IFNβ protein release measured by ELISA</td>
<td>RV-16 (major)</td>
<td>Asthma</td>
<td>Bronchoalveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMCs)</td>
<td>Human</td>
<td>RV induced significant increase of IFNs in cells from healthy subjects; Cells from asthmatics had significantly lower IFN response after 8 and 24h; All IFNs measured significantly increased in PBMCs post-infection. No difference in IFN response between cells from healthy subjects and asthmatics following RV-16 infection.</td>
<td>Sykes et al. [368]</td>
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<tr>
<td>IFNα, IFNβ and IFNλ protein and mRNA measured by ELISA and RT-PCR</td>
<td>RV-16 (major) RV-1B (minor)</td>
<td>Asthma</td>
<td>Human bronchial epithelial cells (HBECS)</td>
<td>Human</td>
<td>RV induced significant release and expression from HBECS; No differences observed between subject groups;</td>
<td>Sykes et al. [370]</td>
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<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
<td>Model</td>
<td>Main findings</td>
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<tr>
<td>RV induces type I and III IFN (ctd.)</td>
<td>IL-8, IP-10 and TNFα measured by ELISA, and MRNA expression of IFNβ and IFNλ measured by RT-PCR</td>
<td>RV-16 (major)</td>
<td>Asthma</td>
<td>Primary bronchial epithelial cells from bronchial brushings</td>
<td>Human</td>
<td>RV induced IFN expression in both cells derived from normal and asthmatic subject; Cells from healthy donors produced more TNFα and IP-10; Significantly lower levels of IFN mRNA were observed in cells from asthmatics.</td>
<td>Bochkov et al. [43]</td>
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<tr>
<td>mRNA expression of IFNβ and IFNλ and virus receptors RG-I and MDA-5 measured by RT-PCR</td>
<td>RV-1B (minor)</td>
<td>NA</td>
<td>Bronchial smooth muscle cells (BSMCs)</td>
<td>Human</td>
<td>Both IFN mRNA levels were significantly increased following RV exposure after 24 and 48h; The effect was dose-dependent; RV also significantly increased the expression of the two RLRs; Chloroquine inhibited both IFN and RLR expression in BSMCs.</td>
<td>Calven et al. [63]</td>
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<tr>
<td>TNFα, IL-6, IL-8, IP-10, neutrophil elastase, IFNα, IFNβ and IFNλ protein release measured by ELISA</td>
<td>RV-16 (major)</td>
<td>COPD</td>
<td>Bronchoalveolar lavage (BAL) cells and in vivo challenge</td>
<td>Human</td>
<td>Only IFNλ was increased in BAL cells after ex vivo infection with RV-16; Cells from COPD patients showed impaired IFN response; Following in vivo challenge, RV did not cause an increase in sputum IL-6, IL-8, TNFα or IP-10.</td>
<td>Mallia et al. [234]</td>
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<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
<td>Model</td>
<td>Main findings</td>
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<tr>
<td>Importance of eosinophils in resolving RV infections</td>
<td>mRNA expression of IL-8, CCL5, MIP-1α, eotaxin and eotaxin-2 measured by RT-PCR Protein levels of IL-8, CCL5, MIP-1α, eotaxin and eotaxin-2 measured by ELISA</td>
<td>RV-16 (major) RV-1B (minor)</td>
<td>NA</td>
<td>BEAS2B cells</td>
<td>Human</td>
<td>IL-8, CCL5, MIP-1α, eotaxin and eotaxin-2 mRNA upregulated following RV infection; Protein levels of all cytokines except for MIP-1α were detectable and were upregulated compared to controls</td>
<td>Papadopoulos et al. [279]</td>
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<tr>
<td>IL-4, IL-5, IL-6, IL-8, IL-12, and IFNγ protein levels measured by ELISA</td>
<td></td>
<td>RV-16 (major)</td>
<td>Allergic rhinitis</td>
<td>Nasal lavage and sputum collected following RV16 in vivo challenge</td>
<td>Human</td>
<td>Delayed onset of symptoms in individuals with allergen exposure prior to RV challenge; Neutrophil count in nasal lavage increased in all patients; Eosinophil number did not increase; IL-6 and IL-8 protein levels were increased in nasal lavage fluid but not in sputum samples; Eosinophil number at day 0, i.e. just before RV infection inversely correlated with the severity of cold symptoms; Only IL-6 and IL-8 levels were upregulated, other cytokines were not.</td>
<td>Avila et al. [13]</td>
</tr>
<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
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<td>Main findings</td>
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<tr>
<td>Importance of eosinophils in resolving RV infections (ctd.)</td>
<td>Looked at RV binding to eosinophils; T cell proliferation and antigen presentation by eosinophils measured by incorporation of tritiated thymidine</td>
<td>RV-16 (major)</td>
<td>Allergic rhinitis and atopic asthma</td>
<td>Eosinophils (purified from peripheral blood samples)</td>
<td>Human</td>
<td>RV-16 did not affect surface expression of ICAM-1; Eosinophils presented RV-16 antigens to RV16-specific T cells promoting T cell proliferation and upregulation of IFNγ</td>
<td>Handzel et al. [147]</td>
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<tr>
<td>RV infects macrophages and monocytes</td>
<td>Viral titers measured using RT-PCR; RV binding to cells measured using radioactively labelled virus; Cell viability – microscopic observation; TNFα protein levels measured by ELISA</td>
<td>RV-16 (major)</td>
<td>NA</td>
<td>Peripheral blood mononuclear cells (PBMCs) Bronchoalveolar macrophages (obtained from BAL) HeLa cells</td>
<td>Human</td>
<td>Viral titers in HeLa cells increased 100-fold, whereas it gradually decreased in monocytes and macrophages; RV bound to HeLa cells and macrophages; RV had no effect on monocyte and macrophage viability; In RV-stimulated macrophages TNFα protein levels were significantly increased compared to controls.</td>
<td>Gern et al. [129]</td>
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<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
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<tr>
<td>RV infects macrophages and monocytes (ctd.)</td>
<td>Intracellular viral RNA measured by PCR; Western blot analysis for RV 3C protease (produced during replication only); TNFα, IFNα and IFNβ protein levels measured by ELISA; Cells transfected with NF-κB-Luc promoter/reporter to assess NF-κB role in response to RV</td>
<td>NA</td>
<td>Macrophages derived from THP-1 – human monocyte cell line</td>
<td>Human</td>
<td>Increased number of viral particles detected in THP-1-derived macrophages; Increased staining of 3C protease in THP-1-derived macrophages; In monocyte-derived macrophages viral replication was limited; Following RV infection, IFNα and IFNβ upregulated in macrophages derived from primary human cells, very low levels of IFN in THP-1-derived cells; Increased luciferase expression indicated induction of NF-κB by RV; TNFα upregulated in both types of cells following RV infection, but peaks earlier in primary cells-derived macrophages; NF-κB was independent of RV serotype; Inhibition of NF-κB and not p38/MAPK abrogated TNFα release</td>
<td>Laza-Stanca et al. [215]</td>
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<tr>
<td>mRNA and protein levels of IL-8 measured by RT-PCR and ELISA, respectively</td>
<td>RV-2 (minor) RV-9, RV-14 (major)</td>
<td>NA</td>
<td>Peripheral blood mononuclear cells (PMBCs) (healthy volunteers) THP-1 cell line</td>
<td>Human</td>
<td>IL-8 released in dose-dependent and time-dependent manner from PMBCs following RV infection; UV-RV also causes IL-8 release (however smaller than live RV); Virus was able to replicate only in THP-1 cells, no virus replication detectable in PMBCs</td>
<td>Johnston et al. [179]</td>
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<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
<td>Model</td>
<td>Main findings</td>
