Candidate role for TLR3 L412F in disease progression in Idiopathic Pulmonary Fibrosis (IPF) during bacterial and viral infection

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I. Declaration of Authorship

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University’s open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.
II. Acknowledgements

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I would like to thank my family. I am so lucky to have such an amazing immediate and extended family. I am so grateful to my mum, Deirdre, my dad, Des and my brother, Conor. Without their unending love and encouragement, none of this would have been possible.

Finally, a special word of thanks to my parents. I can’t express my gratitude for their love and support throughout my life. They have always instilled in my brother and I a love of learning and the self-confidence to take every opportunity that came our way. I hope I have made them proud. I dedicate my thesis to them.
III. Abstract

In this thesis, we investigated the mechanisms by which TLR3 Leu412Phe (L412F) promotes disease progression in idiopathic pulmonary fibrosis (IPF) patients. IPF is a fatal interstitial lung disease of unknown aetiology and currently no cure or specific treatments are available for IPF patients. To date, lung transplant remains the best clinical option for IPF patients. Historically, viral and bacterial infections have been associated with accelerated disease progression and poorer outcomes in patients.

Our research group previously established that TLR3 L412F increased mortality and accelerated lung decline in IPF patients. These findings were associated with a reduced ability of lung fibroblasts from TLR3 L412F-variant IPF patients to induce pro-inflammatory or anti-viral, type I interferon responses to TLR3-activation in cells. In this thesis, we expand on these original findings. Here, we demonstrated that TLR3 L412F-heterozygous lung fibroblasts from IPF patients have attenuated responses to additional TLR agonists, including: LPS (TLR4), Pam3CSK4 (TLR2), flagellin (TLR5), FSL-1 (TLR6) and CpG (TLR9). Furthermore, we demonstrated that variant-TLR3 L412F attenuates responses of IPF lung fibroblasts to non-TLR agonists including: Poly(dA:dT), HT-DNA and PMA. These effects may increase TLR3 L412F-heterozygous IPF patients susceptibility to viral and bacterial infections.

We additionally investigated the role of autophagy in viral clearance in primary lung fibroblasts from IPF and pulmonary sarcoidosis patients. We demonstrated that TLR3 L412F-homozygous sarcoidosis patients have increased transcription of autophagy proteins following IAV H1N1 viral infection. Furthermore, lung fibroblasts from TLR3 L412F-heterozygous IPF patients exhibited increased transcription of autophagy proteins in response to nutrient-starvation. These effects correlated with defective induction of autophagy in IPF fibroblasts following TLR3- and TLR4-activation, and rapamycin treatment, respectively. We also observed persistent mTOR activation in TLR3 L412F-heterozygous IPF fibroblasts, which may play a role in dysregulated autophagy in IPF patients.

In future studies, we will model these TLR3 L412F-mediated effects in vivo using our novel TLR3 L413F knock-in mice, which we have characterised at a
cellular level in this thesis. We believe that our current data provides supportive evidence for the potential use of $TLR3$ L412F as a biomarker in IPF patients.
IV. Awards and Published Abstracts

Best Poster Award Nomination (One of 110 selected posters from 3,151 delegates)

European Congress of Immunology 2018
Toll-like receptor 3 (TLR3) L412F differentially modulates TLR1-9 and non-TLR responses in idiopathic pulmonary fibrosis (IPF) patients: implications for accelerated disease progression.

Published Abstracts

Irish Thoracic Society Scientific Meeting 2017
Toll-like receptor 3 (TLR3) L412F as a candidate biomarker in Idiopathic Pulmonary Fibrosis (IPF) and its role in antibacterial and antiviral responses.

Irish Thoracic Society Scientific Meeting 2016
Toll-like receptor 3 (TLR3) L412F single nucleotide polymorphism as a causative factor in disease progression in idiopathic pulmonary fibrosis (IPF) during bacterial infection in TLR3-defective patients
V. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>4E-BPs</td>
<td>eif4e-binding proteins</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AE</td>
<td>Acute exacebation</td>
</tr>
<tr>
<td>AIP</td>
<td>Acute interstitial pneumonia</td>
</tr>
<tr>
<td>AKAP13</td>
<td>A-kinase anchoring protein 13</td>
</tr>
<tr>
<td>AMD</td>
<td>Age–related macular degeneration</td>
</tr>
<tr>
<td>API</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>Atg</td>
<td>Autophagy related gene</td>
</tr>
<tr>
<td>Atg-5</td>
<td>Autophagy protein 5</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>COP</td>
<td>Cryptogenic organising pneumonia</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CpG ODN</td>
<td>Cpg oligonucleotides</td>
</tr>
<tr>
<td>CRISPR-Cas9</td>
<td>Clustered regularly interspaced short palindromic repeats-CRISPR-associated 9</td>
</tr>
<tr>
<td>DAD</td>
<td>Diffuse alveolar damage</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>DLCO</td>
<td>Diffusing capacity for carbon monoxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic translation initiation factor 2α</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSL-1</td>
<td>Pam2CGDPKHPKS</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
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</table>
gDNA  Genomic DNA
GER  Gastroesophageal reflux
GM-CSF  Granulocyte-macrophage colony-stimulating factor
GSK  Glaxosmithkline
H2RA  Histamine-2 blocker receptor antagonists
HBSS  Hanks’ balanced salt solution
HBV  Hepatitis B virus
HCC  Hepatocellular carcinoma
HET  Heterozygous
HI  Heat-inactivated
HIV  Human immunodeficiency virus
HKLM  Heat Killed Listeria monocytogenes
HOM  Homozygous
HRCT  High-resolution computed tomography
HSV-1  Herpes simplex virus-1
HSV-2  Herpes simplex virus type 2
i.p.  Intra-peritoneal
i.t.  Intra-tracheal
i.v.  Intra-venous
IAV  Influenza A virus
IFN-α  Interferon-α
IFN-β  Interferon-β
IIP  Idiopathic interstitial pneumonia
IL-6  Interleukin-6
IL-8  Interleukin-8
ILD  Interstitial lung disease
IPF  Idiopathic pulmonary fibrosis
IRAKs  IL-1 receptor-associated kinases
IRF3  Interferon regulatory transcription factor 3
IRF7  Interferon regulatory transcription factor 7
IRS1  Insulin receptor substrate 1
ISGs  Interferon-stimulated genes
KC  Also referred to as CXCL1
LAM  Lymphangioleiomyomatosis
LC3  Light chain 3B
LDH  Lactate dehydrogenase
L-Glu  L-glutamine
LPG2  Laboratory of genetics and physiology 2 and a homolog of mouse d11lgp2
LPS  Lipopolysaccharide
LRR  Leucine-rich repeat
MAL  Myd88-adaptor like
MAVS  Mitochondrial antiviral signalling
M-CSF  Macrophage colony-stimulating factor
MDA5  Melanoma Differentiation-Associated protein 5
MEM  Minimum essential medium
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein 2-alpha</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
<tr>
<td>NSIP</td>
<td>Non-specific interstitial pneumonia</td>
</tr>
<tr>
<td>PAMCYS</td>
<td>Pam2CSK4</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PAS</td>
<td>Phagophore assembly site</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>Pen/strep</td>
<td>Penicillin/ streptomycin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase RNA-activated</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>Poly(dA:dT)</td>
<td>Poly(deoxyadenylic-deoxythymidylic) acid</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein pump inhibitor</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T Cell Expressed and Secreted</td>
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<td>RIG-I</td>
<td>Retinoic acid-inducible gene I</td>
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<td>RIP-1</td>
<td>Receptor interacting protein 1</td>
</tr>
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<td>RIPA</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
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<td>RLR</td>
<td>Rig-I like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>Rpm</td>
<td>Revolutions per minute</td>
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<td>RPMI media</td>
<td>Roswell Park Memorial Institute media</td>
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<td>RT</td>
<td>Room temperature</td>
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<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile α- and armadillo- motif containing protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP-A</td>
<td>Surfactant protein A</td>
</tr>
<tr>
<td>SP-D</td>
<td>Surfactant protein D</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tick borne encephalitis</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TERC</td>
<td>RNA component of telomerase</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor – beta</td>
</tr>
<tr>
<td>TICAM 1</td>
<td>TIR domain-containing adapter molecule 1 (also known as TRIF)</td>
</tr>
<tr>
<td>TICAM 2</td>
<td>TIR domain-containing adapter molecule 2 (also known as TRAM)</td>
</tr>
<tr>
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<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R</td>
</tr>
<tr>
<td>TIRAP</td>
<td>Tir domain containing adaptor protein (also known as mal)</td>
</tr>
<tr>
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<td>Toll-like receptor</td>
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<td>Toll-like receptor (1-9)</td>
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<tr>
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<td>TNF receptor associated factor 6</td>
</tr>
<tr>
<td>TRAF 6</td>
<td>TNF receptor associated factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor-inducing interferon-β</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>TSC1/TSC2</td>
<td>Tuberous sclerosis complex 1/2</td>
</tr>
<tr>
<td>UIP</td>
<td>Usual interstitial pneumonia</td>
</tr>
<tr>
<td>ULK-1</td>
<td>Unc-51 like autophagy activating kinase 1</td>
</tr>
<tr>
<td>UVRAG</td>
<td>UV irradiation resistance-associated gene</td>
</tr>
<tr>
<td>VATS</td>
<td>Video-assisted thoracic surgery</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
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<td>Wild-type</td>
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<tr>
<td>β-actin</td>
<td>Beta-actin</td>
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</table>
Chapter 1
Introduction
Chapter 1 Introduction

1.1 Interstitial Lung Disease

1.1.1 Pulmonary Fibrosis

Fibrosis occurs in many different organs and is defined as dysregulation of the wound healing process resulting in physical deformation and loss of organ function (1). There are a number of effector cells responsible for fibrosis: primarily fibroblasts and myofibroblasts which secrete extracellular matrix (ECM) proteins upon activation but also fibrocytes, epithelial cells, immune cells and bone marrow derived cells (1). The normal wound healing process induced by acute inflammation results in the deposition of collagens and ECM proteins which promotes tissue repair. This process is regulated by degradation of collagen and ECM proteins by enzymes known as matrix metalloproteinases (MMPs). During chronic inflammation, constitutively activated effector cells continuously deposit ECM proteins at a rate which cannot be degraded by MMPs, leading to excessive deposition and scarring. Many molecular pathways have also been implicated in fibrosis. Most notably, the TGF-β pathway has been shown to play a major role in fibrosis (2).

Idiopathic pulmonary fibrosis (IPF) is classified under a group of diseases known as Idiopathic interstitial pneumonias (IIPs). Some examples of other members of this group include non-specific interstitial pneumonia (NSIP), cryptogenic organising pneumonia (COP) and acute interstitial pneumonia (AIP) (3). All diseases in this group affect the alveolar epithelium, capillary endothelium and the septal and bronchovascular tissues. Due to inflammation and fibrosis, destruction is seen of the airways, alveolar spaces and surrounding vasculature. Clinically, IIPs are diagnosed by high resolution computed tomography (HRCT), clinical presentation, surgical lung biopsy and histopathology. Many IIPs share similar symptoms in patients (3). Figure 1.1 subdivides members of the interstitial lung disease (ILD) family of diseases. IPF is a well-defined member of the IIPs (4).
Figure 1.1. Subsets of ILD

ILDs can be differentiated into a number of different subsets; lung disease due to a known cause; idiopathic interstitial pneumonia, granulomatous disease and others. IPF is a well-defined member of the idiopathic interstitial pneumonia (IIP) group. Image taken from (4).
1.2 Idiopathic Pulmonary Fibrosis (IPF)

1.2.1 Introduction

IPF is a chronic, progressive fibrosing interstitial pneumonia of unknown etiology with a mean survival rate of less than 3 years. IPF was previously known as cryptogenic fibrosing alveolitis. It is characterised by the histological appearance of usual interstitial pneumonias (UIP) and most commonly seen in older adults (5). It is hypothesised to be a result of aberrant wound healing in the lung, following an unknown injury, in genetically susceptible individuals. In IPF, alveolar epithelial injury drives fibroproliferation, myofibroblast differentiation and excessive collagen and extracellular matrix deposition, leading to impairment of gas exchange, respiratory failure and death (6). The prevalence of IPF is estimated at up to 29 cases per 100,000 individuals, with the incidence and associated mortality currently increasing (5).

1.2.2 Clinical manifestations of IPF in patients

Patients with IPF present with chronic exertional dyspnoea (breathlessness), fine basal late inspiratory crackles, finger clubbing and cough (3). Approximately 5% of patients are asymptomatic. It is rare for IPF to present in adults younger than 50 years old and the median age of presentation is 66 years old (3). IPF is slightly more common in males (5). IPF leads to pulmonary hypertension, right-sided heart failure and eventual respiratory failure.
Figure 1.2. HRCT imaging of IPF patient.

Image A and B are taken using HRCT demonstrating usual interstitial pneumonia (UIP). Images A and B highlight a classical feature of IPF, honeycombing. Image taken from (5).

1.2.3 Possible causes of IPF

Although the cause of IPF remains unknown there are several possible risk factors that have been associated with the condition. These include cigarette smoking, genetic susceptibility, gastroesophageal reflux and environmental exposures (7).

The majority of patients with IPF have a history of cigarette smoking (5). Baumgartener et al. undertook a study in 1997 and showed that a history of smoking increased the risk of development of IPF. Importantly, they found that the time since cessation was also a factor in the development of IPF, with the patients who had most recently quit being at highest risk (8).
Gastroesophageal reflux (GER) is common in IPF patients and may be a risk factor for the development of IPF (5, 9, 10). Gastroesophageal reflux disease (GERD) is a very common chronic disease. It is caused by the reflux or regurgitation of stomach acid which can lead to a number of symptoms. Typical symptoms of GERD include heartburn and regurgitation but GERD can also cause symptoms such as coughing, asthma, hoarseness and chest pain due to the microaspiration of stomach acid into the lungs (11). A high prevalence of abnormal gastroesophageal reflux has been found in IPF patients in a number of separate studies (10, 12, 13). Studies have been conducted into the use of anti-acid treatment in IPF patients. A small study in 2006 by Raghu et al. found that pulmonary function tests (PFTs) in 4 patients stabilised or improved with anti-acid GER therapy (14). Later in 2011 a larger study was undertaken with 242 IPF patients, 124 patients received anti-acid treatment, proton pump inhibitors (PPIs) or histamine-2 blocker receptor antagonists (H2RAs). At 30 weeks it was found that patients receiving anti-acid treatment had a significantly smaller decrease in forced vital capacity (FVC) from baseline than patients receiving the placebo (15). Taken together these studies suggest that the use of anti-acid treatment may decrease the risk of lung injury induced by microaspiration however, further clinical trials are needed to investigate the benefits of anti-acid treatment in IPF patients.

Environmental exposures such as wood and metal dust have been suggested as risk factors for the development of IPF (16, 17). A review of six different case controlled studies have found that smoking, agriculture/farming, livestock, wood dust, metal dust and stone/sand were all significantly associated with increased risk of IPF (18). Interestingly, a recent study looked at the effect of air pollution in disease severity in 25 IPF patients. Patients performed spirometry tests every day for up to 40 weeks and the air pollution levels in their local areas were monitored. It was found that high levels of air pollution were associated with lower lung function but air pollution did not change lung function (19).

Aging has also been suggested to be a factor in the development of IPF (20). Aging is a process in which cells over time lose functionality and capacity to respond and have increased invulnerability to death (21). Exposure over a lifetime to various environmental and occupational risk factors, epigenetic changes and habits such as smoking all are thought to contribute to the aging process. At a cellular level, Lopez-
Otin et al. characterised aging by a number of different hallmarks including: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (21). The fact that the median age of IPF patients at the time of diagnosis is approximately 60 years of age, and IPF is not seen in young people, suggests the aging process is a detrimental factor in the development of fibrosis (20, 22, 23). It has been shown that a number of these hallmarks are present in IPF (24). For example, dysregulation of autophagy in IPF, an essential cellular process, which will be discussed in Section 1.7. Linked with dysregulation of autophagy is cellular senescence. Cellular senescence has been implicated the pathogenesis of IPF (25). In the bleomycin model of pulmonary fibrosis it has been shown that bleomycin injury can induce cellular senescence in fibroblasts and epithelial cells in mice (25). Cellular senescence is characterised by cell cycle arrest, resistance to apoptosis, and secretion of senescence-associated mediators which may play a role in the development of fibrosis (26). Romero et al. has studied the effects of aging in the IPF lung. They found that aging significantly decreased autophagy activity in both old normal lung fibroblasts and IPF fibroblasts compared with young normal lung fibroblasts. This decreased autophagy also correlated with increasing resistance to apoptosis in IPF fibroblasts (27). Another example is shortened telomeres which have been associated with increased risk of developing IPF (28). The gene PINK-1 (PTEN-induced putative kinase 1) has been associated with the aging cell and IPF. Deficiency in PINK-1 leads to dysfunctional mitochondria in the alveolar epithelial cells of aging and IPF lungs and promotes fibrosis (29).

1.2.4 Disease progression and acute exacerbations in IPF

As previously mentioned, the mean survival time for IPF patients from diagnosis is approximately 3 years (9). There are three major disease progression pathways patients can experience following diagnosis. Disease progression in the majority of patients is a slow gradual loss of long function over time. However, some patients experience much more rapid disease progression and loss of lung function which leads to a life expectancy of less than 1 year. Another group of patients can initially experience slow, steady disease progression but, due to an
adverse event known as an acute exacerbation, will experience an accelerated decline (5). Figure 1.3 describes these disease progression pathways. Currently, there is no method to stratify the IPF patients into slow and rapid progressors in clinics. The identification of prognostic markers would be hugely beneficial to separate patients to tailor each patient’s treatment plan to be most effective.

An acute exacerbation (AE) in IPF can occur at any stage in disease progression including at diagnosis. An AE is characterised by the development of diffuse alveolar damage (DAD) (30). Clinically, an AE has been defined as an acute worsening of dyspnoea (shortness of breath) within 1 month, new bilateral ground-glass opacity or consolidation seen by scans using high-resolution computed tomography (HRCT) as well as signs of hypoxemia due to worsened gaseous exchange due to an unexplained cause (30). Patients also experience worsened coughing, fever and/or increased sputum (5). To date, there have been a number of causes suggested for the onset of an acute exacerbation. These include procedures such as a bronchoalveolar lavage or conditions such as pneumonia, pneumothorax and cardiac failure. The role of viral and bacterial infections in AE in IPF will be discussed in Section 1.3.
Figure 1.3. Disease progression pathways in IPF

Disease progression in the majority of patients is a slow gradual loss of lung function over time. However, some patients experience much more rapid disease progression and loss of lung function which leads to a life expectancy of less than 1 year. Image taken from (9).

1.2.5 Treatments available for the treatment of IPF

There are currently two FDA- and EMA - approved drugs available for the treatment of IPF; pirfenidone and nintedanib. FDA approved both drugs for use in the treatment of IPF in 2014. The EMA approved the use of pirfenidone in 2011 and nintedanib in 2015.

1.2.5.1 Pirfenidone

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is an oral therapy used in the treatment of IPF. The promising anti-fibrotic effects of pirfenidone have been shown \textit{in vitro} and in animal models of fibrosis, in particular bleomycin induced pulmonary fibrosis. Bleomycin is the gold standard method for inducing fibrosis in mice. A hamster bleomycin model of fibrosis showed that there was significantly reduced influx of inflammatory cells and macrophages into the lungs at day 7 of
bleomycin challenge in mice given a diet supplemented with pirfenidone compared with a control diet (31). Interestingly, significantly reduced levels of TGF-β were produced in the lungs of hamsters treated with pirfenidone compared with bleomycin alone (31). In the bleomycin model of murine fibrosis, it was found that administration of pirfenidone on day 28 of bleomycin treatment significantly reduced the collagen content seen in the lungs compared with bleomycin treatment alone (32). It has also been shown that pirfenidone treatment significantly reduces the number of circulating fibrocytes in lung tissue of mice treated with bleomycin (32). This is very important as fibrocytes are contributors to lung fibrosis. Fibrocytes are circulating cells in the peripheral blood which secrete a number of different factors which can lead to fibrosis (33). These include collagens I and III, matrix metalloproteinases and other pro-inflammatory cytokines, chemokines and growth factors important for the development of fibrosis (33). There have been three major clinical trials conducted confirming the beneficial effect of this drug in humans.

**Japanese Trial**

The first of the trials in pirfenidone was published in 2010 by Taniguichi et al. 275 Japanese IPF patients were recruited for the phase III trial and were given either high dose pirfenidone, low dose pirfenidone or placebo. The trial lasted 52 weeks and it was found that patients receiving both high and low dose pirfenidone had a significant reduction in the decline in FVC compared with the placebo cohort. It was also shown that patients treated with a high dose of pirfenidone had a significant increase in progression free survival time (34). This was the first human clinical trial evidence to show benefits of pirfenidone as a treatment for IPF.

**CAPACITY Trials**

The second trial to be carried out was a two study multi-national, placebo controlled, double blind Phase 3 clinic trial known as the CAPACITY trials, study 004 and 006 (ClinicalTrials.gov, numbers NCT00287729 and NCT00287716) which was completed in 2008 (35). Study 004 recruited 435 patients and Study 006 recruited 344 patients from Australia, Europe and North America. The trial was conducted for a total of 72 weeks and the patients were assessed at regular intervals throughout. Study 004 consisted of three treatment groups; patients receiving 2403 mg/day pirfenidone, 1197 mg/day pirfenidone or the placebo. Interestingly, at the end of week 72 it was found that patients receiving 2403 mg/day of pirfenidone had
significantly reduced decline in FVC (p= 0.001) compared with the placebo cohort. The mean FVC change in the 2403 mg/day pirfenidone group was -8% while in the placebo cohort it was -12.4% (35). Another important clinical marker is decline of over 10% FVC in IPF patients as it is a predictor of mortality. At week 72, 30 of 174 patients given 2403 mg/day pirfenidone had a decline of more than 10% FVC compared 60 of 174 in the placebo group. It was found that there was significant treatment effect by week 24 which persisted for the duration of the trial. It was shown that treatment with 2403 mg/day of pirfenidone prolonged progression free survival and led to a 36% reduction in the risk of disease progression or death.

In contrast, Study 006 of the CAPACITY trials showed different results. Patients were assigned to one of two groups; 2403 mg/day pirfenidone (171 patients) and placebo (173 patients). At week 72, no significant difference was found in the change in % predicted FVC unlike in Study 004. However, there was a significant treatment effect seen up to week 48. Interestingly, in Study 006 (and not in Study 004) there was a significant reduction in decline in the 6 minute walk test (6MWT) in the 2403 mg/day pirfenidone group compared with the placebo group. The 6MWT was one of several secondary endpoints assessed in the trial. In both studies no significant differences were seen in pulmonary function tests such as dyspnoea (shortness of breath), % predicted DLCO, or worse SpO2 during the 6MWT. Adverse effects were seen in both treatment groups but were more common in the pirfenidone group. Symptoms included nausea, dizziness and rashes most commonly and were mild or moderate in severity with little to no clinical significance.

Following both studies, the results were pooled and overall it was shown that at week 72 treatment with 2403 mg/day of pirfenidone reduced decline in % predicted FVC compared with the placebo. Pirfenidone prolonged progression free survival by 26% compared with the placebo and a 31% difference was seen in the 6MWT between groups. Hazard ratio analysis was also performed in the pooled populations and it was shown that hazard ratios for overall all-cause mortality and mortality related to IPF favoured those treated with pirfenidone.

**ASCEND Trial**

While the results of the CAPACITY trial were very promising, due to the contrasting results in the primary endpoint of the trial (change in % predicted FVC)
in studies 004 and 006, US regulatory bodies would not approve the use of pirfenidone for the treatment of IPF without a final additional trial (36). This trial was known as the ASCEND trial (ClinicalTrials.gov NCT01366209) which was completed in 2014 (36). This phase 3 clinical trial recruited 555 patients in 127 sites from 9 countries. The majority of patients were white males aged 65 years or older and were split into two treatment groups; those receiving the placebo (277 patients) or 2403 mg/day pirfenidone (278 patients) over the course of 52 weeks. As before, the primary endpoint for this trial was change in FVC and other pulmonary function tests such as the 6MWT, progression free survival, dyspnoea and all-cause mortality were assessed. Pirfenidone was found to reduce the percentage of patients who had a decline in FVC of more than 10% at the end of the trial. It was found again that treatment with pirfenidone prolonged progression free survival and reduced decline in the 6MWT. Mortality outcome assessment showed that all-cause mortality showed fewer deaths in the pirfenidone compared with the placebo cohort. Pirfenidone was approved by the FDA in 2014 for the treatment of IPF.

1.2.5.2 Nintedanib and the INPULSIS Trials

The second treatment option available to IPF patients is nintedanib. Nintedanib, previously known as BIBF 1120, is a protein kinase inhibitor which acts intracellulary on a number of protein kinases. The development of fibrosis is dependent on a number of downstream signalling pathways that lead to the expression of pro-fibrotic growth factors and cytokines such as VEGF, PDGF and FGF. Nintedanib works by blocking tyrosine kinase receptors for these growth factors (37, 38). It has been shown in human lung fibroblasts that treatment with nintedanib inhibited TGF-β-stimulated fibroblast differentiation to myofibroblasts in vitro (38). Nintedanib has been tested in animal models of fibrosis, both the bleomycin model and using silica particles to induce fibrosis. In the bleomycin model of fibrosis, it was shown that mice treated with nintedanib showed a reduction in collagen production, inflammation and fibrosis in the lungs compared with bleomycin–only treated animals (38). Similar results were seen in animals with silica-induced lung fibrosis (38).

Following the promising results seen in in vitro studies and in animal models, human clinical trials were undertaken to assess the benefits of nintedanib in the
treatment of IPF. The INPULSIS clinical trials (INPULSIS-1 and INPULSIS-2 ClinicalTrials.gov numbers, NCT01335464 and NCT01335477) were phase III clinical trials conducted to assess the efficacy of 150 mg of nintedanib administered twice daily (39). 1066 patients were split into two separate trials assessed over a 52 week period. As in the clinical trials for pirfenidone, the primary endpoint was a change in FVC. The majority of the IPF patients were males over the age of 66. In both trials it was shown that the rate of decline in change of FVC was significantly reduced in patients given nintedanib compared with the placebo. It was also shown that there was a significantly greater number of patients in the placebo cohort who had a decline of more than 5 or 10% points in % predicted FVC compared with nintedanib group. Acute exacerbations (AEs) were also monitored in this study. It was found that there was no difference in time to first AE or the proportion of patients experiencing acute exacerbations between the nintedanib and placebo cohorts in INPULSIS-1, however in INPULSIS-2 there was a significant increase in time to first AE or the proportion of patients experiencing AEs. However when the data from both trials was pooled for analysis, it could not be determined whether nintedanib had an effect on AE in IPF. Due to the reduced decline in FVC seen in IPF patients given nintedanib treatment, it was concluded that the drug did slow progression of the disease and in 2014 was approved by the FDA for treatment of IPF.
### Table 1.1: Successful Clinical Trials

<table>
<thead>
<tr>
<th>Trial Name</th>
<th>Trial Completion Date</th>
<th>Drug</th>
<th>Length of Trial</th>
<th># of patients per trial</th>
<th>Primary outcome measure</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japanese Trial</td>
<td>2006</td>
<td>Pirfenidone</td>
<td>52 weeks</td>
<td>275</td>
<td>Successful</td>
<td></td>
</tr>
<tr>
<td>CAPACITY Study 004</td>
<td>2008</td>
<td>Pirfenidone</td>
<td>72 weeks</td>
<td>435</td>
<td>Absolute Change in Percent Predicted Forced Vital Capacity (FVC) and Mean Change in Percent Predicted Forced Vital Capacity (FVC) as measured from baseline to week 72</td>
<td>Successful</td>
</tr>
<tr>
<td>CAPACITY Study 006</td>
<td>2008</td>
<td>Pirfenidone</td>
<td>72 weeks</td>
<td>344</td>
<td>Absolute Change in Percent Predicted Forced Vital Capacity (FVC) and Mean Change in Percent Predicted Forced Vital Capacity (FVC) as measured from baseline to week 72</td>
<td>Unsuccessful-no change in primary endpoint of trial</td>
</tr>
<tr>
<td>ASCEND</td>
<td>2014</td>
<td>Pirfenidone</td>
<td>52 weeks</td>
<td>555</td>
<td>Change in Percent Predicted Forced Vital Capacity (%FVC) From Baseline to Week 52</td>
<td>Successful</td>
</tr>
<tr>
<td>INPULSIS-1</td>
<td>2013</td>
<td>Nintedanib</td>
<td>52 weeks</td>
<td>515</td>
<td>Annual Rate of Decline in Forced Vital Capacity (FVC) Over 52 Weeks</td>
<td>Successful</td>
</tr>
<tr>
<td>INPULSIS-2</td>
<td>2013</td>
<td>Nintedanib</td>
<td>52 weeks</td>
<td>551</td>
<td>Annual Rate of Decline in Forced Vital Capacity (FVC) Over 52 Weeks</td>
<td>Successful</td>
</tr>
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</table>
1.2.6 Other clinical trials conducted in IPF patients

1.2.6.1 INSPIRE Trial

To date there have been a number of clinical trials conducted globally to evaluate possible treatments for IPF. As previously discussed, trials to assess the efficacy of pirfenidone (35) and nintedanib as treatments for IPF, were successful, however many others have failed.