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<td>RV induces airway remodelling</td>
<td>mRNA of VEGF measured by RT-PCR; activin A, amphiregulin, VEGF and IP-10 protein levels measured by ELISA; Western blot analysis for induction of MAPK pathways (p38, ERK and JNK);</td>
<td>RV-1A (minor), RV-16 (major)</td>
<td>NA</td>
<td>BEAS-2B – human bronchial epithelial cell line Primary human bronchial epithelial cells Nasal lavage from patients with colds</td>
<td>Human</td>
<td>Activin A, amphiregulin and VEGF were upregulated in both cell types following RV-16 infection; VEGF expression was rapidly induced by RV; Western blot analysis revealed quick activation of all 3 MAPK pathways, but inhibition of p38 and not ERK or JNK attenuated VEGF expression; VEGF production was increased in nasal lavage from patients with RV-induced colds compared to healthy controls</td>
<td>Leigh et al. [219]</td>
</tr>
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<td>Virus RNA measured by RT-PCR; Fibronectin, collagen I, IV and V, perlecan, chondroitin sulphate, tenascin, variscan, elastin and VEGF protein levels measured by ELISA; Imiquimod and poly(I:C) used to assess the role of TLRs</td>
<td>RV-1B, RV-2 (minor) RV-16 (major)</td>
<td>Primary human bronchial epithelial cells Primary human lung fibroblasts</td>
<td>Human and mouse</td>
<td>Perlecan (in bronchial epithelial cell only) and collagen V deposition and VEGF production (in epithelial cells and fibroblasts) increased during exposure to live or UV-RV; Imiquimod exposure resulted in a more pronounced VEGF and collagen V (in fibroblasts) than exposure to poly(I:C); In bronchial epithelial cells perlecan, collagen V and VEGF deposition induced only in response to poly(I:C) and not imiquimod</td>
<td>Kuo et al. [209]</td>
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<td>Effect of or response to RV</td>
<td>Effect measured by VEGF</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
<td>Model</td>
<td>Main findings</td>
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<td>RV induces airway remodelling (ctd.)</td>
<td>VEGF protein levels measured by ELISA and VEGF expression measured by RT-PCR</td>
<td>RV-16 (major)</td>
<td>Asthma</td>
<td>HeLa cells Primary human bronchial epithelial cells MRC5 – foetal fibroblast cell lina Primary airway fibroblasts Nasal aspirates from asthma patients</td>
<td>Human</td>
<td>RV did not upregulate VEGF production in HeLa, primary bronchial epithelial cells or fibroblasts; VEGF levels in nasal aspirates from patients with asthma were not increased during RV-induced exacerbation</td>
<td>De Silva et al. [93]</td>
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<tr>
<td>VEGF, angiopoietin-1 and -2, and IL-6 protein levels measured by ELISA; VEGF expression measured by RT-PCR;</td>
<td>VEGF, angiopoietin-1 and -2, and IL-6 protein levels measured by ELISA; VEGF expression measured by RT-PCR;</td>
<td>RV-1B (minor) RV-9, RV-16 (major)</td>
<td>Asthma</td>
<td>BEAS-2B cells Primary human bronchial epithelial cells HUVECs – human umbilical vein endothelial cells Peripheral blood mononuclear cells (PBMCs)</td>
<td>Human</td>
<td>Significant upregulation of VEGF expression and protein levels in primary and BEAS-2B cells, but angiopoietin-1, and -2 not detectable; Supernatants from RV-infected epithelial cells induced significant increase in HUVEC cell proliferation compared to controls; Addition of supernatants from PBMCs to BEAS-2B cells prior to virus exposure resulted in further upregulation of VEGF production (supernatants from asthmatics induced significantly higher VEGF production from BEAS-2B cells than controls)</td>
<td>Psarras et al. [300]</td>
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<td>Effect of or response to RV</td>
<td>Effect measured by</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
<td>Model</td>
<td>Main findings</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
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</tr>
<tr>
<td>Asthma phenotype predisposes to RV infection</td>
<td>ICAM-1 expression assessed by immunocytochemistry</td>
<td>RV-14 (major)</td>
<td>Asthma</td>
<td>Primary human nasal epithelial cells (from nasal brushings)</td>
<td>Human</td>
<td>Cells from asthmatic patients had higher basal expression of ICAM-1; Allergen challenge increased ICAM-1 expression in atopic patients and not normal controls; RV-14 increased ICAM-1 expression in cells from healthy donors (data for asthma cohort not available).</td>
<td>Bianco et al. [40]</td>
</tr>
<tr>
<td>IFNλ1, IP-10 and CCL5 protein levels measured by ELISA</td>
<td>RV-1B (minor), RV-9, RV-16 (major)</td>
<td>Asthma</td>
<td>BEAS-2B – human bronchial epithelial cell line Primary human bronchial epithelial cells Peripheral blood mononuclear cells (PBMCs) Monocyte-derived macrophages</td>
<td>Human</td>
<td>All RVs induced IFNλ expression and protein release in a dose-dependent manner in all cell types; UV-RVs did not induce IFNλ; IFNλ increased CCL5 and IP-10 in uninfected epithelial cells; Pre-treatment with IFNλ resulted in decreased viral replication and titers (neither IP-10 nor CCL5 had the same effect); In macrophages, UV-RV did not induce IFNλ production; Induction of IFNλ mRNA and protein production significantly reduced in asthamtics compared to normal controls in response to RV infection</td>
<td>Contoli et al. [78]</td>
<td></td>
</tr>
<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by</td>
<td>RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
<td>Model</td>
<td>Main findings</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
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</tr>
<tr>
<td>Asthma phenotype predisposes to RV infection (ctd.)</td>
<td>IFNβ, IL-6, IL-8, CCL5 and TNFα expression and protein levels measured by RT-PCR and ELISA respectively</td>
<td>RV-1B (minor)</td>
<td>Asthma</td>
<td>Primary bronchial fibroblasts</td>
<td>Human</td>
<td>IL-6 and IL-8 and TNF-α mRNAs were significantly induced in both normal and asthmatic at 2h post-infection but returned to normal 8h post infection; CCL5 expression was dependent on viral replication</td>
<td>Bedke et al. [34]</td>
</tr>
<tr>
<td>IL-8, CCL5, IP-10 and IFNγ protein levels measured by ELISA; mRNA of IFNα and IFNβ measured by RT-PCR</td>
<td>RV-16 (major)</td>
<td>Asthma</td>
<td>Primary airway fibroblasts and myofibroblasts</td>
<td>Human</td>
<td>IL-8 significantly upregulated following RV infection; Pre-treatment with transforming growth factor (TGF)-β1 increased viral replication and quantity of viral RNA in fibroblasts from asthmatics; Increased viral replication was observed in fibroblasts from asthmatics compared to normal controls; Fibroblasts from asthmatics had deficient IFN response to RV compared to healthy controls and pre-treatment with TGF-β1 decreased IFN mRNA levels; Exposure to exogenous IFNβ prior to RV infection reduced viral replication rate</td>
<td>Thomas et al. [376]</td>
<td></td>
</tr>
<tr>
<td>Effect of or response to RV</td>
<td>Effect measured by RV serotype used</td>
<td>Disease involved, if any</td>
<td>Cell type used</td>
<td>Model</td>
<td>Main findings</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>Asthma phenotype predisposes to RV infection (ctd.)</td>
<td>The effect on ICAM-1 expression of T helper 2 (Th2) cell chemokines (IL-4, IL-5, IL-10 and IL-13) was assessed by ICAM-1 immunohistochemistry</td>
<td>RV-14 (major)</td>
<td>NA</td>
<td>NCI-H292 – human pulmonary carcinoma cell line</td>
<td>Human \nAll cytokines increased ICAM-1 expression in dose-dependent manner; RV14 treatment upregulates ICAM-1 expression which is further enhanced by pretreatment with Th2 cytokines; Viral titers were higher in supernatants collected from cells retreated with Th2 cytokines</td>
<td>Bianco et al. [39]</td>
<td></td>
</tr>
</tbody>
</table>

IFNβ mRNA measured by RT-PCR; ICAM-1 expression measured by flow cytometry; IFNβ, IL-6 and CCL5 protein levels measured by ELISA

<table>
<thead>
<tr>
<th>Effect of or response to RV</th>
<th>Effect measured by RV serotype used</th>
<th>Disease involved, if any</th>
<th>Cell type used</th>
<th>Model</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNβ mRNA measured by RT-PCR; ICAM-1 expression measured by flow cytometry; IFNβ, IL-6 and CCL5 protein levels measured by ELISA</td>
<td>RV-1B (minor), RV-16 (major)</td>
<td>Asthma</td>
<td>Primary bronchial epithelial cells</td>
<td>Human</td>
<td>ICAM-1 levels prior to RV infection similar in asthma and normal patients; RV induced potent CCL5 and IL-6 release and no significant differences between asthmatics and normal controls found; Viral RNA levels 50x higher in asthmatics than in normal controls; Asthmatics had impaired IFNβ production; Exposure to exogenous IFNβ significantly decreased viral titer in asthmatics</td>
<td>Wark et al. [396]</td>
</tr>
</tbody>
</table>
Appendix II. Effect of smoking status on the release and gene expression of cytokines and interferons
Table II-1 The effect of smoking status on cytokine release in response to poly(I:C) and R848 over time.