Another major clinical trial conducted was the INSPIRE clinical trial. The INSPIRE trial evaluated the efficacy of interferon γ-1 beta as a treatment option for IPF (40). Interferon gamma is known for its anti-proliferative, anti-fibrotic and immunomodulatory effects (41). Narayanan et al. found that interferon γ inhibited proliferation of both normal primary human lung fibroblasts and primary human lung fibroblasts from IPF patients (42). They also found that interferon gamma inhibited collagen synthesis in fibroblasts (42). The INSPIRE trial was a multicentre trial, recruiting a total of 826 IPF patients from Europe, the USA and Canada (40). Eligible patients were aged between 40-79, diagnosed in the last 48 months and given interferon γ-1b (200 μg) or the placebo 3 times per week (40). The trial ended early as it was shown that treatment with interferon γ1 beta did not affect the number of days survival without lung transplantation, survival days without respiratory-related hospital admission, changes in FVC or changes in the 6 minute walk test compared with the placebo. They also showed no significant difference in the occurrence of progression of the disease or acute exacerbation of the disease between treatment and placebo groups (40).

1.2.6.2 Co-trimoxazole

Another trial was conducted assessing the value of the addition of co-trimoxazole to the standard treatment for IPF patients. Co-trimoxazole is a sulphonamide used to treat certain bacterial infections including those that cause pneumonia and bronchitis (43). This small study had 181 participants diagnosed with fibrotic idiopathic interstitial pneumonia (89% probable or definite IPF) who were given 960 mg of co-trimoxazole or the placebo twice daily for 12 months along with their standard care (44). At the end of the trial in 2009, it was found that the addition of co-trimoxazole did not have any effect on lung function tests, such as change in
FVC from baseline, 6 minute walk test, MRC dyspnoea score or change in DL_{CO} and therefore was not recommended to be used as a treatment in fibrotic IIP patients (44). However, the study did show that patients in the treatment group did have an improved quality of life, a significant reduction in the number of infections and fewer patients required oxygen therapy compared with the placebo group (44). Recently, in 2018 another larger clinical trial has begun in order to further validate the use of Co-trimoxazole in IPF treatment. The EME-TIPAC trial has a total of 330 IPF patients and is scheduled to last 4 years (45).

**1.2.7 Genetic susceptibility to IPF**

MUC5B is an established risk factor for IPF (46). Genetic screening of 492 subjects with IPF revealed a single nucleotide polymorphism (SNP) in the promoter region of the MUC5B gene (rs35705950) (46). The SNP causes a G>T substitution on chromosome 11. Among unaffected people the SNP is associated with a 37.4-fold increase in MUC5B expression in healthy lung tissue. In the context of fibrosis, IPF patients who carry at least one copy of the SNP are associated with a 14.4-fold increase in MUC5B expression compared with controls. MUC5B is one of a series of genes that encode gel-forming mucins expressed in the lung. The odds ratio (a measure of likelihood for the development of the disease) among IPF patients who were heterozygous or homozygous for the minor allele of the MUC5B SNP (rs35705950) was 9.0 and 21.8 respectively (46). The rs35705950 polymorphism in located in the promoter region of the MUC5B gene. The presence of this polymorphism confers increased risk of the development of IPF but, interestingly, it has also been to confer a survival benefit in IPF patients. The association between the rs35705950 minor allele and IPF has been replicated in a number of different studies to date.

An association between the engulfment and cell motility (ELMO) domain containing 2 (ELMOD2) gene and IPF was discovered in a familial IPF study in Finland (47). A genome-wide scan was carried out on six families with familial IPF and ELMOD2 was highlighted as a potential candidate for genetic susceptibility in IPF. *In vitro* studies carried out showed that IPF patients had decreased mRNA expression of ELMOD2 in lung tissue compared with healthy controls. They went on
to show in alveolar epithelial cell lines (A549) that ELMOD2 was important in antiviral signalling through TLR3. Inhibition of ELMOD2 lead to abrogation of TLR3 signalling and reduced production of type I interferons (48).

A genome-wide association study (GWAS) was published in 2013 and identified an association between variants in the TOLLIP gene and increased susceptibility to IPF (49). Variants in TOLLIP caused reduced expression of the TOLLIP protein. One variant, rs5743890, was associated with increased mortality in IPF but not with the development of IPF (49). Interestingly, like ELMOD2, TOLLIP is also an important regulator in the immune response induced by TLRs as TOLLIP is involved in MyD88-dependent activation of NF-κB. TOLLIP is also implicated in other pathways associated with fibrosis such as the TGF-β pathway (50, 51).

Mutations affecting different components of telomerase have been associated with IPF (52). Telomerase is a polymerase that adds telomere repeats to the ends of telomeres. Telomerase reverse transcriptase (TERT) and RNA component of telomerase (TERC), two components of telomerase, are required to maintain telomere integrity. Variants in TERT and TERC have been associated with fibrotic idiopathic interstitial pneumonias and IPF (53-55).

Recently, in 2017, another large genome-wide association study was carried out by Allen et al. which found another gene linked to IPF susceptibility (56). In this study IPF patients from European descent were screened and the group identified an association between the gene A-kinase anchoring protein 13 (AKAP13) and IPF. AKAP13 is thought to have an effect on the pro-fibrotic signalling pathways via Rho A. There was no previous known association between AKAP13 and IPF susceptibility. A novel SNP, rs62025270, was also identified in this study. The minor A allele of rs62025270 was associated with increased susceptibility to IPF and an increase in AKAP13 mRNA expression in lung tissue resections from IPF patients (56).

1.2.8 Biomarker requirement for diagnosis and treatment of IPF

Biomarkers have a wide range of uses for clinicians. Biomarkers can help clinicians; identify people at risk of developing a disease, indicate how the disease
will progress, stratify patients by disease severity, monitor disease progression and predict response to treatments (57).

To date there are no diagnostic or prognostic biomarkers in widespread use in the diagnosis or treatment of IPF patients (58). Currently clinicians and researchers rely on clinical data such as physical examinations, radiological data and pulmonary function tests such as FVC and DLCO to divide patients into slow and rapid progressors (57). However these tests do not take into account underlying disease mechanisms (59).

MUC5B is a candidate diagnostic biomarker. MUC5B is a gene encoding the mucin 5 protein. The rs35705950 polymorphism is located in the promoter region of the MUC5B gene. As previously mentioned, the presence of this polymorphism confers increased risk of the development of IPF as well as conferring a survival benefit in IPF patients.

Recently, the PROFILE cohort study, a multi-centre study in the UK, analysed 123 biomarkers to discover potential biomarkers to predict disease progression in IPF patients. The biomarkers assayed included cytokines, chemokines, growth factors, matrix MMPs, extracellular matrix proteins and markers of epithelial injury and apoptosis. Four serum proteins were identified as potential prognostic biomarkers; surfactant protein D, MMP7, CA19-9 and CA-125 (59). Surfactant proteins are secreted by type II alveolar cells and modulate the immune response. Surfactant protein A and D (SP-A and SP-D) have been proposed as diagnostic and prognostic biomarkers in IPF. IPF patients have been described in a number of studies to have increased serum levels of SP-A and SP-D compared with healthy controls. Higher levels of serum SP-A and SP-D have been shown to be predictive of survival in IPF (60, 61). SP-A and SP-D may also be useful in cases of acute exacerbations of IPF as it was found that patients experiencing acute exacerbations have higher levels of serum SP-D proteins compared with stable IPF patients (62).

Another serum biomarker proposed in IPF is matrix metalloproteinase 7 (MMP7). MMP7 has been shown to be increased in the plasma of IPF patients compared with controls (63, 64). In both studies there was increased expression of another MMP, MMP-1, in IPF patients. Rosas et al. also found a significant
association between MMP-7 expression levels and disease severity assessed by forced vital capacity (FVC) and DL_{CO} (63). Interestingly, Song et al. suggested using multivariate analysis that using one biomarker alone, for example 1.27MMP7, may not give as accurate a result when predicting survival compared with when the using a number of biomarkers in conjunction to predict survival (65).

Cancer antigen 19-9 (CA19-9) and Cancer antigen-125 (CA-125) are proteins secreted in small amounts by the bronchial epithelium in normal healthy lung epithelium and secreted in abundance in the metaplastic epithelium of IPF patients. CA19-9 and CA-125 are already validated biomarkers used in the diagnosis of pancreatic cancer and CA-125 in ovarian cancer (66, 67). In the PROFILE study, it was shown that CA19-9 and CA-125 could potentially be used as prognostic biomarker as patients with more progressive disease had significantly higher baseline levels of CA19-9 and CA-125 compared with stable patients. CA-125 can also dynamically reflect disease progression as levels have been shown to increase after 3 months in progressive patients and not in stable patients (59).

1.3 Role of infection in IPF

1.3.1 Putative role of viral infection in the pathogenesis of IPF

The role of viral infection in disease initiation and progression in IPF has been examined robustly in the last number of years. A number of different viruses have been proposed to act as a persistent antigenic stimulus in IPF (68).

Herpesviruses have been proposed by some researchers as a “second-hit” in the development of pulmonary fibrosis (69). The Herpes virus family consists of over 100 known viruses, many of which are known to infect humans and establish latent infections in specific tissues (70). Herpesviruses are dsDNA viruses which have a unique four layer structure. They include viruses such as herpes simplex virus 1 and 2 (HSV), Epstein-barr virus (EBV), cytomegalovirus (CMV) and Human Herpes Virus (HHV)-7 and -8 (70). Different research groups have found the presence of one or more herpesviruses in IPF lung tissue (71-74). One of the initial groups to investigate herpesviruses in IPF pathogenesis assessed the levels of cytomegalovirus (CMV) immunoglobulin G (IgG) in IPF, sarcoidosis, emphysema and control patients. IPF patients had significantly higher levels of CMV IgG in their
serum compared with controls, emphysema and sarcoidosis patients (71). CMV IgM levels however were not higher in IPF patients which indicated that IPF patients were not suffering from active infection but latent infection. Interestingly, they also examined the levels of EBV viral capsid antigen and HSV IgG and found both levels to be higher in IPF patients compared with all other groups (71). This suggested a role for latent herpesvirus infection in IPF pathogenesis. Another study in 2003 also proposed a role for herpesviruses as a source of chronic antigenic stimulation. IPF lung tissue was analysed for the presence of EBV, CMV and HHV-7 and -8. It was shown that EBV, CMV and HHV-8 were detected significantly more frequently in IPF patients compared with controls (73). It was also shown that two or more herpesviruses were present in 57% of IPF patients compared with in 8% of controls. EBV had been previously shown to have a higher prevalence in IPF patients compared with control patients (75, 76). HSV-1 has also been detected in IPF lungs. In vitro experiments demonstrated that HSV-1 infection caused upregulation of expression of the fibrotic growth factors; TGF-β and FGF (72).

The effect of gammaherpes viruses on fibrosis has been examined in murine models of pulmonary fibrosis (77). Bleomycin-induced fibrosis is one of the standard murine models of fibrosis used. Lok et al. infected BALB/c mice, known to be resistant to bleomycin, with murine gammaherpesvirus-68 (γHV68) after which bleomycin was administered interaperitoneally 7 days later. They showed that mice given both bleomycin and γHV68 had significantly greater levels of fibrosis and inflammation in the lungs compared with bleomycin only or γHV68 only mice (77). In 2008 McMillan et al. used another model, fluorescein isothiocyanate (FITC)–induced pulmonary fibrosis, to demonstrate that gammaherpes viruses exacerbated fibrosis in the lung in vivo (78). In contrast to the previous group, fibrosis was established first in the mice following which viral infection was superimposed. Mice were infected with γHV68 during established fibrosis and results were taken at peak lytic cycle of the virus. It was found that there was increased total lung collagen and diminished lung function in the infected mice compared with the controls. Histological changes in the lung following viral infection were also seen (78). These two studies suggest that herpesvirus infection may play a detrimental role in fibrosis patients.
Hepatitis C virus is an enveloped single stranded RNA virus (70). There have been several studies examining the link between HCV and IPF. A study in Japan was the first study to establish a link (79). 66 patients were analysed for serum antibodies to HCV and it was found that 28.8% of IPF patients were positive for serum antibodies to HCV, significantly higher than 3.66% of the controls who tested positive (79). However, a British study which followed the Japanese findings did not find the same results and found that the levels of Hepatitis C in the IPF population were similar to those seen in the general population (80). Interestingly, an Italian study in 1996 later confirmed the Japanese 1992 study. They also found that IPF patients had increased prevalence of anti-HCV antibodies compared with controls (81).

Torque-teno virus (TTV) has also been detected in IPF patients. It is a single stranded circular DNA virus which was first identified in individuals post-transfusion. In one small study, 33 IPF patients were tested for TTV infection, 12 of them were positive (82).

Finally, Influenza A virus (IAV) H1N1 has also been investigated in IPF patients. Fujino et al. hypothesized that infection of fibrotic lung tissue with Influenza A strain, A(H1N1)pdm09, would be more severe than in non-fibrotic lungs (83). In a very small study (5 patients: 3 patients with IPF, 1 patient with cellular nonspecific interstitial pneumonia, and 1 patient with chronic hypersensitivity pneumonitis), type II alveolar epithelial cells were purified from lung tissue and infected with Influenza A strain, A(H1N1)pdm09, for 24 hours. It was found that type II alveolar cells from fibrotic lung tissue had significantly higher viral titres 24h-post infection compared with non-fibrotic lung tissue (83). Additionally, it has been shown that influenza infection promotes collagen deposition in the lungs in vivo by promoting αvβ6 integrin-mediated TGFβ activity and epithelial cell death in human bronchial epithelial cells in vitro (84). There has been one published case study by Umeda et al. suggesting the possible risk of initiation of AE-IPF by the influenza A vaccine (85). They described a 57 year old patient with IPF who was experiencing an acute exacerbation resulting in severe respiratory worsening following administration of the pandemic influenza A vaccine (85). In order to determine the effect of IAV infection in fibrosis, Ashley et al. introduced IAV infection into a bleomycin-model of fibrosis. In this model, it was shown that IAV
infection did not exacerbate fibrosis. Mice were challenged with IAV H1N1 following bleomycin-administration and it was found that IAV infection did not increase fibrotic markers in mice (86).

1.3.2 Putative role of bacterial infection in the pathogenesis of IPF

In the past, the role of infection in the pathogenesis and progression of IPF was thought to be mainly due to viral infection. Currently, there is mounting evidence to suggest that bacterial infection may also play a role in the pathogenesis of IPF. A study was carried out in the UK to examine the role of bacterial infection in IPF (87). In the study IPF patients underwent a bronchoalveolar lavage (BAL) procedure and genomic DNA was isolated. The DNA was subsequently analysed using 16S rRNA gene qPCR and pyrosequencing. Interestingly, the group found that IPF patients had a significantly higher bacterial burden in the BAL fluids compared with healthy controls and chronic obstructive pulmonary disease (COPD) patients. A difference was also shown between IPF patients with progressive disease and stable disease. Patients with progressive disease had a significantly higher bacterial burden compared with IPF patients with stable disease. The results from this study suggested an important role for bacterial infection in IPF as patients with a higher the bacterial burden were at increased risk of mortality compared with patients with a low bacterial burden (87). The composition of the microbiome was also assessed and it was found that IPF patients had four distinct populations of bacteria at significantly higher levels than healthy controls; *Haemophilus* sp., *Neisseria* sp., *Streptococcus* sp. and *Veillonella* sp.

A retrospective study was carried out by Han et al. to examine the relationship between microbiome of IPF patients and IPF progression (88). It was found that there were two different populations of bacteria that were significantly associated with disease progression in IPF; *Streptococcus* and *Staphylococcus* (88). Both pathogens were significantly associated with poorer outcomes. It is worth noting that while the presence of *Streptococcus* and *Staphylococcus* was found in the UK study carried out by Molyneaux et al. they did not find an association between either species and disease progression (87). Han et al. also showed the presence of
the similar bacterial populations to those seen by Molyneaux et al. including *Veillonella* sp. and *Prevotella* sp. (87, 88).

More recently, another modest study (n=34) was conducted with Japanese IPF patients (89). Similar to the other studies, BAL fluids were collected and the microbiome was analysed by 16S rRNA qPCR. In this study it was confirmed again that IPF patients experience increases in *Streptococcus* sp., *Veillonella* sp. and *Prevotella* sp. while there was a decrease in Proteobacteria. These changes in diversity led to decreased bacterial diversity in IPF patients and Takahashi et al., correlated this decrease with progression of IPF and worsening of clinical tests such as 6MWT and FVC. One major drawback of this study was that no healthy controls were used, however a murine model of fibrosis was also used to explain their findings. Mice were treated with bleomycin to induce bleomycin-induced fibrosis (the gold standard murine model of fibrosis) after which their microbiota was analysed. Similar to the results seen in humans, mice given bleomycin treatment suffered a loss in diversity of their bacterial populations.

Huang et al. took a different approach to looking at the relationship between disease progression in IPF and the microbiome (90). As well as examining BAL fluids from IPF patients, peripheral blood mononuclear cells (PBMCs), fibroblasts stimulated with CpG-ODN and leukocytes were all examined. Using micro-array analysis of genes in IPF patients alongside 16S rRNA gene qPCR for bacteria in BAL fluid, the group examined the association between the microbiome and progression-free survival in IPF patients. They found in PBMCs that downregulation of immune pathways such as TLRs and RIG-I pathway correlated with worsened progression free survival (90). This study also linked the presence of certain microbial populations with immune responses, for examples it was found that reduced immune responses by NOD-like receptors were associated with increased abundance of *Streptococcus* which was associated with poorer progression free survival in IPF (90).

It has been suggested that infection could be associated with AE in IPF patients. Recently, a small study was carried out by Molyneaux et al. to examine the microbiome of IPF patients experiencing acute exacerbations in IPF (91). In the study 20 patients with AE-IPF and 15 controls were given BALs. Similar to earlier
studies, the BAL fluid was collected and genomic DNA was analysed using 16S rRNA gene qPCR and pyrosequencing. It was found that total bacterial load was significantly associated with AE-IPF and AE-IPF patients had increased levels of two potentially pathogenic bacteria; *Campylobacter* sp. and *Stenotrophomonas* sp. (91).

**1.3.3 Minor role of fungal infection in the pathogenesis of IPF**

Fungal infection is less frequently associated with IPF and its role in disease pathogenesis in IPF is less well known. Pulmonary aspergillosis is caused by a ubiquitous fungus *Aspergillus* which can cause severe infections in immune-compromised patients. Pulmonary aspergillosis can be a major complication in many lung diseases including sarcoidosis, cystic fibrosis, COPD, interstitial pneumonia as well as patients recovering from TB and lung cancer (92-94). A study was carried out in Japan to examine the infectious pathogens found in lungs from AE-IPF patients after death during autopsy. It was found that 15 patients had died from bronchopneumonia, 7 of those patients had fungal infection; 6 with pulmonary aspergillosis and 1 infected with *Candida Albicans* (95).

**1.4 Innate Immunity**

**1.4.1 Introduction**

The immune response to infection can be divided into two different stages; the innate immune response and the adaptive immune response. The innate immune response mounts the first line of defence in vertebrates to external threats such as bacterial, viral and fungal infection (96). It is characterised by the presence of a number of different cell types which immediately become activated following infection. Examples of these cells include macrophages, neutrophils and dendritic cells as well as natural killer cells, mast cells, basophils and eosinophils. These cells are crucial for both pathogen clearance and presentation of antigens to adaptive immune cells. After several days the adaptive immune response will replace the innate response. This is a highly specific response to the invading pathogen and is characterised by the generation of specific T and B cells as well as memory cells.
which will give the organism future protection against the same infectious pathogen long after the infection is cleared (97).

The innate immune response is initiated through the recognition of pathogens once they enter the body. Crucial proteins involved in this recognition are Pattern Recognition Receptors (PRRs).

**1.4.2 Pattern Recognition Receptors (PRRs)**

PRRs are receptors found on the cell membrane, in the cytosol and on the endosomal membrane which sense both exogenous and endogenous signals in the cell. Exogenous signals come from external threats. PRRs detect components of bacteria, fungi and viruses known as pathogen-associated molecular patterns (PAMPs) which stimulate an inflammatory response. Endogenous signals, known as danger-associated molecular patterns (DAMPs), are internal threats in the cell which also activate PRRs. Some examples of these include ATP, uric acid, DNA and RNA (98). PRRs can be broken down into sub-families which will be explained in detail in the following sections.

**1.4.3 Toll-like receptors (TLRs)**

TLRs are a group of receptors found on cell and endosomal membranes. There are currently 10 known human TLRs and 13 murine TLRs, each with their own specific ligands. TLRs and their respective ligands are summarised in Table 1.2 (99).
<table>
<thead>
<tr>
<th>Human TLRs</th>
<th>Murine TLRs</th>
<th>Ligand</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1/2</td>
<td>TLR1</td>
<td>Bacterial triacylated lipopeptides</td>
<td>Cell membrane</td>
</tr>
<tr>
<td>TLR2/6</td>
<td>TLR2</td>
<td>Bacterial diacylated lipopeptides</td>
<td>Cell membrane</td>
</tr>
<tr>
<td>TLR2</td>
<td>TLR2</td>
<td>Multiple glycolipids, lipopeptides and lipoproteins</td>
<td>Cell membrane</td>
</tr>
<tr>
<td>TLR3</td>
<td>TLR3</td>
<td>Double stranded RNA (dsRNA)</td>
<td>Cell and endosomal membrane</td>
</tr>
<tr>
<td>TLR4</td>
<td>TLR4</td>
<td>Lipopolysaccharide (LPS)</td>
<td>Cell membrane</td>
</tr>
<tr>
<td>TLR5</td>
<td>TLR5</td>
<td>Flagellin</td>
<td>Cell membrane</td>
</tr>
<tr>
<td>TLR7</td>
<td>TLR7</td>
<td>Viral single stranded RNA (ssRNA)</td>
<td>Cell and endosomal membrane</td>
</tr>
<tr>
<td>TLR8</td>
<td>TLR8</td>
<td>Viral single stranded RNA (ssRNA)</td>
<td>Cell and endosomal membrane</td>
</tr>
<tr>
<td>TLR9</td>
<td>TLR9</td>
<td>Unmethylated CpG-containing DNA motifs</td>
<td>Cell and endosomal membrane</td>
</tr>
<tr>
<td>TLR10</td>
<td></td>
<td>Unknown ligand</td>
<td>Cell membrane</td>
</tr>
<tr>
<td>TLR11</td>
<td>T. gondii Profilin</td>
<td>and uropathogenic Escherichia coli</td>
<td>Cell membrane</td>
</tr>
<tr>
<td>TLR12</td>
<td>T. gondii Profilin</td>
<td></td>
<td>Cell membrane</td>
</tr>
<tr>
<td>TLR13</td>
<td></td>
<td>Bacterial 23S rRNA</td>
<td>Endosome</td>
</tr>
</tbody>
</table>
All TLR receptors consist of an extracellular domain, a single transmembrane α-helix and an intracellular Toll/IL1R (TIR) domain which interacts with downstream signalling molecules to induce production of pro-inflammatory cytokines and interferons through pathways such as the NF-κB, MAPK, JNK and IRF pathways. The TLR extracellular domain (or the TLR ectodomain) consists of leucine rich repeat (LRR) structures approximately 24 amino acids in length (100). The LRRs are conserved regions of hydrophobic residues which are responsible for the recognition of ligands by the TLR receptor. The LRR structures fold into β-sheets in the protein to form the characteristic horseshoe (solenoid) shape of the TLR ectodomain (100).

TLRs act as dimers; heterodimers (for example TLR1/2 and TLR2/6) or homodimers (for example TLR3, 4 and 9). Upon ligand binding, TLR receptors undergo a conformational change. This binding brings the C terminal of each TLR monomer into close contact which in turn causes an association of the cytosolic TIR domains allowing downstream adaptor molecules to interact with the TLR receptor (101). The TIR domain contains a conserved motif of approximately 160 amino acids which forms 5 β-sheets and α-helices connected by loops (102). Two important loops are the “BB” and “DD” loops, these loops are essential for protein-protein interactions between the TLRs and adaptor proteins such as MyD88 (102). This process allows signal transduction to occur through protein kinases downstream of the TLR receptor.

Several adaptor proteins are used by TLRs to regulate signal transduction. All adaptor proteins are known to contain a TIR-domain for interactions with the TLRs. There are five main adaptor proteins: MyD88, MyD88-adaptor like protein (Mal, also referred to as TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1), TRIF-related adaptor molecule (TRAM, also called TICAM 2) and sterile α- and armadillo- motif containing protein (SARM) (103).

Signalling through all TLRs except TLR3 relies on signal transduction through the adaptor protein MyD88. MyD88 binds to the cytosolic portion of the TLR receptors and induces a downstream signalling cascade through the IL-1R-associated kinase (IRAK) proteins. TLR2 and TLR4 signalling through MyD88 relies on a second adaptor protein known as Mal (104). Mal has been shown to
facilitate MyD88 recruitment to TLR4 at the plasma membrane to allow for signal transduction through TLR4 to activate transcription factor NF-κB (105).

TLR3 relies on the adaptor protein TRIF for signal transduction (106). TLR3 is the only TLR to rely solely on TRIF for signalling. TLR4 uses both TRIF and MyD88 for signal transduction. TLR4 signalling through TRIF relies on a second adaptor protein known as TRAM (107). TRAM acts as a bridging adaptor protein to allow TLR4 interaction with TRIF (108). TRIF-TRAM interactions are important for IRF3 activation. Following TLR4 activation, delivery of TRAM-TRIF from the plasma membrane to the endosome leads to TLR4-induced IRF3 activation (109, 110). TRAM has also been shown to be important in type I interferon production by TLR2 via IRF7. TRAM allows for endosomal signalling of TLR2 and production of type I interferons (111, 112).

SARM is a negative regulator of TLR signalling. SARM has been found to inhibit TLR signalling through interactions with TRIF and MyD88 (113, 114). Figure 1.4 illustrates TLR receptors, their ligands and adaptor proteins and their subsequent downstream signalling pathway.
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Figure 1.4. Summary of TLR signalling pathways.

TLR-1/2, 2/6, 4, 5, 11 are expressed on the cell membrane while TLR-3, 4, 7, 8, 9 are expressed on the endosomal membrane. In response to the recognition of specific ligands, TLRs induce the activation of many transcription factors resulting in the expression of pro-inflammatory cytokines and type I interferons. Image taken from (99).

TLRs play a very important role in the immune response to infection on all mucosal surfaces including the lungs, the intestines and the reproductive tract. Through production of pro-inflammatory cytokines, type I interferons and anti-microbial factors, TLRs help to protect the mucosal membranes by maintaining the epithelial barrier homeostasis, helping it to function and proliferate properly (115).