<table>
<thead>
<tr>
<th>Time course</th>
<th>Protein</th>
<th>Poly(I:C)</th>
<th>Current smokers (n=3)</th>
<th>Ex-smokers (n=10)</th>
<th>P value</th>
<th>R848</th>
<th>Current smokers (n=3)</th>
<th>Ex-smokers (n=10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1h</td>
<td>0.35 (0.10-0.78)</td>
<td>0.18 (0.18-0.44)</td>
<td>NS</td>
<td>0.82 (0.14-3.99)</td>
<td>4.05 (0.12-5.58)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.78 (1.77-13.73)</td>
<td>3.96 (1.77-10.07)</td>
<td>NS</td>
<td>409.97 (222.96-634.33)</td>
<td>406.10 (207.78-524.32)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.74 (3.51-150.53)</td>
<td>13.24 (8.53-20.37)</td>
<td>NS</td>
<td>322.92 (164.42-571.31)</td>
<td>318.46 (149.34-412.24)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.77 (3.34-129.70)</td>
<td>10.19 (5.77-26.08)</td>
<td>NS</td>
<td>157.06 (83.89-491.86)</td>
<td>168.02 (60.86-173.86)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1h</td>
<td>0.67 (0.27-3.09)</td>
<td>0.43 (0.35-0.79)</td>
<td>NS</td>
<td>0.60 (0.26-1.33)</td>
<td>0.43 (0.34-0.53)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.30 (1.28-4.56)</td>
<td>1.48 (0.92-1.57)</td>
<td>NS</td>
<td>3.37 (1.53-21.20)</td>
<td>2.53 (1.25-2.66)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22.06 (1.91-144.76)</td>
<td>19.43 (1.98-31.49)</td>
<td>NS</td>
<td>7.41 (1.22-17.07)</td>
<td>5.28 (0.88-12.77)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.55 (1.10-140.85)</td>
<td>4.67 (2.88-6.99)</td>
<td>NS</td>
<td>3.40 (1.52-11.42)</td>
<td>1.14 (0.67-4.21)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1h</td>
<td>373 (48-1000)</td>
<td>409 (167-570)</td>
<td>NS</td>
<td>259 (40-830)</td>
<td>386 (116-634)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2442 (341-4431)</td>
<td>3239 (578-3935)</td>
<td>NS</td>
<td>3229 (596-42396)</td>
<td>4928 (1396-5066)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14859 (5973-32970)</td>
<td>26736 (7184-37167)</td>
<td>NS</td>
<td>17360 (5012-30868)</td>
<td>20472 (8663-33566)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>31972 (16692-66101)</td>
<td>43055 (29314-47632)</td>
<td>NS</td>
<td>26610 (15026-40616)</td>
<td>25235 (19911-50134)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as pg/mg of tissue and presented as median (range). There were 3 technical replicates for each patient sample. Current and Ex-smokers were compared using Mann-Whitney tests for each condition.
Table II-2 The effect of smoking status on cytokine gene expression in response to poly(I:C) and R848 over time.

<table>
<thead>
<tr>
<th>Time course</th>
<th>mRNA</th>
<th>Ex-smokers (n=10)</th>
<th>Current smokers (n=3)</th>
<th>P value</th>
<th>Ex-smokers (n=10)</th>
<th>Current smokers (n=3)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poly(I:C)</td>
<td></td>
<td></td>
<td></td>
<td>R848</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>1h</td>
<td>1.14 (0.76-2.03)</td>
<td>1.29 (0.73-1.48)</td>
<td>NS</td>
<td>40.96 (13.69-109.52)</td>
<td>105.06 (21.86-157.04)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>6h</td>
<td>3.72 (2.43-10.45)</td>
<td>3.93 (3.04-4.21)</td>
<td>NS</td>
<td>17.63 (6.75-66.72)</td>
<td>17.15 (14.22-47.01)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>2.51 (1.29-21.19)</td>
<td>2.89 (1.29-6.57)</td>
<td>NS</td>
<td>2.76 (1.53-5.28)</td>
<td>4.64 (3.43-4.92)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48h</td>
<td>2.26 (0.77-13.74)</td>
<td>1.38 (1.19-3.96)</td>
<td>NS</td>
<td>1.78 (1.18-4.53)</td>
<td>1.48 (1.27-3.62)</td>
<td>NS</td>
</tr>
<tr>
<td>CCL5</td>
<td>1h</td>
<td>1.32 (0.70-2.46)</td>
<td>1.16 (0.63-1.45)</td>
<td>NS</td>
<td>1.44 (1.29-2.52)</td>
<td>1.26 (0.97-2.24)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>6h</td>
<td>5.85 (3.11-16.56)</td>
<td>8.22 (1.63-12.00)</td>
<td>NS</td>
<td>3.29 (1.68-8.20)</td>
<td>2.99 (1.19-4.90)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>14.58 (5.86-93.70)</td>
<td>22.24 (4.48-33.47)</td>
<td>NS</td>
<td>3.24 (1.45-4.35)</td>
<td>2.63 (1.72-2.83)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48h</td>
<td>9.41 (2.90-56.89)</td>
<td>6.43 (1.97-30.59)</td>
<td>NS</td>
<td>4.13 (1.17-8.49)</td>
<td>1.44 (1.06-4.59)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6</td>
<td>1h</td>
<td>1.57 (0.70-2.96)</td>
<td>1.15 (1.12-1.50)</td>
<td>NS</td>
<td>1.52 (0.53-3.58)</td>
<td>1.30 (0.60-2.02)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>6h</td>
<td>1.97 (1.08-8.34)</td>
<td>3.75 (2.44-4.77)</td>
<td>NS</td>
<td>2.79 (0.97-12.42)</td>
<td>4.01 (1.53-5.10)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>2.45 (0.74-10.37)</td>
<td>2.73 (1.93-5.74)</td>
<td>NS</td>
<td>2.37 (0.92-7.39)</td>
<td>2.70 (1.58-5.60)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48h</td>
<td>1.81 (0.65-9.95)</td>
<td>1.48 (1.27-2.97)</td>
<td>NS</td>
<td>3.04 (0.86-7.06)</td>
<td>2.06 (1.19-2.34)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as fold-change and presented as median (range). There was one replicate for each patient sample. Current and Ex-smokers were compared using Mann-Whitney tests for each time-point.
Table II-3 The effect of smoking status on IFN gene expression in response to poly(I:C) and R848 over time.

<table>
<thead>
<tr>
<th>Time</th>
<th>mRNA</th>
<th>Poly(I:C)</th>
<th>R848</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ex-smokers (n=10)</td>
<td>Current smokers (n=3)</td>
<td>Ex-smokers (n=10)</td>
<td>Current smokers (n=3)</td>
</tr>
<tr>
<td>1h</td>
<td>0.12 (0.21-4.76)</td>
<td>1.23 (1.21-4.76)</td>
<td>NS</td>
<td>2.00 (0.36-22.55)</td>
</tr>
<tr>
<td>6h</td>
<td>1.87 (0.51-4.59)</td>
<td>1.83 (0.39-2.32)</td>
<td>NS</td>
<td>0.98 (0.64-3.15)</td>
</tr>
<tr>
<td>24h</td>
<td>1.52 (0.30-12.00)</td>
<td>0.98 (0.92-2.53)</td>
<td>NS</td>
<td>1.00 (0.33-2.87)</td>
</tr>
<tr>
<td>48h</td>
<td>1.52 (0.34-3.51)</td>
<td>0.63 (0.51-0.72)</td>
<td>NS</td>
<td>1.72 (0.53-8.82)</td>
</tr>
</tbody>
</table>

IFNβ

<table>
<thead>
<tr>
<th>Time</th>
<th>mRNA</th>
<th>Poly(I:C)</th>
<th>R848</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h</td>
<td>1.25 (0.29-5.43)</td>
<td>0.60 (0.37-1.02)</td>
<td>NS</td>
<td>8.97 (5.43-39.12)</td>
</tr>
<tr>
<td>6h</td>
<td>29.40 (4.72-77.71)</td>
<td>13.09 (10.2-43.71)</td>
<td>NS</td>
<td>1.25 (0.64-2.55)</td>
</tr>
<tr>
<td>24h</td>
<td>13.95 (3.10-56.49)</td>
<td>7.41 (1.79-13.13)</td>
<td>NS</td>
<td>2.10 (1.38-5.98)</td>
</tr>
<tr>
<td>48h</td>
<td>6.05 (0.53-56.69)</td>
<td>2.20 (0.98-12.91)</td>
<td>NS</td>
<td>2.07 (0.66-5.88)</td>
</tr>
</tbody>
</table>

IFNλ

<table>
<thead>
<tr>
<th>Time</th>
<th>mRNA</th>
<th>Poly(I:C)</th>
<th>R848</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h</td>
<td>1.54 (0.81-2.35)</td>
<td>1.57 (0.54-2.17)</td>
<td>NS</td>
<td>1.15 (0.44-4.38)</td>
</tr>
<tr>
<td>6h</td>
<td>333.20 (44.02-701.84)</td>
<td>38.99 (18.19-376.11)</td>
<td>NS</td>
<td>1.36 (0.84-4.45)</td>
</tr>
<tr>
<td>24h</td>
<td>156.68 (22.16-1433.18)</td>
<td>91.14 (6.36-101.83)</td>
<td>NS</td>
<td>2.51 (0.91-10.27)</td>
</tr>
<tr>
<td>48h</td>
<td>72.41 (1.85-207.94)</td>
<td>19.56 (3.75-43.87)</td>
<td>NS</td>
<td>2.52 (1.04-15.03)</td>
</tr>
</tbody>
</table>

Data are expressed as fold-change and presented as median (range). There was one replicate for each patient sample. Current and Ex-smokers were compared using Mann-Whitney tests for each time-point.
Table II-4 The effect of smoking status on cytokine release in response to increasing doses of poly(I:C) and R848 (Smokers and COPD combined).