1.4.4 RIG-I–like receptors (RLRs)

There are three RLR receptors; retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated factor 5 (MDA5) and laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2 (LGP2) which are expressed in the
cytosol. They are DExD/H box–containing RNA helicases which act as intracellular pattern recognition receptors responsible for detecting viral RNA (116). Similar to the TLRs, RLRs once activated induce a signalling cascade resulting in the production of pro-inflammatory cytokines and type I interferons through the transcription factors NF-κB and IRF3. RIG-I and MDA5 are essential for controlling viral infections (117).

RIG-I was the first RLR described and is the best characterised of the RLRs. RIG-I is a 106 kDa highly conserved protein receptor which is activated by viral and synthetic dsRNA in the cytoplasm of the cell. RIG-I consists of three domains; two N-terminal CARD domains, a DExD/H box–containing RNA helicase domain and a C-terminal repressor domain (117). RIG-I activates a downstream signalling cascade through its caspase recruitment domain (CARD) that leads to the activation and translocation of transcription factors NF-κB and IRF3 to the nucleus to induce an anti-viral immune response (117). RIG-I distinguishes viral RNA from human RNA by preferentially recognising RNA sequences marked with 5’ triphosphorylated (5’ppp) end (118). The 5’ triphosphate group alone is not enough to activate the RIG-I receptor, RIG-I also must also recognise motifs within the viral RNA to induce production of anti-viral interferons and interferon stimulated genes (ISGs) (119).
Figure 1.5. Summary of RLR signalling pathways.

RIG-I and MDA5 are intra-cellular viral sensors. In response to the recognition of specific ligands, RLRs act through their adaptor protein MAVS to induce the activation of many transcription factors resulting in the expression of pro-inflammatory cytokines and type I interferons. Image taken from (120).

Mitochondrial anti-viral signalling protein, MAVS, acts as an adaptor molecule in the signal transduction pathway for both RIG-I and MDA5. Seth et al. showed that the MAVS protein (also known as IPS-1, VISA and Cardiff) is essential for the phosphorylation of downstream transcription factors IRF3 and NF-κB and the induction of an anti-viral immune response to infection (121). MAVS contains an N-terminal CARD domain which interacts with the two CARD domains in the RIG-I and MDA5 receptors and a C-terminal transmembrane domain which tethers the MAVS protein to the mitochondria (121). MAVS is essential to RIG-I and MDA5 signalling. Disruption or suppression of MAVS signalling inhibits interferon production and increases viral replication in vitro (121).

MDA5 is the closest relative of RIG-I as they share a very similar structure, LGP2 is different to the other two receptors as it does not have CARD domains.
RIG-I and MDA5 are known to detect different viruses in the cell. RIG-I recognises RNA viruses from the families; Flaviviridae, Paramyxoviridae, Orthomyxoviridae, and Rhabdoviridae. MDA5 is essential for detection of the Picornaviridae family of RNA viruses in the cell (122).

Figure 1.6. Structure of secondary viral sensors; RIG-I, MDA5 and LGP2.

RIG-I and MDA5 share a similar structure containing 2 CARD domains in the N-terminal domain, a DExD/H box–containing RNA helicase domain and C terminal domain. RIG-I and MDA5 interact via their CARD domains with downstream adaptor protein, MAVS, CARD domain to induce activation of a downstream signalling cascade resulting in the expression of pro-inflammatory cytokines and type I interferons. Image taken from (123).

1.4.5 C-type lectin receptors (CLRs) and Nod-like receptors (NLRs)

The C-type lectin receptors (CLRs) and Nod-like receptors (NLRs) are the last two families of PRRs found in the cell. Along with several TLRs which have been implicated in anti-fungal immunity, C-type lectin receptors are essential to anti-fungal immune responses (121, 124). Dectin-1, Dectin-2 and Mincle are all examples of CLRs which recognise different fungal components including β-glucan, α-mannans and α-mannose respectively. Like TLRs, CLRs induce the production of pro-inflammatory cytokines through the transcription factor NF-κB (125).

Intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are responsible for recognising microbial PAMPs and DAMPs (126). NLRs are important for the clearance of intracellular bacteria through their recognition of PAMPs such as peptidoglycan (127). NLRs have several sub-families of intracellular proteins which can be grouped based on their structure or immune
function. Some NLRs including NLRP1b, NLRP3 and NLRC4 assemble into
inflammasome complexes to induce production of pro-inflammatory cytokines via
caspases (128). Other NLRs such as NOD-1 and NOD-2 induce the production of
pro-inflammatory cytokines through the NF-κB and MAPK pathways (127).

1.4.6 dsRNA protein kinase R (PKR)

PKR is an important sensor in the anti-viral innate immune response. It is an
interferon –inducible intra-cellular kinase which is activated by dsRNA, cytokines
and cellular stress. PKR is a member of the eIF2α family of protein kinases which
upon dsRNA binding becomes activated through dimerization and auto-
phosphorylation (129). Activated PKR inactivates eukaryotic translation initiation
factor (eIF)2α by phosphorylation to prevent protein translation in the cell. Activated
PKR can also induce activation of the NF-κB, JNK and P38/MAPK pathways to
induce expression of pro-inflammatory cytokines in the cell (130). Many different
viruses have developed mechanisms to antagonise PKR signalling (131).

1.5 Toll-like receptor 3 (TLR3)

1.5.1 Structure and location of TLR3

TLR3 is a type 1 membrane receptor consisting of an ectodomain, a single
transmembrane α-helix and an intracellular TIR domain that facilitates downstream
cell signalling. The TLR3 receptor consists of two monomers. The extracellular
domain, or the ectodomain, of the TLR3 monomer is a 23 turn coil “horseshoe”
shape consisting of leucine rich repeats (132, 133). Specialised domains are found
within the N and C terminal of the coil (133). It has been found that two TLR3
monomers must bind together through interactions in the N terminal domain to allow
for effective ligand binding (134). Interactions between the leucine rich repeat
regions in the C terminal domain brings the intracellular TIR regions into contact
allowing for signal transduction through the receptor without any conformational
changes to the extracellular domain. This dimerization is essential for both signal
transduction and ligand binding, monomers of TLR3 cannot bind dsRNA or activate
a downstream protein kinase signalling cascade (134).
Intracellularly, the TIR domain of the TLR3 receptor associates with the adapter molecule, TIR-domain-containing adaptor-inducing interferon-β (TRIF).

TLR3 is expressed on the cell surface and endosomal membrane of many different cell types. TLR3 is expressed on immune cells as well as lung fibroblasts, endothelial cells, lung epithelial cells and neurons (135-139). TLR3 is widely expressed in various tissues of the body including all the major organs such as the brain, lungs, heart and spleen. Figure 1.7 was generated by the Human Protein Atlas project and shows the regions of the body in which TLR3 protein is expressed (140, 141).

![Figure 1.7. TLR3 protein expression in humans.](image)

The TLR3 receptor is widely expressed in all major organs and throughout the body. Image produced by the Human Atlas Project (140, 141).

1.5.2 TLR3 signalling

The TLR3 receptor binds dsRNA under slightly acidic conditions such as those found in the endosome. The receptor recognises dsRNA of 45bp or more which interacts with two specific sites in the TLR3 receptor (134). TLR3 has previously been shown to bind dsRNA from viruses, bacteria and helminths, respectively, in addition to mRNA released from necrotic cells (142-145). Once activated the TLR3 receptor activates a downstream signalling cascade through the adaptor molecule TRIF (106). Tyrosine phosphorylation of two tyrosine residues, 759 and 858, in the cytoplasmic domain of the TLR3 receptor is essential for downstream signalling through IRF3 and NF-κB (146).
TRIF associates with a number of downstream signalling proteins such as TNF receptor associated factor 6 (TRAF6), TNF receptor associated factor 3 (TRAF3) and receptor interacting protein 1 (RIP-1). TRIF associates with the downstream molecule TRAF6 through its TRAF6 binding domains, allowing for activation of the transcription factor NF-κB (147). RIP-1 is another downstream signalling protein essential for NF-κB activation by associating with TRIF. In the absence of RIP-1, activation of NF-κB is impaired while JNK and IRF3 pathways remain unaffected (148).

To activate the IRF3 transcription factor, TRIF recruits downstream signalling molecule TRAF3. TRAF3 is a positive regulator for IRF3 activation and type I interferon production (149). TRIF-mediated signalling induces self-ubiquitination of the TRAF3 molecule by non-canonical K63-linked polyubiquitination. TRAF3 subsequently forms a complex with the TANK-binding kinase 1 (TBK1) molecule along with the protein downstream of kinase 3 (DOK3) (150). This complex phosphorylates IRF3 to induce activation leading to its translocation to the nucleus and subsequent type I interferon production (151). Figure 1.8 illustrates the TLR3 signalling pathway.
1.5.3 Role of TLR3 in lung fibrosis

TLR-induced cytokine production plays a role in the wound healing process as well as fighting infection (152). Activation of the TLR3 receptor has been shown to be protective in fibrosis. As previously mentioned, TLR3 once activated induces a pro-inflammatory cytokine and type I interferon response. Pro-inflammatory cytokines and Type I IFNs are important in the resolution of fibrosis. Similarly, IFN-γ has been suggested to be protective in fibrosis through inhibition of TGF-β (152). Th2 cytokines such as IL-4 and IL-13 have been shown to have a detrimental role in fibrosis. In experimental models of fibrosis, it has been demonstrated that these
cytokines exacerbate fibrosis through excessive migration and protease activity in the lungs (153). IL-4 and IL-13 have also been shown to drive fibrosis by driving the differentiation of resident fibroblasts into myofibroblasts in many different tissues. In different models of fibrosis, it has been found that TLR3 responses limit the effects of pro-fibrotic cytokines in fibrosis (154, 155).

In the bleomycin model of fibrosis, TLR3 knockout mice have been shown to have greater levels of fibrosis and increased mortality compared with wild-type mice (154). The absence of TLR3 resulted in increased fibrosis in combination with increased expression of pro-fibrotic cytokines IL-4, IL-13 and TGF-β compared with wild-type mice (154). Type I IFNs have been shown to regulate fibroproliferation. Increased hydroxyproline levels in TLR3−/− mice was indicative of greater collagen deposition and worsened fibrosis in the lungs.

Additionally, Joshi et al. examined the role of TLR3 in a Schistosoma mansoni egg-induced pulmonary granuloma model (155). In this study again, it was shown that TLR3 was protective, as lungs from TLR3−/− mice had larger granulomas and greater collagen deposition compared with wild-type mice. Similar to the bleomycin model of fibrosis, TLR3 knockout mice had significantly higher levels of pro-fibrotic cytokines IL-5 and IL-13. In addition, TLR3−/− mice had reduced levels of IFN-β compared with wild-type mice which was associated with increased collagen expression and TGF-β. This study, therefore, highlighted a role for TLR3 in protection against Th2-driven granulomas and pro-fibrotic Th2 responses (155).

In support of this, TLR4 knockout mice have been shown to have attenuated fibrosis in the bleomycin-induced model of fibrosis (156). It was found that there was reduced fibrosis and inflammatory cell infiltration in TLR4−/− mice compared with wild-type mice. It was found that the reason for this was the associated reduction in expression of many pro-fibrotic mediators, such as IL-13, IL-6, IL-17 and TGF-β, in the TLR4−/− lungs (156).

1.5.4 TLR3 known single nucleotide polymorphisms (SNPs)

Many TLR3 SNPs have been identified and shown to be associated with disease. Ranjith-Kumar et al. characterised 4 SNPs in the TLR3 gene which were thought to affect TLR3 function; L412F, N284I, Y307D and S737T. All four SNPs
caused an amino acid substitution. It was shown that L412F caused partial loss in functionality of the TLR3 receptor, while Y307D and S737T had the same functionality as WT cells (157). N284I caused complete loss of function. The allele frequencies of N284I, Y307D and S737T are very low. An example of this can be seen in a study by Lee et al. It was found that TLR3 N284I SNP was very rare; only 2 of the 613 liver recipients possessed the SNP (158).

Casanova and colleagues found a polymorphism in TLR3 in two unrelated children who have herpes simplex virus 1 encephalitis (159). This SNP is a proline to serine substitution at residue 554 of the TLR3 gene (TLR3 P554S). TLR3 P554S heterozygous lung fibroblasts have reduced responses through NF-κB and IRF3 to Poly(I:C) compared with controls. This weakened response was also seen in other cell types such as NK, monocyte-derived dendritic cells and CD8 T cells in the presence of the SNP.

TLR3 gene polymorphisms have been associated with a number of cancers. Slattery et al. analysed 4 TLR3 single nucleotide polymorphisms (rs5743305, rs11721827, rs3775292 and rs377529) for an association with colon and rectal cancer (160). TLR3 rs11721827 was significantly associated with rectal cancer while TLR3 rs3775292 CG/GG genotypes were associated with slightly improved survival in colon cancer patients who were diagnosed at a more advanced stage of the disease (160).

TLR3 gene polymorphisms have also been associated with hepatocellular carcinoma (HCC) and HBV-related HCC (161). 2 TLR3 SNPs were examined in patients with HCC; rs5743305 (TLR3 T967A) and rs3775291 (TLR3 L412F). It was found that there was no association with rs5743305. However, TLR3 L412F homozygote patients were associated with increased susceptibility to HCC (161).

Another TLR3 polymorphism, TLR3 rs5743312, is associated with oral cancer. rs5743312 is an C>T substitution on chromosome 4 which has been proposed as a potential predictor of worse overall survival in advanced oral cancer (162).

End-stage renal disease (ESRD) is also associated with polymorphisms in the TLR3 gene. ESRD typically presents with immune activation and systemic inflammation (163). Yang et al. screened ESRD patients and healthy controls for 3
polymorphisms in the TLR3 gene; rs3775296 (−7C/A), rs3775290 (1377C/T) and rs3775291 (1234C/T) polymorphisms. The minor allele (C variant allele or risk allele) of the rs3775296 (−7C/A) polymorphism was shown to be associated with a statistically significantly higher risk of ESRD in the Chinese population. Neither of the other two polymorphisms was associated with ESRD (163).

Interestingly, TLR3 polymorphisms have been suggested to influence immunity to vaccinations. In the case of the measles vaccine, it was shown that the heterozygous AT genotype of the TLR3 polymorphism rs5743305 located in the promoter region of the TLR3 gene resulted in low antibody and low lymphoproliferative responses (164). Similarly, the heterozygous variant of the rs3775291 SNP showed significantly lower measles-specific antibody levels compared with homozygote variants (164).

1.6 TLR3 L412F single nucleotide polymorphism (SNP)

1.6.1 Overview

The TLR3 L412F (rs3775291) SNP was first described by Ranjith-Kumar et al. (157) in 2007. The SNP is located on exon 4 of the TLR3 gene. As previously mentioned, TLR3 is a horse shoe solenoid shaped receptor found on the cell surface and endosomal membrane. TLR3 L412F encodes a leucine to phenylalanine substitution at position 412 located in the ectodomain of the TLR3 receptor (Figure 1.9). Ranjith-Kumar et al. predicted that the presence of the SNP could affect ligand binding by destabilising the hydrophobic interaction environment due to the bulky nature of the phenylalanine amino acid and it is thought that the mutation also affects the glycosylation of the neighbouring asparagine residue at position 413 (157). It was found that the SNP is highly conserved through many different species from fish to mammals and is present in the human population at a very high frequency (157). It has been found that in the European and Asian populations, the minor allele frequency of TLR3 L412F is approximately 32 and 26% respectively, while in Sub-Saharan Africa the frequency of the SNP is much less at about 3% (165). Table 1.3 below details the global distribution of the TLR3 L412F (165).
<table>
<thead>
<tr>
<th>Region</th>
<th>Minor allele frequency</th>
<th>Wild-type frequency (Leu/Leu)</th>
<th>Heterozygote frequency (Leu/Phe)</th>
<th>Homozygote frequency (Phe/Phe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
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<td>60.9%</td>
<td>31.7%</td>
<td>7.3%</td>
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<td>32%</td>
<td>44.7%</td>
<td>45.7%</td>
<td>9.5%</td>
</tr>
<tr>
<td>South Asian</td>
<td>26%</td>
<td>54.8%</td>
<td>37.8%</td>
<td>7.4%</td>
</tr>
<tr>
<td>African</td>
<td>3%</td>
<td>94.9%</td>
<td>5%</td>
<td>0.2%</td>
</tr>
</tbody>
</table>
Figure 1.9. *TLR3 L412F* single nucleotide polymorphism

The SNP L412F encodes a leucine to phenylalanine substitution at position 412. It is located in the extracellular domain of the TLR3 receptor. (A). It is a highly conserved SNP which can be found in many species from animals to fish (B). Image taken from (157).
1.6.2 Role of L412F in defective TLR3 function

Many groups, including our lab, have used different methods; including NF-κB luciferase assays, ELISAs and qPCR, to investigate the effect of the SNP on TLR3 responses in vitro (154, 157, 166, 167). As previously mentioned, activated TLR3 receptors activate a downstream signalling cascade leading to the activation and translocation of the transcription factors, NF-κB and IRF3 to the nucleus which leads to the expression of pro-inflammatory cytokines and type I interferons. In the presence of the L412F SNP we have shown that there is reduced activation of transcription factors, NF-κB and IRF3 and subsequently a reduction in the expression of pro-inflammatory cytokines and type I interferons, respectively (154). When analysed by NF-κB luciferase assay it was shown that the L412F had approximately 30% reduction in reporter activity compared with WT TLR3 receptor activity (157).

1.6.3 TLR3 L412F disease associations

1.6.3.1 Idiopathic Pulmonary Fibrosis (IPF)

The Donnelly group previously characterised TLR3 L412F in the context of the interstitial lung diseases, IPF and pulmonary sarcoidosis. As previously described, IPF is a chronic, progressive fibrosing interstitial pneumonia of unknown etiology with a mean survival rate of less than 3 years (5). O’Dwyer et al. in 2013 found a significant association between TLR3 L412F and disease progression in IPF. Two IPF patient cohorts were genotyped for the polymorphism: (i) a UK cohort of 170 IPF patients and (ii) 138 patients from the placebo group of the INSPIRE clinical trial (40, 154). It was found in both cohorts that TLR3 L412F was not associated with the development of IPF, however, it was shown that the presence of the variant allele led to a significantly increased hazard ratio. 412F-heterozygous and homozygous IPF patients had 5 times greater risk of mortality at 12 months and 24 months compared with TLR3 L412F wild-type patients. Additionally, it was found that there was an association between TLR3 L412F and accelerated decline in FVC in 412F-heterozygous and homozygous IPF patients. This highlighted a potential role for TLR3 L412F in disease progression in IPF (154).
Finally, the Donnelly group also investigated TLR3 function in human primary lung fibroblasts in vitro from TLR3 L412F wild-type, heterozygote and homozygote IPF patients using TLR3’s synthetic ligand Poly(I:C). We found that human lung fibroblasts from 412F-heterozygote and homozygote IPF patients had reduced pro-inflammatory cytokine and type I interferon expression in response to Poly(I:C) stimulation. TLR3 receptor mRNA expression was also reduced in TLR3 L412F heterozygote and homozygote IPF patients. We also showed dysregulated fibroproliferation following TLR3 activation (154). These findings led us to propose a role for TLR3 L412F in disease progression in IPF due to defective TLR3 signalling.

1.6.3.2 Pulmonary Sarcoidosis

The Donnelly research group has also identified a role for TLR3 L412F in pulmonary sarcoidosis. TLR3 L412F has been associated with a persistent clinical phenotype in pulmonary sarcoidosis (168). Sarcoidosis is a chronic granulomatous disease of unknown etiology affecting adults between 20 and 60 years of age (169). The diagnosis of sarcoidosis is difficult as many symptoms of sarcoidosis are also seen in a number of different diseases. Lung involvement is typically seen in approximately 90% of sarcoidosis patients. Typically patients present with non-caseating granulomas, inflammation in the lungs and abnormal pulmonary function tests (170). Patients usually follow one of two disease paths, patients either experience an acute form of the disease which will naturally resolve within 1-2 years (85% of patients) or develop a long term persistent disease (171). Standard treatment for sarcoidosis patients, unlike IPF, is the use of steroids. There are now other biological therapies used in the treatment of sarcoidosis. A long term persistent phenotype is seen in about 30-50% of pulmonary sarcoidosis patients (171). Previously in our lab, Cooke et al. have found in two separate sarcoidosis cohorts (Irish cohort: n= 228 and American cohort: n=123) a significant association between the development of a persistent clinical phenotype and the TLR3 L412F polymorphism (168).

TLR3 function was investigated in human primary lung fibroblasts in vitro from TLR3 L412F wild-type and homozygote sarcoidosis patients using TLR3’s synthetic ligand Poly(I:C). It was shown that human lung fibroblasts from 412F-
homozygote sarcoidosis patients had reduced type I interferon expression in response to Poly(I:C) stimulation. Dysregulated fibroproliferation and reduced apoptosis was also shown following TLR3 activation (168). Taken together, these findings suggested a role for the TLR3 L412F polymorphism in the development of a persistent clinical disease in pulmonary sarcoidosis.

1.6.3.3 *Age-related macular degeneration (AMD)*

Age-related macular degeneration (AMD) is the most common cause of irreversible blindness worldwide. AMD presents in two different forms; “dry” AMD or geographic atrophy and “wet” AMD or choroid neovascularisation. TLR3 L412F has been associated with geographic atrophy (172). In two independent studies it was found that the T allele was associated with protection against geographic atrophy (P=0.005) but not choroidal neovascularisation. *In vitro* and *in vivo* studies by Yang *et al.* found that wild type TLR3 L412F primary human retinal epithelial cells stimulated with Poly(I:C) had increased cell death *in vitro* compared with cells carrying the variant allele. In an *in vivo* model TLR3<sup>+/−</sup> mice were injected intravitreally with Poly(I:C). It was found that after two weeks, TLR3<sup>+/−</sup> mice had not developed features of RPE and retinal cell death but the wild-type mice had retinal and RPE cell loss. Together these results suggested that reduced activation of TLR3 due to the mutation TLR3 L412F protected against geographic atrophy (172). Conversely, other groups have been sceptical of the results published by Yang *et al.* and have reported that the SNP is not associated with geographic atrophy (173, 174).

Similarly, a small study in China has shown that there is no association between neovascular age-related macular degeneration and polypodial choroidal vasculopathy and TLR3 L412F (175). Cheng *et al.* identified 6 TLR3 SNPs in total (rs5743303, rs5743305, rs5743312, rs3775291, rs3775290, and rs6830345) and found no association between any TLR3 SNP and neovascular age-related macular degeneration and polypodial choroidal vasculopathy. Neovascular age-related macular degeneration is another form of “wet” AMD (175).

1.6.3.4 *Human Immunodeficiency Virus 1 (HIV-1)*

TLR3 L412F has been shown to confer resistance to HIV-1 infection. Sironi *et al.* genotyped two different populations: (i) Spanish drug users who have remained
seronegative for HIV-1 despite repeated exposures to HIV-1 through injection drug use and (ii) an Italian cohort of patients sexually exposed to HIV-1 yet remained seronegative (176). It was found in the Spanish cohort that the number of patients carrying the variant allele was at a significantly higher frequency in seronegative HIV-1 patients. The odds ratio for protection from HIV-1 was 1.87. A similar result was seen in the Italian cohort with an odds ratio of 1.79 (176). To further confirm their findings Sironi et al. undertook in vitro infection assays with PBMCs from subjects who were wild-type or carrying the variant allele. It was shown that patient’s PBMCs that were carrying at least one copy of the Phe allele have lower viral replication compared with wild type patients (Leu/Leu). These results suggested that TLR3 L412F has a protective effect in HIV-1 infection.

1.6.3.5 *Herpes Simplex virus Type 2 (HSV-2)*

It has been suggested that TLR3 L412F is protective in HSV-2 infection. 239 patients with genital HSV-2 infection and 162 healthy controls were screened for the TLR3 L412F mutation. The results showed that the minor allele frequency was more common among HSV-2 seronegative subjects (35%) compared with infected individuals (28%) (P=0.0272) (177). Svensson et al. also analysed whether TLR3 L412F was associated with disease severity but no association was found (177).

1.6.3.6 *Type I Diabetes Mellitus*

Assman et al. genotyped 448 T1DM and 461 non-diabetic subjects for 5 different TLR3 polymorphisms. They found that TLR3 L412F is associated with the risk of Type 1 diabetes mellitus (T1DM). Patients with at least one copy of the variant allele were associated with increased risk for T1DM with an odds ratio of 2.3 (178).

1.6.3.7 *Human Cytomegalovirus (CMV)*

A study was conducted in children infected with cytomegalovirus (HCMV) and uninfected children. All patients were genotyped for a number polymorphisms in TLR2, 3 7 and 9 genes, including TLR3 L412F. At least one copy of the variant TLR3 L412F allele was found to be significantly associated with increased risk of HCMV infection (179).
1.6.3.8 **Tick-borne encephalitis (TBE)**

A number of studies have analysed whether there is an association between *TLR3* L412F and risk of TBE and disease severity. Tick-borne encephalitis is a zoonotic RNA virus which can cause severe clinical symptoms such as paralysis by targeting the central nervous system (CNS) and in some cases can be fatal (180). It has been shown that there is a significantly higher frequency of wild type (Leu/Leu) in TBE patients compared with healthy controls suggesting that functional TLR3 is a risk factor for TBE (181-183).

1.6.3.9 **Systemic lupus erythematosus (SLE)**

A cohort of age-matched female SLE patients and healthy donors were genotyped for the *TLR3* L412F mutation and a significant association was found between the mutation and SLE (184). The variant allele was found at increased frequency in the SLE patients compared with controls, highlighting a possible role for the SNP in SLE (184).

1.6.3.10 **Hepatitis B virus**

In a large multicentre study of caucasian patients, Fischer et al. found that the 412F-variant allele was more prevalent in chronic hepatitis B virus (HBV) infection patients compared with healthy controls which correlated with an increased risk of developing chronic HBV (185). Furthermore, the group found that 412F-homozygous patients were associated with reduced likelihood of spontaneous hepatitis B surface antigen seroclearance (185).

1.6.3.11 **Enteroviral myocarditis/ cardiomyopathy**

Gorbea et al. screened patients diagnosed with enteroviral myocarditis/cardiomyopathy for mutations in TLR3 (167). It was found that *TLR3* L412F homozygotes were more frequently seen in patients compared with control subjects. *In vitro*, the group analysed TLR3 responses in the presence and absence of the *TLR3* L412F after Poly(I:C) stimulation and Coxsackievirus B3 viral infection. Similar to results obtained by other groups (154, 157, 168) it was found that signalling through NF-κB and IRF3 was reduced in the presence of the SNP. It was also shown that this reduction in pro-inflammatory cytokine and type I interferon
expression was beneficial to Coxsackievirus B3 viral infection. In the presence of the SNP, lower levels of type I interferons correlated with increased viral replication compared with TLR3 L412F wild-type cells (167).

1.7 Autophagy

1.7.1 Forms of autophagy

There are two major degradative mechanisms in the cell: the ubiquitin-proteosome system and autophagy. The ubiquitin-proteosome system tags proteins with chains of ubiquitin molecules for degradation and recycling of amino acids via the 26S proteasome (186). Autophagy is an evolutionary conserved cellular process which is crucial for normal cellular function. Autophagy is important to a number of different cellular processes including protein turnover and recycling, pathogen clearance and antigen presentation. Autophagy consists of three main pathways; macroautophagy, microautophagy and chaperone-mediated autophagy. In general the term “autophagy” is used to describe macroautophagy as it is the best characterised of the three pathways.

Microautophagy is another form of non-selective autophagy where the lysosome directly engulfs the cytoplasmic contents of a cell for degradation (187). Chaperone mediated autophagy (CMA) is the third form of autophagy which selectively degrades single cytosolic proteins in the lysosomes. Both CMA and microautophagy can be induced by nutrient deprivation, CMA is also induced by hypoxia and oxidative stress. CMA is carried out in mammalian cells by heat shock cognate protein 70 (hsc70) along with the LAMP-2 protein which recognises proteins with a penta-peptide motif and delivers them to the lysosome for degradation (188, 189).
The term autophagy refers to a number of different forms of autophagy. Macroautophagy (also classically referred to as autophagy) was the first discovered and most well-defined form of autophagy. Microautophagy and chaperone-mediated autophagy (CMA) are other non-selective forms of autophagy. In selective autophagy; autophagic degradation of specific organelles is referred to by the organelle being degraded. For example, mitophagy refers to autophagic degradation of mitochondria, aggrephagy refers to autophagic degradation of protein aggregates and pexophagy refers to autophagic degradation of peroxisomes. Image taken from (190).