<table>
<thead>
<tr>
<th>Dose response</th>
<th>Protein</th>
<th>Combined COPD and Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyI:C</td>
<td>Ex-smokers (n=12)</td>
<td>Current smokers (n=14)</td>
</tr>
<tr>
<td>0.1</td>
<td>0.75 (0.28-2.12)</td>
<td>0.73 (0.13-1.39)</td>
</tr>
<tr>
<td>1</td>
<td>0.80 (0.40-2.39)</td>
<td>0.71 (0.18-3.83)</td>
</tr>
<tr>
<td>10</td>
<td>1.35 (0.66-19.49)</td>
<td>1.25 (0.31-43.57)</td>
</tr>
<tr>
<td>100</td>
<td>17.15 (3.51-84.24)</td>
<td>11.36 (2.34-169.97)</td>
</tr>
<tr>
<td>1000</td>
<td>59.29 (11.69-134.80)</td>
<td>23.95 (6.63-197.60)</td>
</tr>
<tr>
<td>CCL5</td>
<td>0.1</td>
<td>0.96 (0.20-3.00)</td>
</tr>
<tr>
<td>1</td>
<td>1.47 (0.17-3.54)</td>
<td>1.22 (0.31-4.67)</td>
</tr>
<tr>
<td>10</td>
<td>3.65 (0.71-16.94)</td>
<td>3.40 (0.52-45.85)</td>
</tr>
<tr>
<td>100</td>
<td>24.67 (1.91-144.75)</td>
<td>31.29 (6.51-156.90)</td>
</tr>
<tr>
<td>1000</td>
<td>79.58 (1.94-147.57)</td>
<td>62.46 (13.95-227.59)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.1</td>
<td>7506 ±1391</td>
</tr>
<tr>
<td>1</td>
<td>8951 ±1578</td>
<td>6539 ±1286</td>
</tr>
<tr>
<td>10</td>
<td>9267 ±1507</td>
<td>9207 ±1458</td>
</tr>
<tr>
<td>100</td>
<td>19216 ±3578</td>
<td>16914 ±2493</td>
</tr>
<tr>
<td>1000</td>
<td>20588 ±4087</td>
<td>18870 ±2493</td>
</tr>
</tbody>
</table>

Data are expressed as pg/mg of tissue and presented as median (range) or mean ± SEM. There were 3 technical replicates for each patient sample. Current and Ex-smokers were compared using Mann-Whitney tests (TNFα and CCL5) or Unpaired t-tests (IL-6) for each condition.
### Table II-5

The effect of smoking status on cytokine release in response to increasing doses of poly(I:C) and R848 from patients with COPD only.

<table>
<thead>
<tr>
<th>Dose response</th>
<th>Protein</th>
<th>COPD only</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyI:C</td>
<td>Ex-smokers (n=5)</td>
<td>Current smokers (n=8)</td>
</tr>
<tr>
<td>0.1</td>
<td>1.01 ± 0.32</td>
<td>0.77 ± 0.14</td>
</tr>
<tr>
<td>1</td>
<td>1.30 ± 0.36</td>
<td>1.39 ± 0.49</td>
</tr>
<tr>
<td>10</td>
<td>5.85 ± 3.57</td>
<td>12.90 ± 5.61</td>
</tr>
<tr>
<td>100</td>
<td>26.52 ± 14.53</td>
<td>48.41 ± 20.09</td>
</tr>
<tr>
<td>1000</td>
<td>36.61 ± 9.80</td>
<td>89.16 ± 23.93</td>
</tr>
</tbody>
</table>

| | CCL5 | | |
| 0.1 | 1.86 ± 0.35 | 1.70 ± 0.41 | NS | 0.01 | 1.88 ± 0.29 | 2.80 ± 1.01 | NS |
| 1 | 2.25 ± 0.49 | 2.19 ± 0.53 | NS | 0.1 | 4.65 ± 0.85 | 6.30 ± 2.13 | NS |
| 10 | 7.57 ± 2.68 | 13.80 ± 6.05 | NS | 1 | 11.98 ± 1.53 | 16.38 ± 5.21 | NS |
| 100 | 61.63 ± 25.89 | 60.16 ± 19.29 | NS | 10 | 12.21 ± 1.56 | 17.40 ± 5.33 | NS |
| 1000 | 90.63 ± 14.96 | 119.10 ± 27.07 | NS |

| | IL-6 | | |
| 0.1 | 8291 ± 2754 | 4836 ± 1278 | NS | 0.01 | 9319 ± 4472 | 7392 ± 1734 | NS |
| 1 | 8793 ± 3185 | 5531 ± 1763 | NS | 0.1 | 10362 ± 2481 | 13290 ± 2535 | NS |
| 10 | 9788 ± 2970 | 9688 ± 1869 | NS | 1 | 16756 ± 7381 | 14667 ± 2790 | NS |
| 100 | 14749 ± 6100 | 15023 ± 2829 | NS | 10 | 15503 ± 6056 | 14273 ± 2492 | NS |
| 1000 | 20447 ± 7774 | 19743 ± 3772 | NS |

Data are expressed as pg/mg of tissue and presented as mean ± SEM. There were 3 technical replicates for each patient sample. Current and Ex-smokers were compared using Unpaired t-tests for each condition.
Table II-6 The effect of smoking status on the cytokine release in response to poly(I:C) alone, R848 alone, or the combination in three subgroups: combined Smokers and COPD, Smokers only and COPD only.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Protein</th>
<th>Ex-smokers (n=11)</th>
<th>Current smokers (n=11)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF α</strong></td>
<td>PolyI:(C)</td>
<td>34.00 ±7.76</td>
<td>18.72 ±7.61</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R848</td>
<td>360.80 ±69.16</td>
<td>240.36 ±54.54</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Combination</td>
<td>595.72 ±114.43</td>
<td>349.51 ±82.34</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CCL5</strong></td>
<td>PolyI:(C)</td>
<td>53.07 ±2.56-105.27</td>
<td>25.29 ±7.78-328.02</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R848</td>
<td>7.29 ±0.2-14.82</td>
<td>7.90 ±0.88-33.93</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Combination</td>
<td>55.77 ±18.27-274.99</td>
<td>67.78 ±14.76-508.56</td>
<td>NS</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>PolyI:(C)</td>
<td>27948 ±4463</td>
<td>21204 ±3760</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R848</td>
<td>20967 ±4668</td>
<td>16177 ±9776</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Combination</td>
<td>29823 ±4929</td>
<td>25017 ±4158</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Combination</th>
<th>Protein</th>
<th>Ex-smokers (n=5)</th>
<th>Current smokers (n=5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF α</strong></td>
<td>PolyI:(C)</td>
<td>23.78 ±4.79</td>
<td>16.60 ±8.66</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R848</td>
<td>335.12 ±67.60</td>
<td>173.02 ±39.24</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Combination</td>
<td>592.58 ±118.02</td>
<td>230.38 ±44.40</td>
<td>0.021</td>
</tr>
<tr>
<td><strong>CCL5</strong></td>
<td>PolyI:(C)</td>
<td>8.92 ±2.56-105.27</td>
<td>16.58 (12.40-328.02)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R848</td>
<td>7.29 ±1.51-9.95</td>
<td>6.23 (0.89-18.43)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Combination</td>
<td>24.70 ±18.27-148.87</td>
<td>50.18 ±14.76-508.56</td>
<td>NS</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>PolyI:(C)</td>
<td>27130 ±5907</td>
<td>21123 ±6597</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R848</td>
<td>17281 ±8433</td>
<td>14588 ±5195</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Combination</td>
<td>26358 ±7880</td>
<td>24101 ±7975</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as pg/mg of tissue and presented as median (range) or mean ± SEM. There were 3 technical replicates for each patient sample. Current and Ex-smokers were compared using Mann-Whitney tests (TNFα and CCL5) or Unpaired t-tests (IL-6) for each condition.
Appendix III. Pilot results of poly(I:C)-, R848- and combination-induced activation of p38 MAPK and p65 in human lung tissue.
**Rationale**

In order to evaluate the effects of p38 MAPK and IKK-2 inhibitors in the whole tissue explant model (0) it was necessary to have some evidence that phosphorylation of p38 and p65 occurs in response to TLR ligands.

**Methods**

*Tissue culture*

Whole tissue explants for protein extraction was prepared as before (see section 2.2). However, instead of adding 800μl of fresh medium for overnight incubation, only 784μl were added. The following day, 16μl of 50x poly(I:C), R848 or both were added to the experimental wells while 16μl of fresh medium were added to the control wells. Four time points were chosen: 5, 30, 60 and 120 minutes after the addition of TLR ligand. Additional unstimulated control at time 0 was also included. After the appropriate incubation at 37°C, 5% (v/v) CO₂, the old medium was quickly removed, and, using a pipette tip, tissue was placed on labelled piece of aluminium foil which was then folded and put straight into liquid nitrogen. Tissue in foil was then stored at -80°C for subsequent protein extraction. The reason for not removing media from overnight incubation was to prevent any unwanted protein activation that could be attributed to mechanical activation due to media change rather than activation of TLRs by their agonists.

*Protein extraction*

Proteins were extracted from whole tissue explants as described before [1]. Briefly, tissue explants were placed in a tube and immersed with 1.8ml of ice-cold RIPA buffer (10mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Neonidet P-40) containing phosphatase (Calbiochem, Nottinghamshire, UK) and protease inhibitors (Sigma Aldrich, Dorset, UK) at 1:100 dilution. Tissue was then quickly homogenised on ice using a rotor-stator homogeniser,
then kept in constant agitation for 2 hours at 4°C. Samples were then centrifuged at 12000rpm for 20 mins at 4°C. Subsequently, the supernatant was transferred to a fresh tube and stored at -20°C (15 µl were left to perform Bradford assay – see next section).

**Bradford assay**

Protein concentrations were determined using Bradford reagent (Sigma Aldrich, Dorset, UK) with the use of bovine serum albumin (BSA) (Sigma Aldrich Dorset, UK) as standard. Serial dilutions of BSA in PBS (Sigma Aldrich, Dorset, UK) ranged from 10 to 0.3125 mg/ml. To each well on a 96-well flat-bottomed plate 5 µl of standards (in duplicate) and samples (in triplicate) were added. Then 300 µl of Bradford reagent were added to the wells, mixed, and left to incubate at room temperature for 20 min. Subsequently, optical density at 600 nm wavelength was measured using OPTIMA Plate Reader (BMG Labtech, Aylesbury, UK) and protein concentration was calculated.