1.7.2 Macroautophagy

Macroautophagy (from now on referred to as autophagy) comes from Greek and means “self-eating”. It is a cellular process that has been evolutionarily conserved across many species from yeast to humans. It is a mechanism by which cells remove large cellular targets such as dead and damaged organelles and denatured proteins. Double-membraned vesicles known as autophagosomes are formed in the cell which engulf its “cargo” i.e. proteins and organelles in the cytoplasm. To degrade the cargo the autophagosomes fuse with lysosomes to form autophagolysosomes in the cell and acidification occurs. Enzymes in the autolysosomes breakdown the proteins and organelles into macromolecules so that they can be used again by the cell. Autophagy also functions to assist in the clearance of intracellular infection. One of the primary functions of autophagy is to
ensure cell survival in times of stress and starvation by breaking down organelles and proteins to recycle the macromolecules and generate nutrients and energy for the cell. The autophagic pathway is a long and complex pathway involving many different proteins and stages. Figure 1.11 summarises the various stages and proteins of the autophagy pathway.

**Figure 1.11. Stages of Autophagy**

Autophagy is initiated through the ULK-1 complex. Autophagy can be broken down into a number of stages, initiation, elongation, maturation, fusion with the lysosome and degradation of the autophagosome contents. Image taken from (191).

**Autophagy Pathway**

The autophagy pathway is described in detail in reviews by Kaur et al. and Glick et al.(190, 192). Briefly, the autophagy process starts with formation of a phagophore assembly site (PAS) which is mediated by the ULK1 complex. A double layered crescent shaped lipid bilayer known as a phagophore is initiated by the ULK1 complex when ULK1 conjugates to the class III PI3K protein, VPS34 complexed with Beclin 1 (mammalian ortholog of Atg6). Other proteins form a complex with ULK1 and Beclin 1 such as Atg14L, Atg2 and Atg9. This step of the process is known as autophagic nucleation which forms the phagophore. Atg14L is important for promoting autophagosome-endolysosome fusion during autophagy (193). This induces an Atg conjugation cascade which causes the conjugation of the
Atg5-Atg12-Atg16L complex with the extending phagophore. The Atg5-Atg12 complex mediates vesicle elongation in the autophagosome formation process. Recruitment of LC3B into the phagophore is controlled by the Atg5-Atg12 complex. Once it becomes incorporated into the autophagosome, LC3-I is converted to LC3-II by conjugation with phosphatidylethanolamine. LC3 conjugation to phosphatidylethanolamine is essential for expansion of autophagic membranes and fusion of the autophagosomes with the lysosomes (194, 195). LC3-II protein expression is indicative of increased autophagy in the cell and the ratio of LC3-I: LC3-II protein expression is routinely used in experiments to analyse autophagic flux.

1.7.3 Induction of autophagy

Role of mTOR in autophagy

Autophagy can be initiated by many different stimuli via several different pathways. The mTOR pathway is a major pathway associated with the initiation of autophagy. When nutrients are plentiful in a cell, activated mammalian target of rapamycin (mTOR) prevents the induction of autophagy by inhibiting the ULK1 protein through phosphorylation of its Ser757 residue (196, 197). In times of nutrient stress and starvation activated AMPK inhibits mTOR through phosphorylation of upstream proteins TSC2 and Raptor. One example of this is during infection, the presence of intracellular pathogens leads to competition in the cell for nutrients. Inhibition of mTOR leads to reduced phosphorylation of the ULK1 kinase at the Ser757 residue while AMPK phosphorylates ULK1 at several different sites. This induces autophagy initiation (196). Rapamycin, an inhibitor of mTORC1, is a potent autophagy inducer (197). Two standard methods of inducing autophagy in vitro are rapamycin treatment and Hanks Balanced Salt Solution (HBSS), both of which inhibit mTOR activation.

Role of TLRs in autophagy induction

TLRs can also initiate autophagy. Delgado et al. screened a number of TLR PAMPs to examine autophagy induction (198). They found that TLR7, when activated by imiquimod and ssRNA, induced the greatest levels of autophagy in RAW 264.7 cells. TLR3 and TLR4 activation was also shown to induce autophagy.
in murine macrophages (198). TRAF6, a downstream E3 ligase signalling protein in the TLR pathway, ubiquitinates two major autophagy proteins; Beclin 1 and ULK1 (199). It has been demonstrated that LPS-induced TLR4 activation causes the activation of TRAF6 and subsequent ubiquitination of Beclin 1. This ubiquitination releases Beclin 1 from its inhibitor B cell lymphoma 2 (Bcl-2) and initiates autophagy through formation of a complex with the lipid kinase VPS34, a class III PI3K (200, 201). TRAF6 also poly-ubiquitinates the kinase ULK1 to allow activation and interaction with the Beclin 1 complex (202). Importantly, autophagy is also important for TLR signalling and signalling through other PRRs. It has been discovered that autophagy influences many other cellular pathways including the TLR pathway. In lung cancer cells it was shown that autophagy is important in TLR3 and TLR4 signalling. Inhibition of autophagy results in defective TLR signalling leading to reduced expression of pro-inflammatory cytokines and chemokines such as IL-6 and CCL2 (203). Similarly, Gorbea et al. found that inhibition of autophagy resulted in decreased TLR3-induced type I interferon expression following Poly(I:C) stimulation (167).

Conversely, in 2007 it was shown that autophagy, specifically the Atg5-Atg12 complex, negatively regulates type I interferon production via direct interaction with Rig–I and IFN-β promoter stimulator 1 (IPS1) (204). Inomata et al. found that the autophagy receptor NDP52 negatively regulated TLR signalling by targeting the downstream TLR signalling protein TRAF6 (205). NDP52 causes selective autophagic degradation of TRAF6 through direct binding, resulting in disruption NF-κB signalling.

A number of cytokines can also regulate autophagy (206). Th1 cytokines are thought to induce autophagy while Th2 cytokines inhibit it. The Th1 cytokine, IFN-γ, has been shown to induce formation of autophagosomes in macrophages (207). Similarly, the Th1 cytokine, TNF-α has been shown in a number of cells and species to induce autophagy (206). For example, in vascular smooth muscle cells, TNF-α induces the expression of autophagy genes Beclin-1 and microtubule-associated protein 1 light chain 3 (MAPLC-3) through activation of the JNK pathway. In contrast, during *Mycobacterium tuberculosis (M. tb)* infection it was shown that Th2 cytokines IL4 and IL13 can abrogate autophagy and inhibit clearance of the bacteria (208). IL-4 and IL-13 have also been shown to inhibit starvation induced autophagy
in vitro. Type I interferons also activate autophagy through the JAK/STAT and PI3K/Akt/mTORC1 pathway (209, 210).

### 1.7.4 Role of autophagy in lung disease

Autophagy has been associated with a number of different lung diseases playing either a beneficial or a detrimental role. Autophagy has been associated with diseases like COPD, cystic fibrosis (CF), pulmonary hypertension and respiratory infection (211). In COPD, Chen et al. proposed a detrimental role for autophagy in the disease. There are higher levels of autophagy proteins, LC3-II, Atg5-12 complex, Atg4 and 7, compared with controls. Chen et al. discovered that higher levels of the late phase autophagy protein, LC3-II, in COPD patient lungs correlated with increased levels of cell death (212). In a murine model for COPD, LC3B deficient mice had reduced cigarette smoke extract-induced cell death and emphysematous air space enlargement compared with wild-type mice suggesting a role for autophagy in regulating cell death (212). Similarly, in CF patients, defective autophagy is associated with disease pathogenesis. It has been found that defects in the cystic fibrosis transmembrane conductance regulator (CFTR) lead to defective autophagy resulting in reduced clearance of misfolded protein aggregates characteristic of CF (213).

In contrast, pulmonary hypertension patients have increased levels of LC3B and autophagosomes in lung tissue compared with normal lungs (214). Higher levels of LC3B were associated with a protective effect in hypoxic conditions and therefore in pulmonary hypertension.

### 1.7.5 Role of autophagy in IPF

In IPF the role of autophagy has been investigated by a number of different groups. Patel et al. in 2012 showed that in whole lung homogenate from IPF patients there was decreased autophagic activity when compared with control lung tissue (215). The group also found that TGF-β1 inhibits autophagy in human lung fibroblasts in vitro and therefore postulated that the high levels of TGF-β1 in IPF patients could lead to reduced autophagy (172). Interestingly, another group investigated whether decreased levels of autophagy in IPF patients may play a role in
accelerated epithelial cell senescence and myofibroblast differentiation (216). It was shown that autophagy inhibition induced myofibroblast differentiation and accelerated cellular senescence in epithelial cells but not fibroblasts (216). Similarly, Im et al. also suggested that decreased autophagy could be due to reduced FoXO3a expression which could contribute to IPF pathogenesis (217). As previously mentioned, aging is a factor in the development of IPF (218). During the aging process, dysregulation of cellular processes such as autophagy and mitochondrial dysfunction can occur (21). Specifically, dysregulation of mitophagy (autophagic degradation of mitochondria) can be seen in IPF patients (219). It was found that insufficient mitophagy-induced mitochondrial ROS production results in myofibroblast differentiation and proliferation which could have a role in the pathogenesis of IPF (220). Aging in IPF has also been linked to mTOR activity and autophagy in IPF fibroblasts. Romero et al. has shown that IPF fibroblasts exhibit persistent mTOR activation under starvation conditions which results in apoptosis resistance in IPF fibroblasts (27). They found that IPF fibroblasts had decreased levels of mTOR inhibition following starvation which correlated with reduced induction of autophagy (27).

Interestingly, in a rat model of bleomycin-induced fibrosis, it was found that administration of rapamycin (an mTOR inhibitor/autophagy inducer) with bleomycin reduced levels of fibrosis seen in the lungs compared with bleomycin alone (221). In 2016 a study was published by Mercer et al. describing the effects of a new candidate drug, GSK2126458 (also known as omipalisib), as a treatment for IPF (222). This drug is a PI3K/mTOR inhibitor which is currently under testing for use in cancer treatments. As outlined previously in Section 1.6.3, mTOR inhibition leads to the induction of autophagy. While the group did not measure the levels of autophagy in IPF fibroblasts, in vitro experiments showed that omipalisib reduced serum-induced Akt phosphorylation and proliferation of IPF lung fibroblasts as well as TGF-β-induced collagen production (222). Together these results could suggest a beneficial role for induction of autophagy in IPF. Recently, this PI3K/mTOR inhibitor, omipalisib, has been evaluated in a small study of 17 patients for the treatment of IPF (223). It was found that the drug was able to inhibit PI3K activity and patient’s tolerance of the drug was acceptable. No severe adverse events were
seen. Further clinical trials will be needed to assess omipalisib as a potential treatment for IPF (223).

1.7.6 Role of autophagy in viral infection

Substantial research has been conducted examining the relationship between viruses and autophagy. As previously mentioned, autophagy is a cellular mechanism by which cells eliminate intracellular viral and bacterial infections. Overtime viruses have evolved ways to disrupt or hijack the autophagy pathway to facilitate their survival. Examples of viruses that hijack the autophagy pathway are Influenza A virus, Respiratory Syncytial Virus (RSV), Herpes Simplex 1 (HSV-1), poliovirus and Human Immunodeficiency Virus (HIV) which all exploit autophagy for their survival. Influenza A virus (IAV) is a major respiratory pathogen which also hijacks the autophagy pathway for its survival.

RSV is a single stranded (ss)RNA virus. RSV infection is a major cause of acute lower respiratory tract infection worldwide in children. Recently it has been shown by Li et al. that RSV induces autophagy through production of reactive oxygen species (ROS) (224). ROS activates AMPK signalling which in turn induces autophagy upon infection. Induction of autophagy benefits RSV by inhibiting apoptosis allowing for increased viral replication and lung pathology in vivo (224). However this induction of autophagy by RSV can also be beneficial to the host. Previously it was found that RSV-induced autophagy was critical to dendritic cell maturation and production of pro-inflammatory cytokines in response to infection (225).

HIV targets different proteins of the autophagy pathway. Upon infection of the host, HIV Gag proteins interact with LC3 to augment Gag processing in macrophages (226). HIV expresses the virulent Nef protein which is responsible for HIV pathogenicity. The Nef protein interacts with Beclin-1 to prevent autophagosome fusion with the lysosome, thereby protecting HIV viral particles from degradation (226, 227).

Similarly, it has been shown that the HSV-1 inhibits autophagy through the protein ICP34.5 (228). Like the HIV protein Nef, ICP34.5 interacts with Beclin-1
through the Beclin-1 binding domain leading to the inhibition of Beclin-dependent autophagy induction (228).

It has been suggested by Jackson et al. that the poliovirus uses the host’s autophagic machinery to form double-membraned vesicles during infection. They found that the poliovirus can associate with LC3 and LAMP-1 leading to the formation of these vesicles (229, 230). Furthermore, in contrast to the previous examples, it has been found that poliovirus does not prevent autophagic degradation but induces it during infection (231). For successful viral particle maturation, the acidification of cellular compartments must take place. Richards et al. showed that inhibition of the acidification of the amphisome-like vesicles prevented maturation of the final viral particles into infectious virions (231, 232).

1.7.6.1 Role of autophagy in Influenza A viral infection

Influenza A virus (IAV) affects a number of species including humans, pigs and birds. IAV H1N1 infection has been the cause of pandemics such as the 1918 Spanish Flu which killed over 40 million people (148). IAV is a member of the Orthomyxoviridae family. It is an enveloped virus that consists of a lipid-bilayer capsule in which viral glycoproteins haemaglutinin (HA) and neuraminidase (NA) are embedded (233). Inside the capsule the virus contains 8 short unique segments of negative sense ssRNA surrounded by viral nucleoproteins which encode 11 proteins (233). IAV has evolved over time to manipulate a number of different pathways within the host to replicate and evade the host’s immune response. Autophagy is one of the major pathways that IAV uses to replicate and reproduce. It has been reported that the IAV induces the autophagy pathway to allow for greater viral replication (92, 234). Recently it has been shown that IAV induces autophagy by inhibiting Akt-mTOR pathway in vitro (235). It was also shown that inhibition of autophagy in vitro impaired IAV viral RNA synthesis and viral protein expression including IAV nucleoprotein and M2 protein (235).

Gannagé et al. in 2009 found that the IAV H1N1 M2 ion channel protein was sufficient to compromise macroautophagy by blocking autophagosome degradation. The H1N1 M2 protein prevents fusion of the autophagosome in the host cell with the lysosomes preventing acidification and degradation, leading to perinuclear accumulation of the autophagosomes (236). This is achieved by the M2 protein
binding to the Beclin-1 protein of the host autophagy pathway (227, 237). It was found by another group that the IAV H1N1 M2 protein can disrupt the autophagic machinery by directly interacting with the LC3 protein (238). The M2 protein contains an LC3-interacting region (LIR) which directly interacts with the host LC3 protein and directs localisation of LC3 to the plasma membrane to benefit aspects of the viral life cycle such as budding (238).

1.8 mTOR

1.8.1 mTOR

The mTOR pathway orchestrates many cellular functions including; growth, metabolism and survival (239). It is a conserved multi-component Serine/Threonine kinase which acts downstream by phosphorylating target proteins in response to environmental stimuli. There are two distinct mTOR complexes; mTORC1 and mTORC2. The mTORC1 complex is involved in growth, metabolism and cell survival (239). mTORC2 activity plays a role in actin organisation and cellular growth via activation of downstream protein Akt (240). mTORC1 has been shown to be more sensitive to inhibition by rapamycin compared with mTORC2 which is thought to be relatively insensitive to rapamycin treatment.

In our work we focus on mTORC1 activation and activity and will refer to as mTOR. As previously mentioned mTOR is a negative regulator of autophagy. mTOR acts as a nutrient and stress sensor in the cell which under normal physiological conditions becomes activated in response to nutrients and growth factors. In times of nutrient deprivation or environmental stress such as ER stress and hypoxia, mTOR is inhibited and autophagy is induced through the phosphorylation of the autophagy protein ULK-1 (196).

1.8.2 mTOR and TLR signalling

TLRs can activate the mTOR pathway via activation of class I phosphatidylinositol 3-kinases (PI3Ks). PI3K is activated by numerous stimuli including cytokines, growth factors and TLR activation. PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) to act as a second messenger that
induces the phosphorylation of Akt on Thr308. Akt phosphorylates and inhibits the heterodimer tuberous sclerosis complex 1 (TSC1)/TSC2. This in turn activates Rheb and in turn mTORC1 activation occurs (241).

mTOR is important in the production of pro-inflammatory cytokines and type I interferons through regulation of mRNA transcription and protein translation (242). mTOR controls translation through inhibition of downstream proteins eIF4E-binding proteins (4E-BPs). 4E-BPs are translational repressors which mTOR can phosphorylate releasing the eukaryotic translation initiation factor 4E (eIF4E), which in turn promotes translation (242).

It has been found that the PI3K/Akt/mTOR pathway is important in the expression of IFN-stimulated gene (ISG) expression (243, 244). Type I interferons are known to act back on the cell via the IFNAR1 to induce the downstream Jak/STAT pathway (245). Type I interferons can also activate a separate pathway via the insulin receptor substrate 1 (IRS1) protein to activate PI3K and subsequently mTOR. IFN-induced phosphorylation of Akt is essential for subsequent activation of mTOR and mRNA translation of ISGs (209). In dendritic cells it has been shown that efficient translation of IRF7 is controlled by mTOR through inhibition of 4E-BPs and therefore regulates induction of type I interferons (246).

1.8.3 The effects of mTOR activation in fibrosis

The role of mTOR in the pathogenesis of IPF has been examined by a number of research groups. Previously, Romero et al. found that IPF fibroblasts had decreased levels of mTOR inhibition following starvation compared with normal healthy fibroblasts which correlated with reduced induction of autophagy (27). Furthermore, they have shown that IPF fibroblasts exhibit persistent mTOR activation under starvation conditions which results in apoptosis resistance in IPF fibroblasts (27). Inhibition of the mTOR pathway has also been shown to be beneficial in lung fibrosis murine models. Patel et al. used the bleomycin model of fibrosis in mice to examine the effect of mTORC1 signalling. Rapamycin-induced inhibition of mTOR ameliorated bleomycin-induced lung fibrosis in mice therefore providing evidence that mTORC1 signalling was detrimental in fibrosis (215). Additionally, it was shown that rapamycin had a protective effect in a separate
murine model of pulmonary fibrosis; TGF-α-induced pulmonary fibrosis (247), providing further evidence of the role of mTOR in fibrosis.

Hyperactivation of mTOR and low induction of autophagy has been shown to be detrimental in a number of diseases. Lymphangioleiomyomatosis (LAM) is progressive cystic lung disease seen in females (248). Due to loss of function mutations in the tuberous sclerosis complex (TSC), mTOR remains constitutively active causing autophagy to be kept at low levels (249). Rapalogues (derivatives of rapamycin) targeting mTOR activity have been trialled for the treatment of LAM (249).

Sustained activation of mTOR has also been shown to play a role in fibrosis. Previously Chen et al. found that macrophages and myofibroblasts had persistent mTOR activation during the development of kidney fibrosis in mice. It was shown that rapamycin administration ameliorated kidney fibrosis by inhibiting mTOR activity in myofibroblasts and macrophages (250). Moreover, this group identified that LPS-induced persistent mTOR activation resulted in chronic kidney injury and kidney fibrosis (251). In a murine model for chronic kidney disease and fibrosis, mice were given continuous low dose LPS treatment to model endotoxemia in the mice. LPS treatment induced continuous mTOR activation and the persistent endotoxemia resulted in progressive macrophage infiltration, tubular injury and collagen deposition in the kidneys. Treatment with rapamycin, ameliorated LPS-induced kidney injury and matrix deposition, suggesting that mTOR activation played a significant role in kidney injury and fibrosis (251).
1.9 Aims and Objectives

Hypothesis:

Our central hypothesis is that defective signalling through TLR3 in the presence of the SNP may be detrimental in the immune response to bacterial and viral infections resulting in accelerated disease progression in IPF. We believe that TLR3 L412F dysregulates not only the TLR3 pathway but other TLR and PRR pathways resulting in reduced ability to fight and clear viral and bacterial infections in TLR3 L412F heterozygous and homozygous IPF patients, therefore accelerating their disease progression.

Overall Aim:

The overall aim of this research project is to evaluate the role of the TLR3 L412F polymorphism in disease progression in IPF in the context of anti-viral and anti-bacterial TLR responses.

Research objectives:

- To characterise the role of TLR3 L412F in disease progression in IPF patients in the context of anti-viral TLR responses;

- To characterise the role of TLR3 L412F in disease progression in IPF patients in the context of anti-bacterial TLR responses;

- To characterise the role of TLR3 L412F in autophagy in primary human IPF fibroblasts;

- To characterise the functionality of the novel TLR3 L413F knock-in mouse (generated by CRISPR/Cas9 gene editing).
Chapter 2

Materials and Methods
Chapter 2 Materials and Methods

2.1 Materials

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2.2 Methods for human experiments

2.2.1 Cell Culture

2.2.1.1 Primary human lung fibroblast cell lines

Primary human pulmonary fibroblast cell lines were kindly donated by Professor Cory Hogaboam, Department of Medicine, Cedars Sinai, Los Angeles, USA and Professor Seamas Donnelly, St Vincent’s University Hospital, Dublin, Ireland. All work was undertaken in a sterile laminar flow in a purpose built room. Cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin (pen/strep) (from now on referred to as complete medium). Cells were maintained in a humidified environment (95% relative humidity) containing 5% CO$_2$ at 37°C.

2.2.1.2 Harvesting and subculture of primary cell lines

All fibroblasts were sub-cultured when they reached 80%-90% confluency. Cells were sub-cultured by removing medium and prewashed with sterile pre-warmed PBS. Cells were detached from the flask through incubation of the cells in 5mls of Trypsin-EDTA for 5 minutes at 37°C. Complete medium was added to neutralise the Trypsin-EDTA in the sterile laminar flow hood and the cells were collected. Cells were spun down in a centrifuge at 1200 rpm for 5 minutes at room temperature (RT).

For subculture, the cell pellet was re-suspended in fresh medium and split 1:2 or 1:3 into fresh T175 tissue culture flasks and returned to sterile incubators.

2.2.2 Cryopreservation of primary cells

Primary human lung fibroblasts were cultured in T175 flasks until confluent. Cells were harvested as previously described. The cell pellet was then re-suspended in complete DMEM and the cells were counted. 1.2x10$^6$ cells were frozen down per vial in a solution containing 95% FBS and 5% DMSO. The DMSO was added dropwise to the FBS/cell solution. Cells were frozen down in 1.5 ml cryovials and
stored in the -80°C freezer for 24 hours and transferred to liquid nitrogen for long-term storage.

2.2.3 TLR3 L412F genotyping assay (human)

2.2.3.1 Extraction of genomic DNA from primary human lung fibroblasts

Primary human lung fibroblasts were cultured in T175 flasks until confluent. Cells were harvested as previously stated. Genomic DNA was harvested from the cells using the Macherey-Nagel NucleoSpin® Blood kit (Ref. 740951.250). A maximum of 5x10⁶ cells could be used per each genomic DNA preparation vial. Cells were counted and then spun down at 1,200 rpm at RT for 5 minutes. The resulting cell pellet was reconstituted in 200 µl sterile PBS. 25 µl of Proteinase K was added to the sterile eppendorf tube containing the cells. 200 µl of Buffer B3 was added to each sample and all samples were vortexed. The samples were incubated on a heat block at 70°C for 10-15 minutes while shaking (Eppendorf ThermoMixer C). 210 µl of 100% ethanol was added to each tube and vortexed vigorously. The samples were then loaded into Nucleospin columns fitted with collection tubes and spun at 11,000 x g for 1 minute. The collection tube was discarded and another collection tube was attached to the Nucleospin column. 500 µl of Buffer BW was added to the column. The samples were spun at 11,000 x g for 1 minute. The collection tube was discarded and another collection tube was attached to the Nucleospin column. 600 µl of Buffer B5 was added to the column. The samples were spun at 11,000 x g for 1 minute. The collection tube was discarded and another collection tube was attached to the Nucleospin column. To ensure that all buffer was removed from the samples, the samples are centrifuged again dry at 11,000 x g for 1 minute. The elution buffer was warmed to 70°C. To elute the DNA, the Nucleospin tubes were attached to new labelled sterile eppendorf tubes. To gain the maximum yield of DNA from the samples 50 µl of pre-warmed elution buffer was added to each sample. The samples were left in the elution buffer for 3 minutes at RT before being centrifuged at 11,000 x g for 1 minute. Another 50 µl of pre-warmed elution buffer was added to each sample. The samples were left in the elution buffer for 3 minutes at RT before being centrifuged at 11,000 x g for 1 minute. The DNA samples stored at -20°C.
2.2.3.2 Extraction of genomic DNA from whole blood samples

Whole blood samples were stored at -80°C in EDTA blood tubes. Blood must be stored in EDTA tubes for this experiment as the EDTA acts as an anti-coagulant. Blood was de-frosted at RT on the bench. Genomic DNA was harvested from blood samples using the Macherey-Nagel NucleoSpin® Blood kit (Ref. 740951.250). Once completely thawed, 200 μl of whole blood was placed in a sterile eppendorf tube. If there is less than 200 μl of blood available, sterile PBS can be used to adjust the volume to 200 μl. The minimum volume of whole blood recommended is 5 μl. 25 μl of Proteinase K and 200 μl of Buffer B3 is added to each sample and vortexed thoroughly. At this point the procedure is the same as detailed above in Section 2.2.2.1.

2.2.3.3 TLR3 L412F SNP genotyping assay (human)

The TLR3 L412F single nucleotide polymorphism (SNP) assay is fully validated and provided by Applied Biosystems.

The assay requires three specific components:

1. TaqMan® Universal Master Mix (MM)
2. 40X SNP genotyping assay
3. Genomic DNA (gDNA)

As previously described the genomic DNA was extracted and quantified. This gDNA was then diluted to the necessary working concentration of 10 ng/ml for the SNP Assay. 1.5 μl of gDNA was added to PCR tubes first and a master mix was prepared and added to the tubes. The reaction volumes are as follows for one sample:

Table 2.1: Reaction components for qPCR

<table>
<thead>
<tr>
<th>Components</th>
<th>Reaction Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Universal Master Mix (MM)</td>
<td>6.875</td>
</tr>
<tr>
<td>40X SNP genotyping assay</td>
<td>0.343</td>
</tr>
<tr>
<td>Nuclease-free H2O</td>
<td>5.032</td>
</tr>
<tr>
<td>gDNA</td>
<td>1.5</td>
</tr>
<tr>
<td>Total Volume</td>
<td>13.75 μl</td>
</tr>
</tbody>
</table>
Table 2.2: Thermal cycling protocol for qPCR

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th># cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Taq Activation</td>
<td>95</td>
<td>10 minutes</td>
<td></td>
</tr>
<tr>
<td>2 Denature</td>
<td>92</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>3 Anneal/Extend</td>
<td>60</td>
<td>1 minute</td>
<td>40 Cycles</td>
</tr>
</tbody>
</table>

The ROX filter was selected to amplify the reference dye. The FAM filter was selected to amplify the “C” allele and the VIC filter was selected to amplify the “T” allele in the cDNA samples. The qPCR machine (Stratgene Mx3000P™) used for this experiment did not have a VIC filter, the HEX filter was used instead.

2.2.4 Quantification of cytokine production by enzyme-linked immunosorbent assays (ELISA)

The following cytokines were quantified by ELISA in supernatants from:

Primary human pulmonary fibroblast cell lines: Interleukin 8 (IL-8; human CXCL8; Ref. DY208), RANTES (Regulated and normal T cell expressed and secreted; human CCL5; Ref. DY278) and Interleukin 6 (IL-6; Ref. DY206).

**Human IL-8 ELISA**

96 well plates were coated with 50 μl of capture antibody (4.0 μg/ml) diluted in PBS and left overnight covered at 4°C. Plates were washed 3 times in 0.05% Tween 20 in PBS and blotted. The plates were blocked in 200 μl of blocking buffer (1% BSA in PBS) for 2 hours at RT. Plates were washed again as previously described. 25 μl of sample supernatant was added to the plate with 25 μl of reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-Buffered Saline (TBS)). 50 μl of IL-8 standards in 2-fold serial dilutions were added to the plate in duplicate. The plates were incubated overnight, covered at 4°C. Following this, plates were washed again as previously described. 50 μl detection antibody (20.0 ng/ml) diluted in reagent
diluent was added to the plate and incubated for 2 hours at RT. Plates were washed again as previously described. The plates are covered in 50 μl of Streptavidin-HRP diluted in reagent diluent and incubated for 20 minutes at RT covered to protect Streptavidin-HRP from the light. Plates were washed again as previously described.

50 μl of substrate solution (One OPD gold tablet and one OPD silver tablet in 20 mls dH₂O) was then added to the plate and incubated for 20 minutes at RT covered to protect it from the light. 25 μl of stop solution (2N H₂SO₄) was added to stop the reaction. The plates were read on a microplate reader at 450 nm to determine the optical density.

**Human RANTES (CCL5) ELISA**

96 well plates were coated with 50 μl of capture antibody (1.0 μg/ml) diluted in PBS and left overnight covered at 4°C. Plates were washed 3 times in 0.05% Tween 20 in PBS and blotted. The plates were blocked in 200 μl of reagent diluent (1% BSA in PBS) for 2 hours at RT. Plates were washed again as previously described. 25 μl of sample supernatant was added to the plate with 25 μl of reagent diluent. 50 μl of RANTES standards in 2-fold serial dilutions were added to the plate in duplicate. The plates were incubated overnight, covered at 4°C. Following this, plates were washed again as previously described. 50 μl detection antibody (20.0 ng/ml) diluted in reagent diluent was added to the plate and incubated for 2 hours at RT. Plates were washed again as previously described. The plates are covered in 50 μl of Streptavidin-HRP diluted in reagent diluent and incubated for 20 minutes at RT covered to protect Streptavidin-HRP from the light. Plates were washed again as previously described. 50 μl of substrate solution (One OPD gold tablet and one OPD silver tablet in 20 mls dH₂O) was then added to the plate and incubated for 20 minutes at RT covered to protect it from the light. 25 μl of stop solution (2N H₂SO₄) was added to stop the reaction. The plates were read on a microplate reader at 450 nm to determine the optical density.

**Human IL-6 ELISA**

96 well plates were coated with 50 μl of capture antibody (2.0 μg/ml) diluted in PBS and left overnight covered at 4°C. Plates were washed 3 times in 0.05% Tween 20 in PBS and blotted. The plates were blocked in 200 μl of reagent diluent (1% BSA in PBS) for 2 hours at RT. Plates were washed again as previously described...
described. 25μl of sample supernatant was added to the plate with 25 μl of reagent diluent. 50 μl of IL-6 standards in 2-fold serial dilutions were added to the plate in duplicate. The plates were incubated overnight, covered at 4°C. Following this, plates were washed again as previously described. 50 μl detection antibody (50.0 ng/ml) diluted in reagent diluent was added to the plate and incubated for 2 hours at RT. Plates were washed again as previously described. The plates are covered in 50 μl of Streptavidin-HRP diluted in reagent diluent and incubated for 20 minutes at RT covered to protect Streptavidin-HRP from the light. Plates were washed again as previously described. 50 μl of substrate solution (One OPD gold tablet and one OPD silver tablet in 20mls dH2O) was then added to the plate and incubated for 20 minutes at RT covered to protect it from the light. 25 μl of stop solution (2N H2SO4) was added to stop the reaction. The plates were read on a microplate reader at 450 nm to determine the optical density.

2.2.5 Assessment of mRNA expression from cultured fibroblasts by Quantitative Real Time Polymerase Chain Reaction (qPCR) using SYBR® Green

2.2.5.1 Total RNA extraction

Total RNA was isolated using Tri Reagent© RNA isolation reagent. Fibroblasts were cultured in 24 well plates and treated with the appropriate stimuli. Supernatants were harvested and stored at -20°C to be analysed by ELISA. 1 ml of Trizol was added to the remaining cell monolayer in each well. The cell mono-layer was disrupted using a pipette tip to ensure all the cells were removed and the solution was homogenous. The contents of each well was then transferred to a sterile 1.5 ml microcentrifuge tube and 200 μl of chloroform was added to each sample. The samples were mixed vigorously and left at RT for 15 minutes. The samples were centrifuged at 13,000 rpm for 15 minutes at 4°C to allow phase separation to occur. Once separated into the three layers (RNA, DNA and protein), the top clear layer containing RNA was removed into new sterile 1.5 ml microcentrifuge tubes and 0.5 ml of 2-propanol was added. The samples were mixed vigorously using a vortex and left at RT for 10 minutes. The samples were again centrifuged at 13,000 rpm for 15 minutes at 4°C. Samples were then rested on ice and the supernatant was carefully decanted, leaving the RNA pellet intact. 1ml of 75% ethanol (Sigma Aldrich) was
added to each RNA pellet. The RNA was stored in 75% ethanol at -80°C for later use.

2.2.5.2 *First-strand cDNA synthesis*

First-strand cDNA synthesis was carried out using reagents from Invitrogen (see Section 2.1 Materials). Briefly, previously isolated RNA (stored at -80 ºC) was allowed to thaw. The samples were centrifuged at 13,000 rpm for 15 minutes at 4 ºC. The ethanol was allowed to evaporate off in a laminar flow hood and once dry, the RNA pellet was re-suspended in 15μl of nuclease free water.

In the first stage of cDNA synthesis 8μl of RNA of each sample was aliquoted into the Optical tubes (Agilent Technologies) and a master mix of the following components was added.

**Table 2.3: Mastermix #1 components for first strand cDNA synthesis**

<table>
<thead>
<tr>
<th>Mastermix #1</th>
<th>Components</th>
<th>Reaction volume 1X (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oligo(dT) 20 (200 μg/ml)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>dNTP (10 mM)</td>
<td>1</td>
</tr>
</tbody>
</table>

2 μl of Mastermix #1 was added to each sample and the solution contents was collected by brief micro-centrifugation and then incubated in the thermocycler for 5 minutes at 65°C (lid heated to 95°C to prevent evaporation). Samples were then placed on ice for 1 minute.

**Table 2.4: Mastermix #2 components for first strand cDNA synthesis**

<table>
<thead>
<tr>
<th>Mastermix #2</th>
<th>Components</th>
<th>Reaction volume 1X (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5x First strand buffer</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DTT 0.1M</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>RNase OUT™</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Nuclease Free H₂O</td>
<td>2</td>
</tr>
</tbody>
</table>
9 μl of Mastermix #2 was added to each sample and the solution contents was collected by brief micro-centrifugation and incubated in the thermocycler for 2 minutes at 25°C (lid heated to 95°C to prevent evaporation).

1 μl of Superscript II Reverse Transcriptase™ was added to each sample. The solution contents was collected by brief micro-centrifugation. The samples were then incubated at 42°C for 50 minutes and then at 70°C for 15 minutes in a thermocycler. cDNA was then stored at -20°C for future use.

2.2.5.3 Assessment of mRNA expression by Quantitative Real Time Polymerase Chain Reaction (qPCR) using SYBR® Green

qPCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG along with human gene-specific forward and reverse primers. Table 2.6 lists all primers used for qPCR. All primers were used at a concentration of 10μM. A qPCR reaction mastermix was made with SYBR® Green, target gene forward and reverse primers, RNase free water and cDNA (Table 2.5). Total reaction volume per sample was 20μl.

2μl of cDNA was added to labelled optical tubes with 18μl of qPCR mastermix. Stratagene MX3000P machine was used for qPCR using the thermal profile described in Table 2.8. The house keeping gene, β-actin, was used in all experiments. Optimal annealing temperatures were primer-set specific, described below in Table 2.6.

Table 2.5: Mastermix components for SYBR®-qPCR

<table>
<thead>
<tr>
<th>Mastermix Components</th>
<th>Reaction Volume 1X (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR® Green</td>
<td>10</td>
</tr>
<tr>
<td>Forward Primer (10 μM)</td>
<td>0.2</td>
</tr>
<tr>
<td>Reverse Primer (10 μM)</td>
<td>0.2</td>
</tr>
<tr>
<td>Nuclease Free water</td>
<td>7.6</td>
</tr>
<tr>
<td>Gene</td>
<td>5' Forward 3'</td>
</tr>
<tr>
<td>-------</td>
<td>--------------</td>
</tr>
<tr>
<td>hIL-8</td>
<td>TAG CAA AAT TGA GGC CAA GG</td>
</tr>
<tr>
<td>hRANTES</td>
<td>TCC TGC AGA GGA TCA AGA CA</td>
</tr>
<tr>
<td>hIFN-β</td>
<td>AGC ACT GGC TGG AAT GAG AC</td>
</tr>
<tr>
<td>hβ-actin</td>
<td>GGA CTT CGA GCA AGA GAT GG</td>
</tr>
<tr>
<td>hPKR</td>
<td>TGG CGG TCT TCA GAA TCA ACA TC</td>
</tr>
<tr>
<td>hMDA-5</td>
<td>CTC AGG CCT TAC CAA ATG GA</td>
</tr>
<tr>
<td>hRIG-I</td>
<td>GAG CAC CAG ACC TCC TCT TG</td>
</tr>
<tr>
<td>hBeclin-1</td>
<td>AGC TGC CGT TAT ACT GTT CTG</td>
</tr>
<tr>
<td>hLC3b</td>
<td>GAT GTC CGA CTT ATT CGA GAG C</td>
</tr>
<tr>
<td>hAtg5</td>
<td>AAC TGA AAG GGA AGC AGA ACC A</td>
</tr>
<tr>
<td>hTLR3</td>
<td>AAC CTT TGC CTT CTG CAC GA</td>
</tr>
<tr>
<td>hTLR4</td>
<td>CAG CTC TTG GTG GAA GTT GA</td>
</tr>
<tr>
<td>hTLR9</td>
<td>AAA GAG GAA GGG GTG AAG GA</td>
</tr>
</tbody>
</table>
Table 2.7: Murine Primers for Real Time-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’ Forward</th>
<th>3’ Reverse</th>
<th>Optimal annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRANTES</td>
<td>CAA TCT TGC AGT CGT GTT TG</td>
<td>GGA GTG GGA GTA GGG GAT TA</td>
<td>58</td>
</tr>
<tr>
<td>mMIP-2</td>
<td>CAG ACT CCA GCC ACA CTT CA</td>
<td>TTC AGGGTC AAG GCA AAC TT</td>
<td>58</td>
</tr>
<tr>
<td>mβ-actin</td>
<td>AAG AGC TAT GAG CTG CCT GA</td>
<td>TAC GGA TGT CAA CGT CAC AC</td>
<td>58</td>
</tr>
<tr>
<td>mTLR3</td>
<td>CTG GGT CTG GGA ACA TTT CT</td>
<td>TTG CTG AAC TGC GTG ATG TA</td>
<td>58</td>
</tr>
<tr>
<td>mTLR4</td>
<td>AGA CCT CAG CTT CAA TGG TG</td>
<td>GAG ACT GGT CAA GCC AAG AA</td>
<td>58</td>
</tr>
<tr>
<td>mTLR9</td>
<td>AGC CTC CGA GAC AAC TAC CT</td>
<td>GCT GAG GTT GAC CTC TTT CA</td>
<td>58</td>
</tr>
</tbody>
</table>

Table 2.8: The SYBR®-qPCR thermal cycling protocol is as follow

<table>
<thead>
<tr>
<th>Standard Thermal Cycling Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
2.2.6 Assessment of protein expression by Western blot analysis

2.2.6.1 Cell harvesting for Western blot analysis

Cells were grown and treated as per protocol in section 2.2.1. Cells were seeded at a density of 50,000 cells/well in a 24 well plate. After incubation with relevant treatments, the cells were harvested in RIPA buffer containing protease inhibitors. First, media was removed from all wells in the cell culture plate on ice. Supernatants were stored at -20°C for analysis by ELISA. Cells were washed in 1 ml of ice cold PBS to remove all media. 50 μl of RIPA lysis buffer with protease inhibitors was added to each well. The plates were left on ice for 30 minutes, shaken twice. Each well was scratched with a p200 tip to remove the cells from the plate. Samples were then transferred to labelled eppendorf tubes and stored at -80°C until a BCA assay could be performed and the samples processed for Western blot analysis.

RIPA lysis buffer contained the following components:

Table 2.9: RIPA lysis buffer

<table>
<thead>
<tr>
<th>RIPA lysis buffer solution</th>
<th>Volume</th>
<th>Reagent</th>
<th>Desired concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.88 g</td>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td></td>
<td>1 ml</td>
<td>Triton x-100</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>0.1g</td>
<td>SDS</td>
<td>0.1%</td>
</tr>
<tr>
<td></td>
<td>0.6 g</td>
<td>Tris</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

Make up to 100 ml with distilled H2O

Add 1X solution of Protease Inhibitors to RIPA buffer before use

Store at 4°C
2.2.6.2 *Bicinchoninic acid Assay (BCA) assay*

To perform a BCA assay, all protein samples were defrosted on ice or in the fridge. The samples were spun down at 14,000 rpm for 30 minutes at 4°C. The supernatants were decanted from the samples into new labelled eppendorf tubes. The pellet was discarded. The supernatant contains the proteins needed for the BCA assay and Western blot analysis.

First, the standard solutions were prepared. 10 μl of each protein sample and standard was added to a 96 well plate in duplicate. Each sample and standard was vortexed vigorously before addition. The BCA reagent mix was prepared in a ratio of 50:1 BCA reagent A: BCA reagent B. 200 μl of the BCA reagent mix was added to each well. The 96 well plate was incubated for 30 minutes at 37°C. The plate was read on a microplate reader at 562 nm. Using the standard curve produced, the protein concentration was calculated for each sample.

2.2.6.3 *Western blot preparation for autophagy proteins, receptor proteins and mTOR*

The protein samples were equilibrated to the desired concentration (8 μg protein/ sample was used for all blots) using the loading buffer. The loading buffer was prepared with 900μl 4X loading buffer and 100μl β-mercaptoethanol. The diluted samples were boiled at 90°C for 5 minutes. The samples were spun down in a microcentrifuge and stored at -20°C at this point until needed. The Western blot method used changed depending on the size of the protein probed.

*Autophagy protein: LC3-II*

The proteins were run on a 4-15% gradient gel in the running buffer in Table 2.10 below. A molecular weight ladder was run as a reference with the samples on all gels (Bio-Rad, Ref. 1610394). The gel was run at 100 V for 15 minutes and 150-200 V for 45-60 minutes. The wet transfer method was used to transfer the proteins to a PVDF membrane at 32V for 70 minutes. The PVDF membrane was blocked in 5% non-fat dried (marvel) milk in TBS-0.1% Tween 20 for 1 hour. The membranes were incubated in the primary antibody at 4°C overnight and the secondary antibody at RT for 1 hour. Primary antibodies were diluted in 5% BSA in TBS-0.1% Tween 20 and secondary antibodies were diluted in 5% non-fat dried (marvel) milk in TBS-
0.1% Tween 20. Following blocking, incubation in primary antibody and incubation in secondary antibody the membranes were washed in 1X TBS with 0.1% Tween 20 for 10 minutes 3 times. The bands were visualised using chemiluminescent HRP substrate (Millipore Immobilon™ Western).

**Receptor proteins: RIG-I, MDA5, PKR and TLR3**

These proteins are larger and heavier than LC3B. Therefore, there is a change in the transfer step of the Western blot process to allow for more efficient transfer of bigger proteins. The wet transfer method was used to transfer the proteins to a PVDF membrane at 200 mA for 90 minutes at RT. To avoid the transfer overheating, the tank is surrounded by ice in a Styrofoam box for the duration of the transfer. The protocol follows as described for LC3B for running the gel, blocking the membrane, incubating the membrane in primary and secondary antibodies and visualising the proteins. Primary antibodies were diluted in 5% BSA in TBS-0.1% Tween 20 and secondary antibodies were diluted in 5% non-fat dried (marvel) milk in TBS-0.1% Tween 20.

**Total mTOR and phospho-mTOR proteins**

The mTOR protein is 289 kDa in weight. This protein is much heavier than all other proteins probed and therefore the protocol was adapted to suit this molecular weight. For larger proteins a lower percentage gel was used (6% resolving gel with 4% stacking gel). See Table 2.12 below for gel composition. A high molecular weight ladder (30-460 kDa) (Invitrogen™, Ref. LC5699) was used instead of the standard ladder (10-250 kDa). Samples were prepared to a concentration of 8 μg protein/ sample in 4X Laemmmli Buffer.

The gel was run at 80V until the ladder was sufficiently separated, approximately 20 minutes. The wet transfer method was used to transfer the proteins to a PVDF membrane at 70V for 3 hours on ice. The transfer buffer was made up in advance and stored for a couple of hours at 4°C. Transfer buffer was made up with 5% methanol instead of the standard 10% methanol. The PVDF membrane was blocked in 5% non-fat dried (marvel) milk in TBS-0.1% Tween 20 for 1 hour. The membranes were incubated in the primary antibody at 4°C overnight and the secondary antibody at RT for 1 hour. Primary antibodies were diluted in 5% BSA in TBS-0.1% Tween 20 and secondary antibodies were diluted in 5% non-fat dried
(marvel) milk in TBS-0.1% Tween 20. Following blocking, incubation in primary antibody and incubation in secondary antibody, the membranes were washed in 1X TBS with 0.1% Tween 20 for 10 minutes 3 times. The bands were visualised using chemiluminescent HRP substrate (Millipore Immobilon™ Western).

**Table 2.10: Western Blot Buffers**

<table>
<thead>
<tr>
<th>Western Blot buffers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Running Buffer – 10X</strong></td>
</tr>
<tr>
<td>60.4 g Trizma Base</td>
</tr>
<tr>
<td>288 g Glycine</td>
</tr>
<tr>
<td>20 g SDS</td>
</tr>
<tr>
<td>Make up to 2 litres with distilled H₂O</td>
</tr>
</tbody>
</table>

**Table 2.11: Antibody dilutions**

<table>
<thead>
<tr>
<th>Antibody Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Dilutions</strong></td>
</tr>
<tr>
<td>LC3-II</td>
</tr>
<tr>
<td>RIG-I</td>
</tr>
<tr>
<td>MDA5</td>
</tr>
<tr>
<td>PKR</td>
</tr>
<tr>
<td>β-actin</td>
</tr>
<tr>
<td>TLR3</td>
</tr>
<tr>
<td>mTOR</td>
</tr>
<tr>
<td>Phospho-mTOR</td>
</tr>
<tr>
<td><strong>Secondary Dilutions</strong></td>
</tr>
<tr>
<td>Anti-Rabbit IgG – HRP linked antibody</td>
</tr>
</tbody>
</table>
Table 2.12: Gel preparations

<table>
<thead>
<tr>
<th>Reagents</th>
<th>4% gel</th>
<th>6% gel</th>
<th>10% gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>2.72 ml</td>
<td>8.5 ml</td>
<td>3.966 ml</td>
</tr>
<tr>
<td>Tris*</td>
<td>0.5 ml</td>
<td>4 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>0.68 ml</td>
<td>3.2 ml</td>
<td>3.33 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>40 μl</td>
<td>160 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>APS</td>
<td>40 μl</td>
<td>160 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 μl</td>
<td>16 μl</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

* 4% stacking gel needs 0.5M Tris and 6% resolving gel need 1.5M Tris – keep both at 4°C

2.2.6.4 Western Blot Stripping

The stripping solution up was made up as per Table 2.13. The PVDF membrane was stripped with 10 mls of the stripping solution. The membrane was placed on a rocker for 7-8 minutes to strip the membrane. The membrane were then washed in 1X TBS with 1% Tween 20 for 10 minutes 3 times. The membrane was blocked in 5% non-fat dried (marvel) milk in TBS-0.1% Tween 20 for 1 hour. The membrane was incubated in the primary antibody at 4°C overnight and the western was continued as per the protocol in Section 2.2.6.3.

Table 2.13: Stripping Solution

<table>
<thead>
<tr>
<th>Western blot stripping solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 g Glycine</td>
</tr>
<tr>
<td>0.5 g SDS</td>
</tr>
<tr>
<td>5 mls Tween 20</td>
</tr>
</tbody>
</table>
2.2.7 Assessment of cell proliferation in primary human lung fibroblasts by MTT assay

Primary human lung fibroblasts were cultured as previously described. Cells were seeded at a density of 10,000 cells/200 μl in a 96 well cell culture flat-bottomed plate and stimulated with the relevant treatment. To begin the MTT assay, the cells were washed in complete DMEM without phenol red once to remove DMEM containing phenol red. 100 μl of complete DMEM without phenol red was added to each well. A stock solution of 12 mM MTT was prepared by adding 1 ml sterile PBS to 5 mg MTT and 10 μl of the resulting solution was added to each well. A well containing 100 μl of complete DMEM without phenol red and 10 μl MTT stock solution in the absence of cells was included as a negative control. The samples were incubated at 37°C for 4 hours. A second solution was prepared containing 10 mls of 0.01 M hydrochloric acid (HCl) and 1g of sodium dodecyl sulphate (SDS). After 4 hours 100 μl of the SDS-HCl solution was added to each well and mixed thoroughly using a pipette. The 96 well plate was returned to the incubator for 4-18 hours at 37°C. Before reading the plate, the samples were mixed again using a pipette. The absorbance of each sample was read at 570 nm.

2.3 Methods for murine experiments

2.3.1 TLR3 L413F genotyping assay (murine)

2.3.1.1 Extraction of genomic DNA from mouse ear punch biopsies

Ear punch biopsies must first undergo a 16-18 hour lysis. To begin lysis, a DNA lysis buffer/Proteinase K solution must be made. This should only be done on the day of the experiment. 20 μl of proteinase K was needed per 1 ml of DNA lysis buffer, described in the table below. 500 μl of DNA lysis buffer with proteinase K was added to each ear punch in a 1.5 ml eppendorf. The samples were shaken overnight (350-400 rpm) at 55°C in a thermomixer (Eppendorf Thermomixer C).
Table 2.14: DNA lysis buffer components

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
<th>Desired concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ml</td>
<td>1M Tris pH8</td>
<td>0.1 M</td>
</tr>
<tr>
<td>2.5 ml</td>
<td>0.5 M EDTA</td>
<td>5 mM</td>
</tr>
<tr>
<td>10 ml</td>
<td>5 M NaCl</td>
<td>0.2 M</td>
</tr>
<tr>
<td>2.5 ml</td>
<td>20% SDS</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

Make up to 250 ml with distilled H₂O

Store at RT

After 16-18 hours incubation, 1 ml of 100% ethanol was added to each sample and mixed by inversion a number of times. The samples were centrifuged at RT for 10 minutes at 14000 rpm in a micro-centrifuge. The supernatant was decanted and 500 μl 70% high grade ethanol was added to each tube to wash the pellet. The samples were inverted a number times and centrifuged at RT for 5 minutes at 14000 rpm in a micro-centrifuge. The supernatant was decanted and the samples were centrifuged at RT for 1 minute at 14000 rpm. The remaining ethanol was removed from the samples using a p200 pipette and all samples were air-dried for 2 minutes at RT. The resulting DNA was re-suspended in 60 μl ultra-pure water. The samples were incubated for a further 2 hours at 55°C in a thermomixer, shaking at 350-400 rpm. After 2 hours, the samples were centrifuged 14000 rpm at RT and stored at 4°C until ready for PCR.

2.3.1.2 PCR

First, a mastermix was prepared as described in Table 2.15. 48 μl of mastermix was added to each labelled PCR tube. 2 μl of DNA was added into each
tube. The tubes were vortexed and centrifuged to collect the solution. The thermal cycle protocol was run as described in Table 2.16.

**TLR3 L413F Primers**

Forward Primer: 5’ CAAAAGCAATACCTTACG 3’

Reverse Primer: 5’ CGACTGGGATTTCATCTAGGC 3’

**Table 2.15: TLR3 L413F genotyping mastermix #1**

<table>
<thead>
<tr>
<th>Mastermix Components</th>
<th>1X (µl)</th>
<th>20X (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>My Taq Red mix, 2X</td>
<td>25</td>
<td>500</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>21</td>
<td>420</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>960</td>
</tr>
</tbody>
</table>

**Table 2.16: Thermal cycling protocol for TLR3 L413F PCR**

<table>
<thead>
<tr>
<th>Program Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5 minutes</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>30 seconds</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>30 seconds</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1 minute</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>10 minutes</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>Pause</td>
</tr>
</tbody>
</table>
2.3.1.3 *Digestion*

Following PCR, the samples were digested. A second mastermix was prepared. The components are listed in Table 2.17. 21 μl of mastermix #2 was added into new labelled PCR tubes and 20 μl of the PCR reaction mixture prepared in section 2.2.9.2.

**Table 2.17: Mastermix #2 for digestion**

<table>
<thead>
<tr>
<th>Mastermix Ingredients</th>
<th>1X reaction (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>18</td>
</tr>
<tr>
<td>10x Buffer</td>
<td>2</td>
</tr>
<tr>
<td>Hph I</td>
<td>1</td>
</tr>
<tr>
<td>PCR reaction</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>31</strong></td>
</tr>
</tbody>
</table>

**Table 2.18: Thermal cycling protocol for digestion**

<table>
<thead>
<tr>
<th>Program Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>16 hours</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>20 minutes</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>Pause</td>
</tr>
</tbody>
</table>

Following the digestion, the samples were run on a 2% agarose gel in 1X TAE. 20μl of each sample and 10 μl of DNA ladder were loaded into the gel and the gel was run at 90-100 V for approximately 30-40 minutes until the dye front had run down 75% of the gel. Imaging software was used to image the DNA bands.
Figure 2.1. **Image of TLR3 L413F genotyping DNA gel.** A 2% agarose gel is used to separate the DNA bands. Digestion of the PCR product differentiates TLR3 L413F wild-type, heterozygote and homozygote genotypes. The TLR3 L413F wild-type genotype has two distinct bands at 344 bp and 188 bp, the TLR3 L413F heterozygote genotype has three distinct bands at 344 bp, 269 bp and 188 bp and the TLR3 L413F homozygote genotype has two distinct bands at 269bp and 188bp.

2.3.2 **Cell culture (murine cell lines)**

2.3.2.1 **Primary murine lung fibroblasts isolation and culture**

Primary murine lung fibroblasts were isolated by dissecting the lungs from mice on the bench top. The lungs were sprayed with 70% ethanol and added to pre-warmed sterile complete DMEM. The lungs were then transferred to a sterile petri dish containing 10 mls sterile complete DMEM in the sterile laminar flow hood and cut into small fragments using a sterile scalpel. Each set of lungs were added to a separate sterile T75 cell culture flasks in 10 mls of media to allow lung fragments sit on the bottom of the flask and adhere. Lung fragments were left 24-48 hours after which 10 mls of pre-warmed sterile complete DMEM was added. Flasks were routinely cultured in sterile complete DMEM. After 7-8 days, fibroblasts have grown out of the lung fragments and begun forming a monolayer on the tissue culture flask. At this stage lung fragments were removed as the fibroblasts had grown into foci. Once fibroblasts in a T75 tissue culture flask had reached 80% confluency, cells were subcultured, as described in 2.2.1.7 and added into a sterile T175 flask. Again, once cells had reached 80% confluency in the sterile T175 flask. Cells were subcultured as described in 2.2.1.7 again into a number of T175 flask in preparation for plating and treatment.
2.3.2.2 **Primary murine splenocyte isolation and culture**

Primary murine splenocytes were isolated by dissecting spleens from TLR3 L413F mice on the bench top. The spleens were sprayed with 70% ethanol and added to sterile PBS on ice. In a laminar flow hood, spleens were transferred to a sterile BD Falcon cell strainer. Using the plunger of a 1ml plastic syringe, the spleen was homogenised using a cell strainer to obtain a single cell suspension in sterile PBS. The strainer was washed twice with sterile PBS to guarantee maximum cell yield. The resulting cell suspension was centrifuged at 1200 rpm for 5 minutes at RT to obtain a pellet. The resulting pellet was washed using pre-warmed sterile PBS. The PBS was removed and the pellet was re-suspended using a 3.5 ml Pasteur pipette in RPMI supplemented with 10% penicillin/ streptomycin and L-Glutamine. The cells were once again centrifuged at 1200 rpm for 5 minutes at RT to obtain a pellet. The cell pellet was re-suspended and the cell count was obtained using a haemocytometer. The cells were then plated at a concentration of $2 \times 10^6$ cell/ml and treated with Poly (I:C) or LPS as described in section 2.2.3.