**Polyacrylamide gel electrophoresis**

Protein samples were diluted 1:3 in sample buffer (62.5 mM Tris, 10% glycerol, 1% SDS, 1% beta-mercaptoethanol, 0.1% bromphenol blue, pH 6.8). Samples were then boiled for 10 min. At the same time, stacking and separating gels were prepared (stacking gel: 10 mL H₂O, 3.75 mL buffer A (0.53 M Trizma base, 0.01 M SDS, pH 6.8), 1.13 mL acrylamide bis solution, 150 µl 10% APS in H₂O and 15 µl TEMED; separating gel: 18 mL H₂O, 9.375 mL buffer B (1.56 M Trizma base, 0.01 M SDS, pH 8.8), 9.375 mL acrylamide bis solution, 375 µl 10% APS in H₂O, 37.5 µl TEMED; all reagents were bought from Sigma Aldrich (Dorset, UK)). Samples were then loaded onto the gels using the volume containing exactly 20 µg of protein. Once the proteins were separated, they were transferred to Hy-bond ECL membranes (Whatman, Kent, UK). After the transfer, the membrane was immersed in blocking buffer (5% milk in Tris-buffered saline with Tween (TBSt)) for 1 h at room temperature on a shaker. Then, the membrane was incubated with 1:1000 dilution of primary antibody (phospho-p38 MAPK and
phospho-p65, both from Cell Signalling (Bioscience, Cambridge, UK), and beta-actin (Abcam, Cambridgeshire, UK)) in blocking buffer overnight at 4°C on a shaker. The following day, membranes were washed in TBSt and incubated with secondary antibody conjugated with peroxide for 1h at room temperature on a shaker. Then the membranes were washed again and incubated in lumigen TMA-6 at room temperature for 5min. Membranes were then visualised using Bio-Rad ChemiDoc System (Hertfordshire, UK).

Membranes were then stripped by immersing them in stripping buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 10mM 2-beta-mercaptoethanol) for 40min at 60°C. Membranes were then washed in H2O and the protocol continued as above, i.e. membrane was blocked and incubated with a different primary antibody.

Results

Pilot results showed that both p38 and p65 were activated by poly(I:C), R848 and combination. Phosphorylated p38 was visible in the blots from early time points and remained activated up to 120min (Figure III-1, A). Phosphorylated p65, however, was visible early and then gradually decreased (Figure III-1, B). The blots in Figure III-1 A and B had no beta-actin loading control. This is due to the fact that, at the time, the research group had technical difficulties with beta-actin antibody. However, to show that loading was equal in each well, an earlier Western blot is included in the Figure III-1 C. This blot shows an initial attempt to visualise phosphorylated p38 in the whole tissue explant (left) and the control beta-actin loading control (right). Samples in A and B were loaded in exactly the same way as samples in C which suggests that the results in A and B are accurate and not distorted by protein loading differences.

Conclusion

Having established that p38 and p65 are activated in the whole tissue explants by TLR ligands, it was justified to proceed to experiments that aimed to inhibit activation of p38 and p65 (see Chapter 4).
**A Activation of p38 by TLR ligands**

Time (mins):

```
--0-- 5 30 60 120
```

Condition

20μg of protein loaded to each well (as measured by Bradford assay); beta-actin control for these blots unavailable.

U=unstimulated; P=Poly(I:C) 100μg/ml; R=R848 10μg/ml; C=Combination

**B Activation of p65 by TLR ligands**

Time (mins):

```
--0-- 5 30 60 120
```

Condition

20μg of protein loaded to each well (as measured by Bradford assay); beta-actin control for these blots unavailable.

U=unstimulated; P=Poly(I:C) 100μg/ml; R=R848 10μg/ml; C=Combination

**C Beta-actin control on previous Western blot:**

P-p38 (20μg protein loaded as measured by Bradford assay)  Beta-actin loading control

---

Figure III-1 Effect of TLR ligands on p38 and p65 activation in the human lung tissue.

The effect of poly(I:C), R848 and combination on activation of p38 (A) and p65 (B) and the accuracy of loading control (C).
Appendix IV. Haematoxylin and eosin stain of human lung tissue explants.
**Rationale**

To visualise the types of cells present in the whole tissue explants.

**Methods**

Several 30mg WTE samples were taken at the time of tissue processing, after overnight incubation and after 48h in tissue culture with medium only. The samples were fixed in formalin and embedded in paraffin.

Tissue sections were dewaxed in xylene 2 times for 5min. Then the slides were put in alcohol solutions: (1) 100% for 3min, (2) 90% for 3min, (3) 75% for 2min and (4) 50% for 1min. The slides were then immersed in Mayer’s Haematoxylin (Sigma Aldrich, Dorset, UK) for 15min. Sections were then washed in tap water for 10min. Tissue was then immersed in eosin for 2min and washed again in tap water for 5min. After that tissue was put again in alcohol solutions: (1) 50% for 1min, (2) 75% for 2min, (3) 90% for 3min and (4) 100% for 3min. Slides were then placed in xylene for 5min (2x) and finally were mounted with DPX (Sigma Aldrich, Dorset, UK). Slides were stored at room temperature.

**Results**

Samples from the same donor (ex-smoker without COPD) are shown in Figure IV-1. The pictures show a variety of cells present in the WTE samples, including bronchial and alveolar epithelial cells, smooth muscle cells, macrophages and lymphocytes.

**Conclusion**

From the slides it can be concluded that there is a variety of cells present in the samples which reflect the variety in the human lung. In addition, cells look viable in the first two stages however, they start to look less healthy after 48h in culture.
Figure IV-1 WTE samples stained with haematoxylin and eosin.

All samples are from the same donor (ex-smoker without COPD). Tissue is shown at 100x (A,C,E) and 200x magnification (B,D,F). Samples were fixed just after tissue processing (A,B), at the time of stimulation (after overnight incubation) (C,D), and after 48h in tissue culture in medium only (E,F).
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