2.3.2.3 **Primary murine intraperitoneal (I.P.) macrophage isolation and culture**

For i.p. macrophage preparation, the skin was removed from around the abdomen using a scissors and forceps on the bench top, taking care not to pierce the peritoneum. A 5 ml syringe was filled with sterile PBS and a rubber catheter with an attached needle was attached. The needle was inserted into the peritoneal cavity through the peritoneum. 5 ml sterile PBS was injected into the cavity and the needle was removed leaving the rubber catheter in place. PBS was massaged around the abdomen and then carefully withdrawn through the catheter into the syringe. The cell suspension was placed in a 50ml falcon tube and spun at 1200 rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 5ml red blood cell lysis buffer and left for 4 minutes on ice, shaking every 30 seconds. 15 ml of RPMI supplemented with 10% penicillin/ streptomycin and L-Glutamine was added to the 50ml falcon to neutralise the lysis buffer. The cells were spun down again for 5 minutes at 1200 rpm. If the cell pellet was still red in colour, another 5mls of red blood cell lysis buffer was added to re-suspend the pellet and left for 4 minutes on ice, shaking every 30 seconds. 15ml in RPMI was added to the 50ml falcon to neutralise the lysis buffer and spun down again for 5 minutes at 1200 rpm. The cell
pellet was reconstituted in 5 ml RPMI and counted. I.P. macrophages were plated at a density of 100,000 cells/ well in a 48 well plate and placed in the incubator overnight before treatment.

2.3.2.4 Primary murine bone marrow derived macrophages (BMDM) isolation and culture

For BMDM preparation, the skin was removed using a scissors and forceps on the bench top. Skin, muscles and tissues were removed from around the hind legs. Each hind leg was removed from the body from high on the hip bone and any remaining tissue was stripped away. The bones were sprayed in 70% ethanol and placed in a 50ml falcon tube with 5mls sterile PBS before being brought into the sterile laminar flow hood. The legs were placed on a sterile petri dish with 10 mls of fresh complete DMEM to prevent the legs drying out.

The leg bones were separated at the hinge joint, taking care not to snap either bone. Using a scalpel the ends of each bone were removed taking care to not take too much bone. A syringe filled with complete DMEM fitted with a 25 gauge needle was inserted into the top of the bone. The media was injected (flushed) through the bone and the throughput is collected in a sieve placed over a sterile 50ml Falcon tube. The legs were flushed once or twice more quickly as the legs will dry out and can no longer be used. Cell were spun at 1200 rpm for 5 minutes.

The supernatant was discarded and the pellet was re-suspended in 5ml red blood cell lysis buffer and left for 4 minutes on ice, shaking every 30 seconds. 15ml complete DMEM was added to the 50ml falcon to neutralise the lysis buffer. The cells were spun down again for 5 minutes at 1200 rpm. If the cell pellet was still red in colour, another 5mls of red blood cell lysis buffer was added to re-suspend the pellet and left for 4 minutes on ice, shaking every 30 seconds. 15ml complete DMEM was added to the 50ml falcon to neutralise the lysis buffer and spun down again for 5 minutes at 1200 rpm. The pellet was re-suspended in 10ml complete DMEM and transferred to a sterile T175 cell culture flask. The bone marrow from both legs of one mouse was added to 1 sterile T175 cell culture flask. Another 30mls of complete media supplemented with 20% L929 media was added to the flask (24mls complete DMEM + 6mls L929 media). L929 media is used during BMDM
prep as L929 cells secrete Macrophage colony-stimulating factor (M-CSF) which is needed for BMDM differentiation. Culturing of L929 cells is described in 2.3.2.5.

BMDMs were incubated for 3 days after which most cells should adhere to the bottom of the flask. The supernatant containing cells that have not adhered was removed from the flask and placed directly into a new T175 flask. Another 20% L929 media was added to the new flask. The original flask was given 40mls of complete media supplemented with 20% L929 media. Both flasks were incubated for 4-5 days until confluent. BMDMs were plated at a density of 100,000 cells/well in a 48 well plate and placed in the incubator overnight before treatment.

2.3.2.5 L929 cell culture

The L929 cell line is a murine connective tissue cell line. L929 supernatants are routinely used in BMDM cell culture as L929 cells secrete M-CSF and GM-CSF to promote differentiation of BMDMs (252, 253). Cells were seeded into T175 flask at a density of 20x10^6 cells per flask. Cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin (pen/strep). Cells were maintained in a humidified environment (95% relative humidity) containing 5% CO₂ at 37°C. After 10 days, supernatant was removed and filter-sterilised. Aliquots of L929 media were stored at -20°C for use in BMDM cell culture.

2.3.3 Quantification of cytokine production by enzyme-linked immunosorbent assays (ELISA)

The following cytokines were quantified by ELISA in supernatants from:

Primary murine lung fibroblasts, splenocytes and intraperitoneal (I.P.) macrophages:
KC (CXCL1; Ref. DY453) and RANTES (Ref. DY478).

Murine KC (CXCL1) ELISA

96 well plates were coated with 50 μl of capture antibody (2.0μg/ml) diluted in PBS and left overnight covered at 4°C. Plates were washed 3 times in 0.05% Tween 20 in PBS and blotted. The plates were blocked in 200 μl of reagent diluent (1% BSA in PBS) for 2 hours at RT. Plates were washed again as previously
described. 25 μl of sample supernatant was added to the plate with 25 μl of reagent diluent. 50 μl of KC standards diluted in reagent diluent in 2-fold serial dilutions were added to the plate in duplicate. The plates were incubated overnight, covered at 4°C. Following this, plates were washed again as previously described. 50 μl detection antibody (200.0 ng/ml) diluted in reagent diluent was added to the plate and incubated for 2 hours at RT. Plates were washed again as previously described. The plates are covered in 50 μl of Streptavidin-HRP diluted in reagent diluent and incubated for 20 minutes at RT covered to protect Streptavidin-HRP from the light. Plates were washed again as previously described. 50 μl of substrate solution (One OPD gold tablet and one OPD silver tablet in 20mls dH₂O) was then added to the plate and incubated for 20 minutes at RT covered to protect it from the light. 25 μl of stop solution (2N H₂SO₄) was added to stop the reaction. The plates were read on a microplate reader at 450 nm to determine the optical density.

**Murine RANTES (CCL5) ELISA**

96 well plates were coated with 50 μl of capture antibody (2.0 μg/ml) diluted in PBS and left overnight covered at 4°C. Plates were washed 3 times in 0.05% Tween 20 in PBS and blotted. The plates were blocked in 200 μl of reagent diluent (1% BSA in PBS) for 2 hours at RT. Plates were washed again as previously described. 25 μl of sample supernatant was added to the plate with 25 μl of reagent diluent. 50 μl of RANTES standards diluted in reagent diluent in 2-fold serial dilutions were added to the plate in duplicate. The plates were incubated overnight, covered at 4°C. Following this, plates were washed again as previously described. 50μl detection antibody (12.5 ng/ml) dilute in reagent diluent was added to the plate and incubated for 2 hours at RT. Plates were washed again as previously described. The plates are covered in 50 μl of Streptavidin-HRP diluted in reagent diluent and incubated for 20 minutes at RT covered to protect Streptavidin-HRP from the light. Plates were washed again as previously described. 50 μl of substrate solution (One OPD gold tablet and one OPD silver tablet in 20mls dH₂O) was then added to the plate and incubated for 20 minutes at RT covered to protect it from the light. 25 μl of stop solution (2N H₂SO₄) was added to stop the reaction. The plates were read on a microplate reader at 450nm to determine the optical density.
2.3.4 Generation of TLR3 L413F knock in mouse by CRISPR/CAS

We commissioned the generation of the TLR3 L413F knock in mouse by CRISPR/Cas9 mediated gene editing. Mice were generated on a C57BL/6NTac background. The target gene was located on chromosome 8. The human Leu412Phe amino acid substitution corresponds to Leu413Phe in mice (TLR3 L413F knock-in mouse). Fig 2.2 below illustrates the position of the SNP in human and mouse TLR3 gene. Validation studies were carried out by Taconic Biosciences. The mice were genotyped as per protocol in Section 2.3.1.

Fig. 2.2. Alignment of human and mouse TLR3 gene with introduction of the TLR3 L413F SNP in mice

The TLR3 L412F SNP (L413F in mice) was introduced into the murine TLR3 gene by CRISPR/Cas9 gene editing. It involved a single base pair change in C > T. The above diagram illustrates the position at which the mutation was introduced and alignment of both murine and human TLR3 genes. Image generated by Taconic Biosciences.

2.3.5 Statistical Analysis

All statistical analyses were carried out using GraphPad Instat Software (GraphPad Software Inc. CA, USA). In ELISA and qPCR analyses, One-way analysis of variance (ANOVA) was used to test for statistical significance (two-tailed analysis) between experimental groups of three or more. Multiple comparisons between groups were then assessed using the Tukey-Kramer post-hoc test (for parametric analysis) or Dunn’s post-hoc test (for non-parametric analysis).
For data sets with only two experimental groups with equal standard deviations (SDs), unpaired T tests were used to compare data sets. Unpaired T tests with Welch’s correction were used for analysis if the data sets did not have equal SDs (two tailed analysis). Non-parametric data was analysed using a Mann-Whitney test for statistical significance (two tailed analysis). Statistical significance was recorded at p<0.05.
Chapter 3
Initial genotyping and characterisation of primary IPF lung fibroblasts from diagnostic and explanted lung tissue
Chapter 3 Initial genotyping and characterisation of primary IPF lung fibroblasts from diagnostic and explanted lung tissue

3.1 Introduction

A key cell type in the progression of pulmonary fibrosis is the fibroblast. Fibroblasts are a member of the connective-tissue family of cells which under normal circumstances provide cells and organs with structural support through the excretion of collagenous extra-cellular matrix (ECM) (254). Fibroblasts play an important role in the wound-healing process through their ability to migrate to the site of injury and aide in the repair of damaged tissue through proliferation and excretion of ECM (254). However, dysregulation of this wound healing process is detrimental to the body and leads to the onset of fibrosis. In IPF, fibroblasts, along with epithelial cells, play a pivotal role in disease pathogenesis. IPF is clinically characterised by a pattern of fibrosis known as “honeycombing” in the lung tissue (255). Fibroblasts become activated and remain chronically active in IPF, leading to the production excessive amounts of ECM and abberant remodelling (255). Fibroblasts can induce a phenotypic change from normal lung fibroblasts to myo-fibroblasts through pro-fibrotic stimuli such as TGF-β, a key cytokine in fibrosis (256).

Previously, our laboratory has demonstrated that defective TLR3 is associated with a more aggressive clinical phenotype in IPF patients and in patients with pulmonary sarcoidosis (154, 168). TLR3 is activated by viral dsRNA or the synthetic equivalent, Poly(I:C). TLR3 activates a downstream signalling cascade leading to the activation and translocation of the transcription factors, NF-κB and IRF3, respectively, to the nucleus which leads to the production of pro-inflammatory cytokines and type I interferons, respectively (257). Specifically, IPF patients who were heterozygous or homozygous for the TLR3 L412F single nucleotide polymorphism had a greater risk of mortality and an accelerated decline in lung function (FVC) compared with TLR3 L412F wild-type IPF patients. Furthermore, patients with pulmonary sarcoidosis who were homozygous for TLR3 L412F had a significantly increased risk of developing a persistent clinical phenotype compared with TLR3 L412F wild-type IPF patients.
In these earlier studies completed by the Donnelly Research Group, we established that primary human lung fibroblasts from TLR3 L412F-variant IPF or pulmonary sarcoidosis patients produced attenuated TLR3-induced pro-inflammatory cytokines and type I interferon responses. In addition, TLR3 L412F-variant pulmonary fibroblasts from these patients had dysregulated proliferative responses compared with TLR3 L412F wild-type patients.

In this chapter, and in subsequent chapters, we have examined TLR3 responses following stimulation with the synthetic agonist for TLR3, Poly(I:C). We have used Poly(I:C) to mimic double-stranded RNA found in the IPF lung. In the context of IPF, there are a number of potential sources of dsRNA in the lung which can activate TLR3. TLR3 can be activated by a number of stimuli including, viral dsRNA, mRNA from necrotic cells and mRNA from a limited number of bacteria and helminths (142-145). There are many different potential sources of TLR3 stimuli in the IPF lung including non-infectious and infectious sources. In fibrosis, repetitive injury to the lung tissue by environmental hazards, occupational hazards or infection leads to damage of the lung tissue. In the normal healthy lung, cell necrosis resulting in DAMP release induces an inflammatory response in the lung in order to clear cell debris. Excessive DAMP signalling can lead to detrimental inflammation in the lungs (258). Epithelial cells in the lungs undergo necrosis in IPF patients resulting in the release of DAMPs. Type II alveolar epithelial cell death is an early feature of pathogenesis in the IPF lung. DAMPs including mRNA from necrotic cells have the ability to activate TLR3 on the surface of lung fibroblasts. dsRNA has previously been detected in BAL fluids collected from IPF patients highlighting a potential activator for TLR3 in the IPF lung (258).

Another potential source of TLR3 activation in the lungs is by infectious agents. Infectious agents such as active and latent viral infections have an established role in disease pathogenesis in IPF (259). The presence of infectious agents in the lung leads to sustained activation of the PRRs including TLRs and RLRs resulting in chronic inflammation. In addition to the presence of DAMPs in IPF BAL fluid, the presence of herpesviruses from chronic/latent infection has been detected in the IPF lung (71-73, 75). Herpesviruses lead to the activation of TLR3 and the production of type I interferons (260, 261). Re-activation of latent herpes infections have been suggested as a trigger for disease progression in IPF (69).
The aim of this thesis was to investigate the mechanism of action of TLR3 L412F further in disease progression in IPF patients. In order to facilitate this at a cellular level, we obtained primary human lung fibroblasts from IPF patients both at diagnosis and prior to lung transplant (i.e. explanted lung tissue). Previously, in the Donnelly Research Group, we characterised the TLR3 responses in IPF lung fibroblasts cultured from video assisted thoracic surgery (VATS) biopsy samples taken at diagnosis (154). However, there is recent evidence to suggest that the VATS biopsy procedure (previously used in the diagnosis of IPF) is associated with a significant risk of mortality in patients. Therefore, the use of this procedure at diagnosis has become unpopular and diagnostic IPF lung tissue samples are rarely taken. Currently, explanted lung tissue is used primarily to culture primary human lung fibroblasts from IPF patients for mechanistic studies.

In this chapter, a total of twenty five IPF patient lung fibroblasts were cultured, genotyped and characterised functionally for use in this study in the context of Poly(I:C)-induced TLR3- and LPS-induced TLR4 responses.
3.2 TLR3 L412F genotyping and characterisation of IPF primary human lung fibroblasts

Primary human IPF fibroblast cell lines were cultured and genotyped as described in Chapter 2. Genotypes were determined using a Taqman genotyping SNP assay for TLR3 L412F through examination of the amplification plots for the wildtype (Leu/Leu) (Fig. 3.1A), heterozygous (Leu/Phe) (Fig. 3.1B) and homozygous (Phe/Phe) (Fig. 3.1C) genotypes. Previously, a total of 21 primary cell lines were obtained at diagnosis and grown from VATS biopsies from IPF patients in St. Vincent’s University Hospital and University of Michigan. IPF fibroblasts were genotyped and it was found that 3/21 (14.3%) cells lines were Leu/Leu, 16/21 (76.2%) were Leu/Phe and 2/21 (9.5%) were Phe/Phe (Fig. 3.2A). For this study, a total of 14 primary IPF fibroblast cell lines grown from explanted lung tissue from IPF patients in Cedars Sinai Hospital, Los Angeles, USA, were used for all experiments (Fig. 3.2B). 8/14 (57.14%) cells lines were Leu/Leu and 6/14 (42.86%) were Leu/Phe. No Phe/Phe IPF patients were found. An additional 8 primary IPF fibroblast cell lines were obtained at diagnosis from the University of Edinburgh. However, IPF patients who underwent surgical biopsies were known to have a mild form of IPF, and due to this all cell lines were Leu/Leu which therefore were not used in this study (Fig. 3.2C). Images of Leu/Leu and Leu/Phe IPF fibroblasts were taken using a Motic Image Plus software (Fig. 3.3). Morphological differences were seen between the two genotypes. Leu/Phe fibroblasts had a longer, more stretched appearance while the Leu/Leu IPF fibroblasts were shorter.
Figure 3.1. Amplification plots for TLR3 L412F single nucleotide polymorphism (SNP) assay. Genomic DNA was isolated from IPF lung fibroblasts and genotyped. The “C” allele was detected by the Taqman specific probe tagged with the FAM fluorescent dye (green). The “T” allele was detected by the Taqman specific probe tagged with the HEX fluorescent dye (red). ROX dye was used as a reference (blue). TLR3 L412F wild-type “CC” (A), TLR3 L412F heterozygote “CT” (B) and TLR3 L412F homozygote “TT” (C).
Figure 3.2. Distribution of TLR3 L412F wild-type (Leu/Leu), heterozygote (Leu/Phe) and homozygote (Phe/Phe) genotypes in primary human lung fibroblasts from IPF patients. IPF fibroblasts were cultured from tissue taken by VATS biopsy at St Vincent’s Hospital, Dublin and University of Michigan at diagnosis (A). IPF fibroblasts were cultured from explanted lung tissue in Cedars-Sinai Hospital, Los Angeles (B) and University of Edinburgh from tissue taken at diagnosis (C).
Figure 3.3. Images of primary human lung fibroblasts from wild-type and heterozygote IPF patients. Lung samples were obtained from explanted lung tissue. Tissue was genotyped and images were taken using Motic Image Plus 2.0 ML software and at 10X magnification.
3.3 Characterisation of the responses of TLR3 L412F wild-type and heterozygous primary human lung fibroblasts from IPF patients to Poly(I:C) and LPS treatment

Initial characterisation studies were undertaken to establish the responsiveness of Leu/Leu and Leu/Phe IPF primary human cell lines to appropriate stimuli including TLR3 and TLR4 synthetic agonists. All cells including those cultured from explanted lung tissue and at diagnosis were characterised. Primary IPF human lung fibroblasts from Leu/Leu and Leu/Phe patients were cultured in vitro. Cells were subsequently treated with increasing doses of Poly(I:C) for 24 h (0.1, 1, 10 μg/ml) and LPS for 24 h (1, 10, 100 ng/ml). The cytokine IL-8 was used as a readout for NF-κB activation and RANTES protein as an IRF3 readout. RANTES has been strongly associated with the activation of transcription factor IRF3 (262). However, it is worth noting that RANTES can also be induced via NF-κB activation (263). RANTES is an important chemokine in the immune response to infection, as it attracts many different cell types including monocytes, NK cells, T cells and eosinophils to the site of infection. It has been shown during viral infection that RANTES expression can be induced by transcription factors IRF3, IRF7 and NF-κB (263). Leu/Leu IPF fibroblasts cultured from explanted lung tissue samples produced a robust pro-inflammatory cytokine and RANTES response following stimulation with both Poly(I:C) and LPS. IL-8 protein production was significantly upregulated following stimulation with the highest concentration of Poly(I:C) (10 μg/ml) compared with untreated cells (**p<0.01, Fig. 3.4A). Similarly, RANTES protein production was significantly upregulated (**p<0.01, Fig. 3.4C) after 24 hours. Leu/Phe IPF fibroblasts had an attenuated NF-κB and IRF3 response following Poly(I:C) stimulation compared with untreated cells (Fig. 3.4B, D).

Similar results were observed following treatment with LPS. IL-8 (**p<0.01, Fig. 3.5A) and RANTES protein (**p<0.01, Fig 3.5C) production was significantly upregulated in Leu/Leu IPF fibroblasts upon LPS stimulation (100 ng/ml) compared with media only. Conversely, upregulation of IL-8 and RANTES protein production was not observed in Leu/Phe IPF fibroblasts (Fig. 3.5B, D).

Diagnostic primary IPF human lung fibroblasts were also characterised. In addition to the IPF fibroblasts from explanted lung tissue, Leu/Leu IPF fibroblasts
cultured from VATS biopsies at diagnosis significantly upregulated IL-8 (**p<0.001, Fig. 3.6A, C) and RANTES (**p<0.001, Fig. 3.6B, D) protein expression following 24 hour stimulation with Poly(I:C) (10 μg/ml) and LPS (100 ng/ml) stimulation compared with untreated cells.

From this experiment we established which cell lines could be used in the study. Interestingly, it was found by ELISA that primary IPF human lung fibroblasts from Leu/Phe patients have higher basal levels of cytokines IL-8, IL-6 and RANTES compared with Leu/Leu IPF patients (Fig. 3.7B, D, E). Pulmonary fibroblasts were cultured in a 24 well plate unstimulated for 24 h, basal levels of IL-8, IL-6 and RANTES were quantified by ELISA. Variability in basal levels of cytokines and RANTES between IPF patients is seen in both genotypes (Fig. 3.7A, C, E). However, overall primary IPF human lung fibroblasts from Leu/Phe patients have significantly higher basal levels of IL-8 (**p<0.001, Fig. 3.7B), IL-6 (**p<0.001, Fig. 3.7D) and RANTES (**p<0.001, Fig. 3.7E). These characterisation studies revealed a clear difference between primary IPF human lung fibroblasts from Leu/Leu patients and Leu/Phe patients at the protein level.
Figure 3.4. Characterisation of the effect of TLR3 L412F on Poly(I:C)-induced IL-8 and RANTES protein expression in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients obtained from explanted lung tissue. TLR3 L412F attenuates Poly(I:C)-induced IL-8 and RANTES protein expression in primary human lung fibroblasts from Leu/Phe IPF patients (B, D) compared with (A, C) Leu/Leu wild-type IPF patients at 24 h post-treatment, as quantitated by ELISA. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: treatment with Poly(I:C) compared with medium-only treatment. Representative Leu/Leu and Leu/Phe cell line are shown. Results shown are the mean +/- S.E.M of six replicates. [Poly(I:C) gradient; 0.1,1 and 10 μg/ml].
Figure 3.5. Characterisation of the effect of TLR3 L412F on LPS-induced IL-8 and RANTES protein expression in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients obtained from explanted lung tissue. TLR3 L412F attenuates LPS-induced IL-8 and RANTES protein expression in primary human lung fibroblasts from Leu/Phe IPF patients (B, D) compared with (A, C) Leu/Leu IPF patients at 24 h post-treatment, as quantitated by ELISA. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: treatment with LPS compared with medium-only treatment. Representative Leu/Leu and Leu/Phe cell line are shown. Results shown are the mean +/- S.E.M of six replicates. [LPS gradient; 1, 10 and 100 ng/ml].
Figure 3.6. Characterisation of the effect of TLR3 L412F on TLR3- and 4-induced IL-8 and RANTES protein expression in TLR3 L412F wild-type (Leu/Leu) primary human lung fibroblasts from IPF patients obtained at diagnosis from the University of Edinburgh. Poly(I:C) (A,B) and LPS (C,D) -induced IL-8 and RANTES protein expression in primary human lung fibroblasts from Leu/Leu wild-type IPF patients at 24 h post-treatment, as quantitated by ELISA. No Leu/Phe lung fibroblast samples were found in the University of Edinburgh cohort. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: treatment with Poly(I:C) or LPS compared with medium-only treatment. Representative Leu/Leu cell line is shown. Results shown are the mean +/- S.E.M of six replicates. [Poly(I:C) gradient (0.1, 1, 10 μg/ml) and LPS gradient (1, 10 and 100 ng/ml)].
Figure 3.7. Effect of TLR3 L412F on basal levels of IL-8, IL-6 and RANTES protein production in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. Higher levels of IL-8, IL-6 and RANTES protein production are seen in primary human lung fibroblasts from Leu/Phe IPF patients compared with Leu/Leu wild-type IPF patients at 24 h post-treatment, as quantitated by ELISA. Results shown are the mean +/- S.E.M of six replicates (A, C, E). Results shown are the mean +/- S.E.M of six cell lines per genotype with five to six replicates. Mann-Whitney test was used to test for statistical significance (non-parametric unpaired t test) *** p<0.001 (B, D, F).
3.4 Characterisation of the effects of TLR3 and TLR4 activation over time in primary IPF human lung fibroblasts from diagnostic and explanted lung tissue and fibroblast cell lines

In order to examine TLR3 and TLR4 cytokine and RANTES responses in TLR3 L412F IPF fibroblasts over a period of time, a time course study was undertaken in vitro for 24 h. Diagnostic and explanted Leu/Leu and Leu/Phe IPF fibroblasts were stimulated with three concentrations of Poly(I:C) (10, 20 and 50 \( \mu \)g/ml) and LPS (1 \( \mu \)g/ml). All treatments were calculated relevant to media only (control) at each timepoint and upregulation of IL-8, IL-6 and RANTES expression was assessed using fold increase. As a comparison the human fibroblast cell line, CCD-19lu was also included in the time course. TLR3 and TLR4 activation of downstream signalling pathways was assessed by measuring IL-8, IL-6 and RANTES protein production by ELISA at 3 h, 6 h, 12 h and 24 h.

IL-6 protein production was assessed as a readout for transcription factor NF-kB activation in the time course. In Leu/Leu fibroblasts grown from diagnostic VATS biopsies, IL-6 protein production was induced at all three concentrations of Poly(I:C) (purple line; Fig. 3.8A, B, C). IL-6 protein levels did not increase with increasing concentrations of Poly(I:C). Highest levels of IL-6 protein production in diagnostic Leu/Leu fibroblasts were seen at 24 h following stimulation with 10, 20 or 50 \( \mu \)g/ml Poly(I:C) (Fig. 3.8A, B, C). In Leu/Leu fibroblasts grown from explanted IPF lung tissue, IL-6 protein levels increased steadily over 24 h (blue line; Fig. 3.8A, B, C). Highest levels of IL-6 protein production in explanted Leu/Leu IPF fibroblasts were seen at 24 h following stimulation with 10, 20 or 50 \( \mu \)g/ml Poly(I:C). Diagnostic Leu/Leu IPF fibroblasts had higher levels of IL-6 production after 24 h compared with explanted Leu/Leu IPF fibroblasts when stimulated with 10 \( \mu \)g/ml Poly(I:C). However, at higher concentrations of Poly(I:C) (20 or 50 \( \mu \)g/ml Poly(I:C)) similar levels of IL-6 were produced by diagnostic and explant Leu/Leu fibroblasts. In Leu/Phe fibroblasts from explant lung tissue, IL-6 protein production showed a slight increase following Poly(I:C) stimulation after 6 h (red line; Fig. Fig. 3.8A, B, C). IL-6 protein production was not induced further at any time point or at any concentration of Poly(I:C). Finally, CCD-19lu fibroblasts induced a modest
increase of IL-6 following treatment with Poly(I:C) (10 μg/ml) (green line; Fig. 3.8A). At high concentrations of Poly(I:C) CCDs induced the highest levels IL-6 of all cell lines which continually increased over 24 h (green line; Fig. 3.8C).

IL-8 expression was also assessed by ELISA. Leu/Leu IPF fibroblasts cultured from explanted IPF lung tissue showed a sharp spike in IL-8 cytokine expression at 6 h post treatment with Poly(I:C) at all concentrations which dropped off between 6 h and 12 h (blue line; Fig. 3.9A, B, C). Interestingly, Leu/Leu fibroblasts cultured from IPF lung tissue at diagnosis did not have a spike of IL-8 production at 6 h but instead a steady increase in IL-8 expression over the 24 h period (purple line; Fig. 3.9A, B, C). IL-8 production showed minimal upregulation in diagnostic Leu/Leu IPF fibroblasts with increasing concentrations of Poly(I:C). Leu/Phe IPF fibroblasts cultured from explanted IPF lung tissue had attenuated IL-8 expression at all concentrations of Poly(I:C) at all timepoints (red line; Fig. 3.9A, B, C). CCD-19lu had low levels of IL-8 production in the first 12 h of treatment but upregulated production after 12 h to the same levels as diagnostic and explant Leu/Leu IPF fibroblasts (green line; Fig. 3.9A, B, C).

Finally, IRF3 activation over 24 h was analysed using RANTES protein production as a readout. As seen in IL-8 and IL-6 protein production, Leu/Phe IPF fibroblasts cultured from explanted IPF lung tissue had attenuated responses at all concentrations of Poly(I:C) at all timepoints compared with media only. Explanted Leu/Phe IPF fibroblasts express minimal levels of RANTES compared with all other cell types (red line, Fig. 3.10A, B, C). Leu/Leu IPF fibroblasts cultured from explanted IPF lung tissue had a steady increase in RANTES protein expression over 24 h at all concentrations of Poly(I:C). Highest levels of RANTES protein were seen at a concentration of 50 μg/ml Poly(I:C) at 24 h (blue line, Fig. 3.10A, B, C). Leu/Leu fibroblasts cultured from IPF lung tissue at diagnosis expressed higher levels of RANTES over 24 h compared with explant Leu/Leu IPF fibroblasts at all concentrations of Poly(I:C) (purple line, Fig. 3.10A, B, C). Diagnostic Leu/Leu fibroblasts steadily increased RANTES protein production over 24 h. CCD19-lu showed the greatest induction of RANTES protein expression between 12 and 24 h compared with all other cell lines (green line, Fig. 3.10A, B, C). At 0 to 12 h CCDs at all concentrations of Poly(I:C) expressed low levels of RANTES, however, between 12 and 24 h there was a sharp increase in the amount of RANTES produced.
IPF fibroblasts and CCD-19lu fibroblasts were also treated with LPS (1 μg/ml) for 24 h. IL-6, IL-8 and RANTES levels were analysed by ELISA at 3 h, 6 h, 12 h, and 24 h. In contrast to Poly(I:C) stimulation, highest induction of IL-6 expression was in the Leu/Phe IPF fibroblasts cultured from explant IPF lung tissue over 24 h when treated with LPS (red line; Fig. 3.11A). High levels of IL-6 were produced after 3 h and these levels did not change significantly over time. Similarly, Leu/Leu fibroblasts cultured from diagnostic IPF lung samples showed no upregulation of IL-6 production over the 24 h period (purple line; Fig. 3.11A). Both Leu/Leu explant IPF fibroblasts and CCD-19lu fibroblasts had a modest induction of IL-6 up to 12 h which then decreased until 24 h (blue and green line respectively; Fig. 3.11A).

IL-8 production was analysed following LPS treatment as well in all fibroblasts. As previously seen following Poly(I:C) treatment, Leu/Leu IPF fibroblasts from explant lung tissue has a spike of IL-8 production after 6 h which tapered off sharply at 12 h (blue line; Fig. 3.11B). Leu/Phe fibroblasts cultured from explanted IPF lung tissue had induced IL-8 expression following LPS treatment for 6 h but no further upregulation was seen over the remaining 18 h (red line; Fig. 3.11B). The same was seen in diagnostic Leu/Leu IPF fibroblasts or CC-19lu, IL-8 expression following LPS treatment for 6 h but no further upregulation was seen over the remaining 18 h (purple and green line respectively; Fig. 3.11B). Leu/Leu and Leu/Phe IPF fibroblasts cultured from explant IPF lung tissue samples had a sharp increase in RANTES protein production after 6 and 12 h, respectively (blue and red line; Fig. 3.11 C). Explant Leu/Phe IPF fibroblasts had a steady increases in RANTES expression between 12 and 24 h while explant Leu/Leu cells spiked at 12 h stimulation with LPS and decreased until 24 h. Leu/Leu fibroblasts cultured from diagnostic IPF lung samples showed a modest increase in RANTES production over the 24 h period (purple line; Fig. 3.11C).CCD-19lu fibroblasts had a steady increase of RANTES expression over the 24 h period (green line; Fig. 3.11C).

These results are significant as they prove that the differences in responses to TLR3 and TLR4 activation seen in Leu/Leu and Leu/Phe IPF fibroblasts can been seen at different time-points and different concentrations of Poly(I:C) and LPS. Time-point studies also revealed that the CCD-19lu immortalised cell line could not be used as a control cell line for comparison between IPF and healthy/ normal
fibroblasts as CCD-19lu’s had similar cytokine responses following Poly(I:C) stimulation to the IPF cells.
Figure 3.8. Time course of Poly(I:C)-induced IL-6 protein production. Explant Leu/Leu (blue) and explant Leu/Phe (red) primary human lung fibroblasts from IPF patients, diagnostic Leu/Leu (purple) primary human lung fibroblasts from IPF patients and CCD19-LU (green) normal lung fibroblasts. IL-6 protein levels were assessed at 3, 6, 12 and 24 hrs. All fibroblast cell lines were stimulated with 10 μg/ml Poly(I:C) (A), 20 μg/ml Poly(I:C) (B) and 50 μg/ml Poly(I:C) (C). Results shown are the mean +/- S.E.M of one experiment, each cell line with six replicates. [Poly(I:C) (TLR3) 10, 20 and 50 μg/ml].
Figure 3.9. Time course of Poly(I:C)-induced IL-8 protein production. Explant Leu/Leu (blue) and explant Leu/Phe (red) primary human lung fibroblasts from IPF patients, diagnostic Leu/Leu (purple) primary human lung fibroblasts from IPF patients and CCD19-LU (green) normal lung fibroblasts. IL-8 protein levels were assessed at 3, 6, 12 and 24 hrs. All fibroblast cell lines were stimulated with 10 μg/ml Poly(I:C) (A), 20 μg/ml Poly(I:C) (B) and 50 μg/ml Poly(I:C) (C). Results shown are the mean +/- S.E.M of one experiment, each cell line with six replicates. [Poly(I:C) (TLR3) 10, 20 and 50 μg/ml].
Figure 3.10. **Time course of Poly(I:C)-induced RANTES protein production.** Explant Leu/Leu (blue) and explant Leu/Phe (red) primary human lung fibroblasts from IPF patients, diagnostic Leu/Leu (purple) primary human lung fibroblasts from IPF patients and CCD19-LU (green) normal lung fibroblasts. RANTES protein levels were assessed at 3, 6, 12 and 24 hrs. All fibroblast cell lines were stimulated with 10 μg/ml Poly(I:C) (A), 20 μg/ml Poly(I:C) (B) and 50 μg/ml Poly(I:C) (C). Results shown are the mean +/- S.E.M of one experiment, each cell line with six replicates. [Poly(I:C) (TLR3) 10, 20 and 50 μg/ml].
Figure 3.11. Time course of LPS-induced IL-6, IL-8 and RANTES protein production. Explant Leu/Leu (blue) and Leu/Phe (red) primary human lung fibroblasts from IPF patients, diagnostic Leu/Leu (purple) primary human lung fibroblasts from IPF patients and CCD-16Lu (green) normal lung fibroblasts were assessed at 3, 6, 12 and 24 hrs for IL-6 (A), IL-8 (B) and RANTES (C) protein production. Results shown are the mean +/- S.E.M of one experiment, each cell line with six replicates. [LPS (TLR4) 1 μg/ml.]
3.5 Discussion

In this chapter, we genotyped and characterised a total of 25 explanted and diagnostic IPF lung fibroblast samples. Specifically, we examined the effect of TLR3 L412F on IPF fibroblast cytokine responses to TLR3- and TLR4-activation following Poly(I:C) and LPS treatment, respectively. IPF fibroblasts cultured from explanted lung tissue were the main cell type used throughout these studies. Genotyping analysis of IPF fibroblasts cultured from explanted lung tissue revealed that 42.86% of patients were wild-type (6/14) for the TLR3 L412F SNP while 57.14% of patients were heterozygous (8/14). This high frequency of heterozygous patients is in keeping with the original genotyping studies carried out by us previously and in addition, by Ranjith-Kumar et al. who analysed the geographic distribution of the TLR3 L412F SNP worldwide and found that the SNP has been kept at a relatively high frequency globally with the highest frequency in populations with European ancestry, followed by Asian populations and was very rare in the African population (154, 157, 168). In Europe, the heterozygosity for the SNP is 45.7% (165). In our diagnostic IPF fibroblasts it is even more striking; 76.2% of IPF patients (16/21) are heterozygous for the SNP. Wild-type and homozygous patients made up 14.3% and 9.5% of the cohort respectively.

Interestingly, in our genotyping of IPF fibroblasts cultured from explanted lung tissue we did not find any patients that were homozygous for the TLR3 L412F SNP. The absence of a homozygote patient in the explanted IPF lung tissue cohort may be because these patients had a more severe form of IPF and had died before they could receive a lung transplant. In 2013, O’Dwyer et al. analysed the placebo cohort of the INSPIRE clinical trial and found that at 12 and 24 months patients that were heterozygous or homozygous for TLR3 L412F were 5 times more likely to die than L412F- wild-type IPF patients (154). Here, we suggest that the reason no homozygotes were found in the explant IPF lung tissue cohort was due to the fact that TLR3 L412F homozygote patient’s disease progression is more severe and therefore, these patients had died before lung transplantation was possible. This theory is backed-up by the fact that genotyping of IPF fibroblasts cultured from diagnostic lung tissue revealed 2 homozygous patients out of a total of 21 patients. Moreover, in the small cohort of IPF patient lung fibroblasts cultured from VATS
biopsies at diagnosis from the University of Edinburgh, 100% TLR3 L412F wild-type patients (8/8) were found. Following personal communication with the respiratory consultant who obtained the samples it was revealed that only IPF patients with mild disease progression were referred for VATS biopsy due to the significant risk of morbidity or mortality associated with the procedure. This added further evidence to hypothesis that homozygote patients were dead prior to lung transplantation and thus, we could not access any TLR3 L412F IPF fibroblasts cultured from explant lung tissue.

Following genotyping, all IPF patient fibroblasts were cultured in vitro and cytokine responses to Poly(I:C)-induced TLR3 activation were assessed. Activation of TLR3 on the cell surface and the endosome induces a downstream signalling cascade resulting in the activation of transcription factors, NF-κB and IRF3. These transcription factors are critical for the expression of pro-inflammatory cytokines and type I interferons. Previous work completed in the Donnelly Research Group by O’Dwyer et al. examined TLR3-induced NF-κB and IRF3 activation in TLR3 L412F wild type and heterozygote IPF primary lung fibroblasts (154). Their work showed that TLR3-induced activation of IRF3 was reduced in both L412F-heterozygous and -homozygous IPF lung fibroblasts compared with TLR3 L412F wild-type fibroblasts. At the mRNA level, IFN-β and RANTES, and at the protein level, RANTES (readouts for IRF3) had attenuated expression in both L412F-heterozygous and homozygous IPF lung fibroblasts following Poly(I:C)-induce activation of TLR3 compared with TLR3 L412F wild-type fibroblasts (154). Similarly, Poly(I:C)-induced activation of NF-κB was affected in the presence of the SNP. IL-8 mRNA expression and protein production, a readout of NF-κB, was reduced in L412F-heterozygous and homozygous IPF lung fibroblasts compared with TLR3 L412F wild-type fibroblasts.

In this chapter, we confirm and extend these results in a new cohort of IPF patients. Moreover, we had added to the observations of O’Dwyer et al. All published work in the past have examined differences in TLR3 activation in IPF fibroblasts following 24 h stimulation with Poly(I:C) in vitro. Here, we have shown that differences in TLR3 activation in IPF fibroblasts between TLR3 L412F wild-type and heterozygote genotypes can be seen as early as 6 h post treatment. Importantly, we have shown that the results previously found in fibroblasts cultured
from diagnostic IPF fibroblasts can be replicated in fibroblasts cultured from explanted lung tissue. This suggests that that differences seen in IPF patients in the presence of the SNP can be observed throughout the course of the disease, from initial diagnosis through to end-stage disease.

The effect of the SNP has been published in the context of other diseases and cell types. Importantly, work published by one of the initial groups to look at the SNP backs-up our findings. Ranjith-Kumar et al. transfected the immortalised cell line; HEK293T, with plasmids for TLR3 L412F. They showed using a luciferase assay that in the presence of the L412F-plasmids Poly(I:C)-induced TLR3 activation was attenuated in comparison to HEK293T with transfected wild-type TLR3 (157). Similarly, Gorbea et al. also used luciferase assays to confirm that L412F-expressing Cos7 cells had reduced luciferase activity following Poly(I:C) stimulation compared with Cos7 cells expressing exogenous TLR3 (167). This supports the results we have found in IPF primary lung fibroblasts, however in both studies, research was conducted using immortalised cell lines. In our lab we have shown effect of TLR3 L412F on TLR3 signalling in two independent primary cells lines from IPF patients and pulmonary sarcoidosis patients. Specifically, the Donnelly Research Group has previously shown that TLR3 L412F homozygous primary lung fibroblasts from pulmonary sarcoidosis patients had defective signalling through TLR3 and reduced downstream activation of NF-κB and IRF3 activation (168). This study affirms our findings in primary pulmonary fibroblasts in a different disease with lung involvement.

In addition to lung fibroblasts, other research groups have analysed other primary cell types in the presence of the SNP. PBMCs were isolated from patients with chronic candidiasis, recurrent infections and multiple autoimmune manifestations and stimulated with Poly(I:C) (264). It was shown that IFN-γ and TNF-α levels were reduced in PBMCs expressing the TLR3 L412F mutation compared with wild-type PBMCs (264).

In Chapter 4, we employ the primary human lung fibroblasts from IPF patients genotyped and characterised in this chapter to investigate the role of TLR3 L412F in the context of: (i) TLR3-independent responses (i.e. TLR1-9), (ii) secondary-IFN responses and (iii) non-TLR-dependent responses.
Chapter 4

Characterisation of anti-bacterial and anti-viral TLR responses in primary human lung fibroblasts from TLR3 L412F wild-type and heterozygote
Chapter 4 Characterisation of anti-bacterial and anti-viral TLR responses in primary human lung fibroblasts from TLR3 L412F wild-type and heterozygote IPF patients

4.1 Introduction

TLRs are vital immune sensors which detect both bacterial and viral PAMPs, in addition to other pathogenic moieties. TLR expression is important at the mucosal surfaces which are constantly exposed to the environment (115). TLR3 has been found to be highly expressed in the lungs and at other mucosal sites (140, 141). TLR3 is expressed on the cell surface and the endosomal membrane of many immune cells as well as fibroblasts, endothelial cells, epithelial cells and neurons (135-138). Once activated TLRs initiate a downstream signalling cascade resulting in the expression of pro-inflammatory cytokines and type I interferons which initiate the immune response to infection.

TLRs share the same signalling pathway to activate transcription factors NF-κB, IRFs, activator protein 1 (AP1), and mitogen-activated protein kinases. Once activated most TLRs initiate a signalling cascade through their adaptor protein MyD88. TLR3 uses a different adaptor protein TRIF while TLR4 can use both MyD88 and TRIF (106). For the production of pro-inflammatory cytokines via transcription factor NF-κB, all TLRs converge and signal through the downstream protein TRAF6 (265, 266). For type I interferon expression, TLRs 3 and 4 act through TRAF3 to activate transcription factor IRF3 (149, 267). Interestingly, other non-TLR cytosolic sensors such as RIG-I and MDA5 also share this pathway and activate the same downstream transcription factors (268).

Using the primary human lung fibroblasts from IPF patients which were genotyped and characterised in chapter 3, the aim of this chapter was to investigate the role of TLR3 L412F in the context of: (i) TLR3-independent anti-viral and anti-bacterial responses (i.e. TLR1-9 responses), (ii) secondary-IFN responses and (iii) non-TLR-dependent responses. Using IL-8 and RANTES as readouts for NF-κB and IRF3 activation, respectively, we assessed downstream signalling capabilities of Leu/Leu and Leu/Phe IPF fibroblasts in these experiments. Our overarching
hypothesis was that TLR3 L412F may have additional effects on responses to anti-viral and anti-bacterial TLR-PAMPs, given the high degree of homology that exists within the signalling pathways of TLR superfamily members.

The role of viral infection in disease progression in IPF has been extensively studied and is believed to be of major significance in disease progression in IPF. As previously discussed, many different viral infections have been associated with IPF including herpesviruses, influenza A virus, hepatitis C virus and torque tenue virus (71, 72, 79, 82, 85). There is conflicting evidence regarding the role of viral infection in acute exacerbations of IPF (AE-IPF). One study carried out using nasopharyngeal swabs in AE-IPF and stable patients found that AE-IPF patients had higher viral titres than stable IPF patients. Interestingly, HHV human herpesvirus and influenza virus A were the two most commonly detected viruses in AE-IPF patients (269). However, others analysing bronchoalveolar lavage (BAL) fluids and serum have shown that viruses are present at low levels and not necessarily associated with AE-IPF (270).

Recently, there is mounting evidence to suggest that bacterial infection also plays a role in IPF disease progression. Previously, BAL fluid collected from IPF patients revealed that IPF patients had a higher bacterial load than healthy people and COPD patients. Four bacterial strains, Streptococcus sp., Veillonella sp., Haemophilus sp. and Neisseria sp. were found at higher levels in IPF patients than controls and COPD patients. This increased bacterial load correlated with increased risk of mortality in IPF patients (271). Similarly, bacterial infection has also been implicated in disease progression during AE-IPF (91, 269). IPF patients undergoing an acute exacerbations were shown to have higher levels of anti-microbial IgM compared with stable IPF patients, however they showed no significant difference between bacterial detection rates in stable and AE-IPF patients (269).

Our results suggest that the presence of the TLR3 L412F SNP may have a detrimental impact on disease progression in IPF patients during both viral and bacterial infection. Here, we show that TLR3 L412F affects signalling not only through TLR3 but in addition, through the bacterial TLRs 2, 4, 5, 6 and 9. Furthermore, we have shown that the IFN-induced secondary response is attenuated in Leu/Phe IPF patients. Leu/Phe IPF patients have dysregulated expression of the
anti-viral cytosolic receptors RIG-I, MDA5 and PKR. In addition, we have demonstrated that treatment with recombinant IFN-β does not restore TLR responses in Leu/Phe IPF fibroblasts. Finally, we have shown TLR3 L412F also attenuates responses of IPF fibroblasts to TLR-independent PAMPs, Poly(dA:dT), PMA and Herring-testes (HT)-DNA. Therefore, the experiments described in this chapter confirm that TLR3 L412F can exert effects in IPF fibroblasts from 412F-heterozygote patients which are TLR3- and TLR-independent, respectively. This data provides novel evidence which may help to explain why 412F-heterozygote IPF patients experience an accelerated clinical phenotype, which is associated with enhanced mortality, compared with TLR3 L412F wild-type IPF patients.
4.2 Primary human lung fibroblasts from Leu/Phe IPF patients have a reduced IL-8, IFN-β and RANTES responses to TLR3, TLR4 and TLR9 agonists

We investigated TLR3 function in primary human fibroblasts from TLR3 L412F wild-type (Leu/Leu), heterozygous (Leu/Phe), and homozygous (Phe/Phe) patients with IPF after TLR3 activation. We previously demonstrated that TLR3 L412F conferred defective NF-kB- and IRF3-function in primary human lung fibroblasts from Leu/Phe and Phe/Phe IPF patients in response to Poly(I:C) treatment (154). In this study, we examine the effect of TLR3 L412F on functionality of additional TLR family members. Here, we compared TLR3, TLR4 and TLR9 activation in primary human lung fibroblasts from Leu/Leu IPF patients and Leu/Phe heterozygote IPF patients with untreated cells. Treatment of primary human lung fibroblasts from Leu/Leu IPF patients with Poly(I:C)(10 μg/ml) significantly upregulated IL-8 mRNA transcription (Fig. 4.1A; NF-kB-readout; *** p<0.001), IFN-β mRNA (Fig. 4.1C; IRF3-readout, *** p<0.001) and RANTES mRNA (Fig. 4.1E; IRF3-readout, *** p<0.001) expression but this was not observed in cells from Leu/Phe IPF patients (Fig. 4.1B, D, E), respectively.

Similarly, treatment of primary fibroblasts from Leu/Leu IPF patients with LPS (100 ng/ml) significantly upregulated IL-8 mRNA (Fig. 4.1A), IFN-β mRNA (Fig. 4.1C) and RANTES mRNA (Fig. 4.1E, *p<0.05) expression compared with medium only cells but this was not observed in cells from Leu/Phe IPF patients (Fig. 4.1B, D, F), respectively. Treatment of Leu/Leu IPF primary lung fibroblasts with CpG, resulted in a more modest increase in IL-8, IFN-β and RANTES transcription (Fig. 4.1A, C, E) which was attenuated in cells from Leu/Phe IPF patients (Fig. 4.1B, D, F). These results demonstrate that TLR3 L412F attenuates TLR3, TLR4 and TLR9 signalling in primary human lung fibroblasts from Leu/Phe IPF patients which is not seen Leu/Leu IPF patients.
Figure 4.1. Effect of TLR3 L412F on TLR3-, TLR4- and TLR9-induced IL-8, IFN-β and RANTES mRNA expression in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. TLR3 L412F attenuates Poly(I:C), LPS and CpG-induced IL-8 mRNA, IFN-β mRNA and RANTES mRNA expression in primary human lung fibroblasts from (B, D, F) Leu/Phe IPF patients compared with (A, C, E) Leu/Leu wild-type IPF patients at 24 h post-treatment, as quantitated by qPCR analysis. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: treatment with respective TLR agonist compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments. [Poly(I:C), 10 µg/ml; LPS, 100 ng/ml; CpG, 5 µM].
4.3 TLR3 L412F attenuates secondary interferon responses in primary human lung fibroblasts from Leu/Phe IPF patients

Type I interferons act through their cell membrane receptor, IFNAR, to upregulate the expression of many other genes involved in the secondary interferon response (257). Cytosolic sensors RIG-I, MDA5 and PKR are among those genes whose expression is induced (257). RIG-I is a highly conserved protein receptor which is activated by viral and synthetic dsRNA (272). MDA5 is the closest relative of RIG-I as they share a very similar structure. RIG-I and MDA5 are known to detect different viruses in the cell. RIG-I recognises RNA viruses from the families; Flaviviridae, Paramyxoviridae, Orthomyxoviridae, and Rhabdoviridae. MDA5 is essential for detection of the Picornaviridae family of RNA viruses in the cell (122).

As we have shown that Leu/Phe IPF patients show reduced IFN-β mRNA and RANTES mRNA expression (Fig. 4.1D, F). Here, we investigated the effect of this on secondary interferon-induced cytosolic sensor expression. Following 24 h stimulation with Poly(I:C), Leu/Leu IPF fibroblasts significantly upregulated RIG-I (Fig. 4.2A, *** p<0.001), MDA5 (Fig. 4.2C, *** p<0.001) and PKR (Fig. 4.2E, * p<0.05) mRNA expression compared with media only. Similarly, LPS treatment upregulated RIG-I, MDA5 and PKR expression compared with medium only (Fig 4.2A, C, E). RIG-I, MDA5 and PKR mRNA expression in remained unchanged Leu/Leu IPF fibroblasts following CpG treatment (Fig. 4.2A, C, E).

Treatment of primary human lung fibroblasts from Leu/Phe IPF patients with Poly(I:C) resulted in a modest increase in RIG-I (Fig. 4.2B), MDA5 (Fig. 4.2D, *p<0.05) and PKR (Fig. 4.2F) mRNA expression compared with unstimulated Leu/Phe IPF fibroblasts. However, greater RIG-I, MDA5 and PKR upregulation was seen in Leu/Leu fibroblasts compared with Leu/Phe fibroblasts. LPS and CpG treatments did not induce secondary interferon receptor expression in Leu/Phe IPF fibroblasts (Fig. 4.2B, D, F).

These results show that TLR3 L412F attenuates secondary interferon responses in Leu/Phe IPF patients which was not seen in Leu/Leu IPF patients in response to TLR3 and TLR4 activation.
Figure 4.2. Effect of TLR3 L412F on TLR3-, TLR4- and TLR9-induced RIG-I, MDA5 and PKR mRNA expression in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. TLR3 L412F attenuates Poly(I:C), LPS and CpG-induced RIG-I, MDA5 and PKR mRNA expression in primary human lung fibroblasts from Leu/Phe IPF (B, D, F) patients compared with Leu/Leu patients (A, C, E) at 24 h post-treatment, as quantitated by qPCR analysis. *p<0.05, **p<0.01, ***p<0.001: treatment with respective TLR agonist compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments. [Poly(I:C), 10 µg/ml; LPS, 100 ng/ml; CpG, 5 µM].
4.4 TLR3 L412F effects RIG–I and MDA5 protein expression in primary human lung fibroblasts from Leu/Phe IPF patients

Intracellular receptors; RIG-I, MDA5 and PKR protein levels were also analysed by Western blot analysis. Fibroblasts were treated in vitro for 12 hours with Poly(I:C) (10 μg/ml) and LPS (100 ng/ml).

RIG-I

RIG-I is an intracellular viral sensor important for the detection of specific viruses and preferentially activated by 5′-ppp dsRNA (118). Activation of RIG-I leads to the production of pro-inflammatory cytokines and type I interferons which act back on the cell, to induce further upregulation of RIG-I and other viral sensor expression (117). In earlier studies we have shown by qPCR that RIG-I mRNA expression is upregulated in Leu/Leu IPF fibroblasts following both Poly(I:C) and LPS stimulation. Here, we have shown that Leu/Leu IPF fibroblasts have low basal RIG-I protein expression. Following 12 h stimulation with Poly(I:C), RIG-I expression is upregulated in Leu/Leu IPF fibroblasts (Fig. 4.3).

In contrast, previously we have shown that upregulation of RIG-I mRNA expression was attenuated in Leu/Phe IPF fibroblasts following Poly(I:C) and LPS treatment. Interestingly, by Western blot we have shown that minimal upregulation in RIG-I expression is seen in Leu/Phe IPF fibroblasts following Poly(I:C) stimulation. Leu/Phe IPF fibroblasts have varying basal levels of RIG-I expression. It was found in Fig. 4.3 that some Leu/Phe IPF fibroblasts have high basal levels of RIG-I while no expression of RIG-I was seen in others.

A similar pattern is seen following LPS treatment in Leu/Leu and Leu/Phe IPF fibroblasts (Fig. 4.4). Leu/Leu IPF fibroblasts have low basal RIG-I protein expression. Following 12 h stimulation with LPS, RIG-I expression is upregulated in Leu/Leu IPF fibroblasts (Fig. 4.4). Basal levels of RIG-I expression are low in Leu/Phe IPF fibroblasts and minimal upregulation is seen following LPS stimulation in two heterozygote patient fibroblast lines. One Leu/Phe IPF patient showed upregulation of RIG-I expression.
**Figure 4.3.** RIG-I protein expression levels in TLR3 L412F wild-type and heterozygote IPF fibroblasts following Poly(I:C) stimulation. TLR3 L412F wild-type cells express low levels of the viral sensor RIG-I basally. Stimulation with Poly(I:C) for 12 h induces expression of RIG-I. TLR3 L412F heterozygote IPF fibroblasts vary in expression of RIG-I. HET#1-2 have low basal levels of RIG-I while HET#3-4 express high levels of RIG-I. All heterozygote cells show minimal upregulation of protein expression following stimulation with Poly(I:C). Protein expression levels were analysed by Western blot following 12 hr treatment with Poly(I:C) (10 μg/ml). ImageJ software was used to obtain densitometry data. WT: TLR3 L412F wild-type IPF patients.
Figure 4.4. **RIG-I protein expression levels in TLR3 L412F wild-type and heterozygote IPF fibroblasts following LPS stimulation.** TLR3 L412F wild-type IPF fibroblasts express low levels of RIG-I basally. Stimulation of TLR3 L412F wild-type IPF fibroblasts with LPS for 12 h induces expression of RIG-I. TLR3 L412F heterozygote fibroblast line #1 shows low basal levels of RIG-I which become upregulated following LPS stimulation. TLR3 L412F heterozygote fibroblast lines HET#2 and HET#3 show minimal upregulation of protein expression following stimulation with LPS. Protein expression levels were analysed by Western blot following 12 h treatment with LPS (100 ng/ml). ImageJ software was used to obtain densitometry data. WT: TLR3 L412F wild-type IPF patients.
MDA5

MDA5 is another intracellular anti-viral receptor which activates the same downstream signalling cascade as TLR3 and RIG-I in response to infection (122). In earlier studies, we have found that, like RIG-I, MDA5 mRNA expression is induced in Leu/Leu IPF fibroblasts following Poly(I:C) and LPS stimulation for 24 h. This was not seen in Leu/Phe IPF fibroblasts by qPCR, MDA5 expression was attenuated following Poly(I:C) and LPS treatment (Fig. 4.2). By Western blot, we have found that Leu/Leu IPF fibroblasts have low basal MDA5 expression. Following 12 h stimulation with Poly(I:C), MDA5 expression is upregulated in Leu/Leu IPF fibroblasts (Fig. 4.5). Similar to the results found in RIG-I, by Western blot we have shown that MDA5 expression in Leu/Phe IPF fibroblasts has minimal upregulation following Poly(I:C) stimulation. Leu/Phe IPF fibroblasts have varying basal levels of MDA5 expression. It was found in Fig. 4.5 that some Leu/Phe IPF fibroblasts have high basal levels of MDA5 while no expression was seen in others.

Following LPS-induced TLR4 activation, MDA5 expression is upregulated in Leu/Leu IPF fibroblasts (Fig. 4.6). Basal levels of MDA5 expression are low in Leu/Phe IPF fibroblasts and no upregulation is seen following LPS stimulation in two Leu/Phe patient fibroblast lines. One Leu/Phe IPF patient showed upregulation of MDA5 expression.

These results are important as it shows an inability of Leu/Phe IPF fibroblasts to mount appropriate anti-viral response following TLR3 and TLR4 activation. In earlier studies we have already shown blunted NF-κB and IRF3 activation in primary human lung fibroblasts following TLR3 and TLR4 activation. These results confirm that the downstream secondary interferon response is affected by the presence of the SNP in IPF fibroblasts at both the mRNA and protein level.
Figure 4.5. MDA5 protein expression levels in TLR3 L412F wild-type and heterozygote IPF fibroblasts following Poly(I:C) stimulation. TLR3 L412F wild-type cells express low levels of the viral sensor MDA5 basally. Stimulation with Poly(I:C) for 12 h induces expression of MDA5. TLR3 L412F heterozygote IPF fibroblasts vary in expression of MDA5. HET#1-2 have low basal levels of MDA5 while HET#3-4 express high levels of MDA5. All heterozygote cells show minimal upregulation of protein expression following stimulation with Poly(I:C). Protein expression levels were analysed by Western blot following 12hr treatment with Poly(I:C) (10 μg/ml). ImageJ software was used to obtain densitometry data. WT: TLR3 L412F wild-type IPF patients.
Figure 4.6. MDA5 protein expression levels in TLR3 L412F wild-type and heterozygote IPF fibroblasts following LPS stimulation. TLR3 L412F wild-type and heterozygote IPF fibroblasts express low levels of MDA5 basally. Stimulation of TLR3 L412F wild-type IPF fibroblasts with LPS for 12 h induces expression of MDA5. TLR3 L412F heterozygote fibroblast line #1 show upregulation of MDA5 protein expression following LPS stimulation. TLR3 L412F heterozygote fibroblast lines HET#2 and HET#3 show minimal upregulation of protein expression following stimulation with LPS. Protein expression levels were analysed by western blot following 12 hr treatment with LPS (100 ng/ml). ImageJ software was used to obtain densitometry data. WT: TLR3 L412F wild-type IPF patients.
4.5 TLR3 L412F dysregulates PKR protein expression in primary human lung fibroblasts from Leu/Phe IPF patients

PKR is another cytosolic anti-viral sensor whose expression is controlled by type I interferon expression. PKR initiates an anti-viral response through activation of the transcription factors NF-κB and IRF3 (273, 274). By qPCR we have shown that like RIG-I and MDA5, PKR expression is induced in Leu/Leu IPF fibroblasts following Poly(I:C) and LPS stimulation after 24 h. However, TLR3 L412F attenuated PKR mRNA expression in Leu/Phe cells (Fig 4.2). Here we show by western blot that Leu/Leu IPF fibroblasts upregulate expression of PKR after 12 h stimulation with Poly(I:C) (Fig. 4.7). Interestingly, we show here that Leu/Phe IPF fibroblasts express high basal levels of PKR and show minimal upregulation of protein expression following Poly(I:C) treatment.

Similar results are seen following LPS treatment (Fig. 4.8). Leu/Leu IPF fibroblasts upregulate expression of PKR after 12 h stimulation with LPS. Leu/Phe IPF fibroblasts express high basal levels of PKR and show minimal upregulation of protein expression following LPS treatment.

Previously we have shown that Leu/Phe cells produce high levels of RANTES basally. RANTES is a readout for the IRF3 arm of the TLR3 signalling pathway. It could be suggested that high basal RANTES is indicative of high type I interferon basal expression in Leu/Phe cells. As PKR expression is induced by type I interferons (274), it could be suggested that the high basal levels of PKR seen in these fibroblasts is due to constitutively high levels of IFNs in the cell preventing upregulation upon activation of TLRs.
Figure 4.7. PKR protein expression levels in TLR3 L412F wild-type and heterozygote IPF fibroblasts following Poly(I:C) stimulation. TLR3 L412F wild-type cells express low levels of the viral sensor PKR basally. Stimulation with Poly(I:C) for 12 h induces expression of PKR. TLR3 L412F heterozygote IPF fibroblasts vary in expression of PKR. HET#1 has low basal levels of PKR while HET#2-4 express high levels of PKR. All heterozygote cells show minimal upregulation of protein expression following stimulation with Poly(I:C). Protein expression levels were analysed by western blot following 12 h treatment with Poly(I:C) (10μg/ml). ImageJ software was used to obtain densitometry data.
Figure 4.8. PKR protein expression levels in TLR3 L412F wild-type and heterozygote IPF fibroblasts following LPS stimulation. TLR3 L412F wild-type cells express low levels of the viral sensor PKR basally. Stimulation of TLR3 L412F wild-type IPF fibroblasts with LPS for 12 h induces expression of PKR. TLR3 L412F heterozygote IPF fibroblasts (HET#1, 3, 5) express high basal levels of PKR basally. All heterozygote cells show minimal upregulation of protein expression following stimulation with LPS. Protein expression levels were analysed by western blot following 12 h treatment with LPS (100 ng/ml). ImageJ software was used to obtain densitometry data. WT: TLR3 L412F wild-type IPF patients.
4.6 Primary human lung fibroblasts from Leu/Phe IPF patients have reduced TLR3 mRNA expression following Poly(I:C), LPS and CpG treatment, respectively

In earlier studies we have found that TLR3 activity is attenuated in the presence of the SNP in IPF human lung fibroblasts. In order to investigate whether the TLR3 L412F affects activity because of reduced or defective TLR3 mRNA and protein expression in IPF fibroblasts, we analysed TLR3 mRNA and protein by qPCR and western blot, respectively. In a related ILD, pulmonary sarcoidosis, it was shown that following TLR3 activation by Poly(I:C) treatment, Phe/Phe fibroblasts also had blunted TLR3 activity and mRNA expression compared with wild-type fibroblasts (168). Here, we have shown that in IPF fibroblasts there is a difference in TLR3 mRNA expression in Leu/Phe heterozygous cells compared with Leu/Leu cells. We stimulated Leu/Leu and Leu/Phe IPF fibroblasts with Poly(I:C) (10 μg/ml), LPS (100 ng/ml) and CpG (1 μM) for 24 h. Leu/Leu fibroblasts significantly induced TLR3 mRNA expression following Poly(I:C) stimulation (**p<0.001, Fig. 4.9A) compared with untreated cells. LPS and CpG also induced TLR3 mRNA expression to a lesser extent in Leu/Leu IPF fibroblasts (Fig. 4.9A) compared with untreated cells.

TLR3 mRNA expression was induced in Leu/Leu IPF fibroblasts following Poly(I:C) treatment compared with media only (Fig. 4.9A), however upregulation was reduced in Leu/Phe IPF fibroblasts in the presence of the SNP (Fig. 4.9B). Following TLR4 and TLR9 activation in the Leu/Phe IPF fibroblasts, we did not see upregulation of TLR3 expression (Fig. 4.9B).

4.7 Primary human lung fibroblasts from Leu/Phe IPF patients have reduced TLR3 protein expression Poly(I:C), LPS and CpG treatment, respectively

We have shown that TLR3 L412F affects TLR3 mRNA expression. Following on from this, we investigated whether TLR3 expression was also affected at the protein level. It was found that Leu/Leu IPF fibroblasts had low basal TLR3 protein expression which was upregulated after 12 h stimulation with Poly(I:C) (Fig. 4.10).
Here, we show that 2/3 Leu/Phe IPF patient fibroblasts had low basal expression of TLR3 protein which was not upregulated after Poly(I:C) stimulation. One Leu/Phe patient had high basal levels of TLR3 protein which were upregulated following Poly(I:C) stimulation.

Similarly, Leu/Phe- IPF fibroblasts also had attenuated upregulation of TLR3 protein expression following LPS stimulation after 12 h. Again, 2/4 Leu/Phe patients had higher basal levels of TLR3 expression while the other 2 did not. Leu/Phe fibroblasts had reduced expression of TLR3 compared with Leu/Leu fibroblasts following LPS stimulation (Fig. 4.11).

Variation in protein expression between different IPF patient fibroblasts was seen in each genotype throughout all western blots. Interestingly, similar to the results found with the intracellular receptors; RIG-I, MDA5 and PKR, we observed variation in TLR3 expression levels between each Leu/Phe IPF fibroblast cell line. One heterozygous patient had higher basal levels of TLR3 than others, however, most TLR3 L412F heterozygous fibroblasts showed defective upregulation of receptor expression upon stimulation.

This result is important as it shows that the presence of the TLR3 L412F SNP greatly effects both mRNA and protein expression of the receptor in IPF fibroblasts. Lack of upregulation of TLR3 expression in Leu/Phe IPF fibroblasts following TLR3 and 4 activation most likely plays a role in the defective downstream signalling resulting in attenuated levels of pro-inflammatory cytokines and type I interferons. To continue this work we wanted to analyse the responses of TLRs 1-8 in the presence and absence of the SNP.
Figure 4.9. Effect of TLR3 L412F on Poly(I:C)-, LPS- and CpG-induced TLR3 mRNA expression in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. TLR3 L412F attenuates Poly(I:C), LPS and CpG-induced TLR3 mRNA expression in primary human lung fibroblasts from Leu/Phe IPF patients (B) compared with Leu/Leu patients (A) at 24 h post-treatment, as quantitated by qPCR analysis. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. ***p<0.001: treatment with respective TLR agonist compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments. [Poly(I:C), 10 µg/ml; LPS, 100 ng/ml; CpG, 5 µM].
Figure 4.10.  TLR3 protein expression levels in TLR3 L412F wild-type and heterozygote IPF fibroblasts following Poly(I:C) stimulation. TLR3 protein expression was upregulated in TLR3 L412F wild-type IPF fibroblasts following 12 h stimulation with Poly(I:C). TLR3 L412F heterozygote IPF fibroblasts #1 and #2 have low basal expression of TLR3 and no upregulation following stimulation. HET#3 has high basal levels compared with both wild-type and heterozygote #1-2 cells and increased expression of TLR3 following stimulation. TLR3 protein expression levels were analysed by western blot following 12 hr treatment with Poly(I:C) (10 μg/ml). ImageJ software was used to obtain densitometry data. WT: TLR3 L412F wild-type IPF patients.
**Figure 4.11.** TLR3 protein expression levels in *TLR3* L412F wild-type and heterozygotes IPF fibroblasts following LPS stimulation. TLR3 protein expression was upregulated in *TLR3* L412F wild-type IPF fibroblasts following 12 h stimulation with LPS. HET#1 had low basal expression of TLR3 which was upregulated following LPS stimulation. HET#2 had low basal expression of TLR3 and no upregulation following stimulation. HET#3 and #5 had higher basal levels of TLR3 expression but no upregulation. TLR3 protein expression levels were analysed by western blot following 12 h treatment with LPS (100 ng/ml). ImageJ software was used to obtain densitometry data. WT: *TLR3* L412F wild-type IPF patients.
4.8 Primary human lung fibroblasts from Leu/Phe IPF patients have attenuated NF-κB activation to specific anti-bacterial and anti-viral TLR1-8 agonists

In initial studies in IPF primary human lung fibroblasts we have demonstrated that Poly(I:C) and LPS-induced activation of NF-κB through TLRs 3 and 4 respectively is attenuated in Leu/Phe IPF fibroblasts. All TLRs (with the exception of TLR3) use the adaptor protein MyD88 to initiate a downstream signalling cascade resulting in the activation of many transcription factors including NF-κB and IRF3 (99). Due to the many common downstream signalling proteins which all TLRs share, we hypothesise that TLR3 may not be the only TLR signalling pathway affected by the presence of the SNP in Leu/Phe IPF fibroblasts. In support of this, we have already shown that there is defective upregulation of pro-inflammatory cytokines and RANTES through TLR4 as well as TLR3 (Fig. 4.1). To test whether other TLR signalling pathways are affected in primary human lung fibroblasts from Leu/Phe IPF patients, we stimulated wild-type and heterozygous fibroblasts with a panel of TLR synthetic ligands which activate TLR receptors 1-8. TLRs 1-8 sense many different bacterial and viral PAMPs on the cell surface and in the endosome (99). TLRs 2, 4, 5, 6, and 9 recognise specific bacterial PAMPs, while TLRs 3, 7, 8 and 9 recognise viral PAMPs. Gram-positive bacteria are recognised by TLR2 which dimerises with either TLR1 or 6. Gram-negative bacteria are recognised by TLR4 (275). TLR5 recognises bacterial flagellin (276). TLRs 7 and 8 recognise single stranded RNA (277-279). TLR9 recognises CpG motifs from both bacterial and viral pathogens (280, 281).

IL-8 protein production was significantly induced in Leu/Leu IPF fibroblasts following TLR2, TLR3 (**p<0.001), TLR4 (**p<0.001), TLR5 (**p<0.001) and TLR6 (**p<0.001) activation with their respective agonists (Fig. 4.12A). TLR7 and 8 activation did not induce IL-8 production in Leu/Leu IPF fibroblasts (Fig. 4.12A).

In Leu/Phe IPF fibroblasts a more modest induction of IL-8 protein expression was seen following TLR2, TLR3 (**p<0.01), TLR4 (**p<0.001), TLR5 (**p<0.01) and TLR6 (**p<0.001) activation with their respective agonists (Fig. 4.12B). Again we see the presence of TLR3 L412F attenuates IL-8 production
following activation of TLR 3 and 4 as well as now TLRs 2, 5 and 6 in Leu/Phe fibroblasts.

**4.9 Primary human lung fibroblasts from Leu/Phe IPF patients have attenuated IRF3 activation to specific anti-bacterial and anti-viral TLR1-8 agonists**

In this study, we previously demonstrated reduced RANTES mRNA expression and defective IRF3 signalling through TLR3 and TLR4 in Leu/Phe IPF patients (Fig. 4.1). Here, we demonstrated that in primary human lung fibroblasts from IPF patients, TLR3 and TLR4 were the only TLRs to induce RANTES protein in Leu/Leu and Leu/Phe fibroblasts. RANTES protein expression was significantly induced in Leu/Leu IPF fibroblasts following TLR3 (*p<0.05) and TLR4 (*p<0.05) activation (Fig. 4.13A). In Leu/Phe IFP fibroblasts TLR3 L412F attenuates RANTES protein expression following TLR3 and TLR4 activation. RANTES protein was not induced by any TLR agonist (Fig. 4.13B).

**4.10 Recombinant IFN-β treatment in primary human lung fibroblasts from Leu/Phe IPF patients does not reconstitute pro-inflammatory cytokine or type I interferon responses.**

Previously we have shown in Fig. 4.1C that IFN-β mRNA expression was attenuated in Leu/Phe fibroblasts compared with Leu/Leu fibroblasts. The aim of this study was to elucidate whether recombinant IFN-β treatment could restore IL-8 and RANTES protein production in primary human lung fibroblasts from Leu/Phe IPF patients. Leu/Phe fibroblasts were stimulated for 24 h with TLR1-8 agonists in the presence and absence recombinant IFN-β. IL-8 and RANTES protein levels were analysed by ELISA and it was found that neither IL8 (Fig. 4.12C) nor RANTES (Fig. 4.13C) showed significant upregulation of protein expression following TLR activation in the presence of recombinant IFN-β.
Figure 4.12. Effect of TLR3 L412F on TLR1-8-induced IL-8 protein production in Leu/Leu and Leu/Phe IPF primary human lung fibroblasts and the effect of IFN-β treatment on TLR1-8 responses in Leu/Phe IPF fibroblasts. TLR3 L412F attenuates Pam3CSK4, Poly(I:C), LPS, Flagellin and FSL-1-induced IL-8 production in primary human lung fibroblasts from Leu/Phe IPF patients (B) compared with Leu/Leu patients (A). Co-incubation of IFN-β with TLR1-8 agonists in TLR3 L412F heterozygous fibroblasts did not significantly increase IL-8 in primary human lung fibroblasts from Leu/Phe IPF patients (C). A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. **p<0.01, ***p<0.001: treatment with respective TLR agonist compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments. [Pam3CSK4 (TLR1/2), 1 µg/ml; HKLM (TLR2), 10⁶ cells/ml; Poly(I:C) (TLR3), 10 µg/ml; LPS (TLR4), 100 ng/ml; flagellin (FLAG; TLR5), 1 µg/ml; FSL-1 (TLR6/2), 1 µg/ml; imiquimod (IMIQ; TLR7), 1 µg/ml; ssRNA40 (TLR8), 1 µg/ml, IFN-β, 1000 I.U./ml].
Figure 4.13. The effect of TLR3 L412F on TLR1-8-induced RANTES protein production in Leu/Leu and Leu/Phe IPF primary human lung fibroblasts and the effect of IFN-β treatment on TLR1-8 responses in Leu/Phe IPF fibroblasts. TLR3 L412F attenuates Poly(I:C) and LPS-induced RANTES production in primary human lung fibroblasts from Leu/Phe IPF patients (B) compared with Leu/Leu patients (A) at 24 h post-treatment, as quantitated by ELISA. Co-incubation of IFN-β with TLR1-8 agonists in TLR3 L412F heterozygous fibroblasts did not significantly increase RANTES protein production (C) at 24 h post-treatment, as quantitated by ELISA. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05: treatment with respective TLR agonist compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments. [Pam3CSK4 (TLR1/2), 1 µg/ml; HKLM (TLR2), 10^6 cells/ml; Poly(I:C) (TLR3), 10 µg/ml; LPS (TLR4), 100 ng/ml; flagellin (FLAG; TLR5), 1 µg/ml; FSL-1 (TLR6/2), 1 µg/ml; imiquimod (IMIQ; TLR7), 1 µg/ml; ssRNA40 (TLR8), 1 µg/ml; IFN-β, 1000 I.U./ml].
4.11 Primary human lung fibroblasts from Leu/Phe IPF patients have reduced responses to non-TLR agonists

To elucidate whether the effect of TLR3 L412F was specific to the TLR family of receptors, we examined the effect of the SNP on the functionality of non-TLR receptors. The aim of this experiment was to analyse whether signalling through transcription factors NF-κB and IRF3 was also defective upon stimulation with non-TLR receptors. Specifically, we examined responses to non-TLR receptor agonists; Poly(dA:dT), Herring-Testes DNA (HT-DNA) and non-specific NF-κB activators; PMA and ionomycin using Poly(I:C) as a positive control. Poly(dA:dT) is a synthetic double stranded DNA agonist used to indirectly activate the intracellular viral sensor RIG-I leading to the induction of pro-inflammatory cytokines and type I interferons (282). Poly(dA:dT) is used by RNA polymerase III to generate a 5’ triphosphate RNA intermediate which can be recognised by RIG-I (282). Poly(dA:dT) has also been shown to activate other sensors such as the AIM2 inflammasome (283). HT-DNA is another double-stranded DNA agonist. It activates the cytosolic DNA sensing pathway cGAS-cGAMP-STING pathway via the generation of the second messenger cGAMP (284, 285). PMA and ionomycin are NF-κB activators (286, 287). Primary IPF human lung fibroblasts from Leu/Leu and Leu/Phe heterozygous patients were cultured in vitro. Cells were subsequently treated for 24 h with increasing doses of Poly(I:C) (0.1, 1, 10 μg/ml), Poly(dA:dT) (0.5, 0.75, 1 μg/ml), PMA (10, 100, 1000 ng/ml), HT-DNA (0.5, 0.75, 1 μg/ml) and ionomycin (0.1, 1, 10 μg/ml).

Here, we found that Leu/Leu IPF fibroblasts significantly induced expression of IL-8 protein following stimulation with Poly(I:C) (1 and 10 μg/ml; ***p<0.001, Fig. 4.14A), Poly(dA:dT) (all concentrations; ***p<0.001, Fig. 4.14C) and PMA (all concentrations; **p<0.01 and ***p<0.001, Fig. 4.14E) for 24 h compared with untreated fibroblasts. IL-8 protein expression did not increase in a dose dependent manner following both Poly(dA:dT) and PMA stimulation (Fig. 4.14C, E).

Leu/Phe IPF fibroblasts also significantly induced expression of IL-8 protein following Poly(I:C) (10 μg/ml; ***p<0.001, Fig. 4.14B), Poly(dA:dT) (all concentrations; ***p<0.001, Fig. 4.14D) and PMA (all concentrations; ***p<0.001,
Fig. 4.14F) treatment for 24 h compared with media only. However, we observed following 24 hours stimulation TLR3 L412F attenuates Poly(I:C), Poly(dA:dT) and PMA-induced IL-8 production in Leu/Phe IPF fibroblasts (Fig. 4.10 B, D, F).

IRF3 activation and RANTES protein production were also assessed by ELISA. We have found that Leu/Leu IPF fibroblasts significantly induced expression of RANTES protein following stimulation with Poly(I:C) (1 and 10 μg/ml; *p<0.05, ***p<0.001, Fig. 4.15A) and Poly(dA:dT) (all concentrations; ***p<0.001, Fig. 4.15C) compared with media only. RANTES protein expression did not increase in a dose dependent manner following Poly(dA:dT) (Fig. 4.15C). Leu/Phe IPF fibroblasts also significantly induced expression of RANTES protein following treatment with Poly(I:C) (10 μg/ml; ***p<0.001, Fig. 4.15B) and Poly(dA:dT) (all concentrations; ***p<0.001, Fig. 4.15D) compared with media only. TLR3 L412F attenuated Poly(dA:dT) upregulation of RANTES protein production in Leu/Phe IPF fibroblasts which is not seen in Leu/Leu IPF fibroblasts. As expected, PMA did not induce RANTES protein production in either genotype as it is a NF-κB activator (Fig. 4.15E, F).

Ionomycin treatment induced similar levels of IL-8 protein production in both Leu/Leu and Leu/Phe IPF fibroblasts (Fig. 4.16A, B, *p<0.05, ***p<0.001). HT-DNA transfection induced IL-8 protein production in both Leu/Leu, however levels of IL-8 were not significant (Fig. 4.16C). HT-DNA transfection also upregulated IL-8 production Leu/Phe IPF fibroblasts, however upregulation was greater in Leu/Leu IPF fibroblasts (Fig. 4.16D).

Ionomycin did not induce RANTES protein production in either genotype as it is a NF-κB activator (Fig. 4.16A, B). However, HT-DNA transfection significantly induced IL-8 protein expression in Leu/Leu IPF fibroblasts (Fig. 4.17C) but this was not seen in Leu/Phe IPF fibroblasts (Fig. 4.17D).

Here, we demonstrate that defective intracellular signalling seen in the Leu/Phe IPF fibroblasts is not TLR specific as non-TLR family intracellular receptors also have attenuated responses upon stimulation.
Figure 4.14. Effect of TLR3 L412F on Poly(I:C), Poly(dA:dT) or PMA-induced IL-8 protein production, respectively, in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. TLR3 L412F attenuates Poly(I:C), Poly(dA:dT) and PMA-induced IL-8 production in primary human lung fibroblasts from Leu/Phe IPF patients (B, D, F) compared with Leu/Leu patients (A, C, E) at 24 h post-treatment, as quantitated by ELISA. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: treatment with Poly(I:C), Poly(dA:dT) and PMA compared with medium-only treatment. Leu/Leu results shown represent the mean +/- S.E.M of 3 cell lines with n=3 to 6 replicates. Leu/Phe results shown represent the mean +/- S.E.M of 5 cell lines with n=3 to 6 replicates. [Poly(I:C) (TLR3) gradient 0.1, 1 and 10 μg/ml; Poly(dA:dT) (RIG-I) gradient 0.5, 0.75 and 1 μg/ml; PMA (NF-κB activator) gradient 10, 100, 1000 ng/ml.]
Figure 4.15. Effect of TLR3 L412F on Poly(I:C), Poly(dA:dT) or PMA-induced RANTES protein production, respectively, in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. Poly(I:C) expression was similar in Leu/Leu (A) and Leu/Phe (B) IPF fibroblasts. TLR3 L412F attenuates Poly(dA:dT)-induced RANTES production in primary human lung fibroblasts from Leu/Phe IPF patients (D) compared with Leu/Leu patients (C) at 24 h post-treatment, as quantitated by ELISA. PMA did not induce RANTES protein production in human lung fibroblasts in Leu/Leu (E) or Leu/Phe (F) IPF patients. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, ***p<0.001: treatment with Poly(I:C), Poly(dA:dT) and PMA compared with medium-only treatment. Leu/Leu results shown represent the mean +/- S.E.M of 3 cell lines with n=3 to 6 replicates. Leu/Phe results shown represent the mean +/- S.E.M of 5 cell lines with n=3 to 6 replicates. [Poly(I:C) (TLR3) gradient 0.1, 1 and 10 µg/ml; Poly(dA:dT) (RIG-I) gradient 0.5, 0.75 and 1 µg/ml; PMA (NF-κB activator) gradient 10, 100, 1000 ng/ml].
Figure 4.16. Effect of TLR3 L412F on Ionomycin and HT-DNA-induced IL-8 protein production, respectively, in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. TLR3 L412F attenuates HT-DNA-induced IL-8 production in primary human lung fibroblasts from Leu/Phe IPF patients (D) compared with Leu/Leu patients (C) at 24 h post-treatment, as quantitated by ELISA. Ionomycin induced similar levels of IL-8 protein production in human lung fibroblasts in Leu/Leu (A) and Leu/Phe (B) IPF patients. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: treatment with Ionomycin and HT-DNA compared with medium-only treatment. Results shown are the mean +/- S.E.M. of two separate experiments with five replicates. [Ionomycin gradient 0.1, 1 and 10 μg/ml; HT- DNA (Herring Testes DNA) gradient 0.5, 0.75 and 1 μg/ml.]
**Figure 4.17.** Effect of *TLR3* L412F on Ionomycin and HT-DNA-induced RANTES protein production, respectively, in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. *TLR3* L412F attenuates HT-DNA-induced RANTES production in primary human lung fibroblasts from Leu/Phe IPF patients (D) compared with Leu/Leu patients (C) at 24 h post-treatment, as quantitated by ELISA. Ionomycin did not induce RANTES protein production in human lung fibroblasts in Leu/Leu (A) or Leu/Phe (B) IPF patients. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p*<0.05, **p**<0.01, ***p**<0.001: treatment with Ionomycin and HT-DNA compared with medium-only treatment. Results shown are the mean +/- S.E.M of two separate experiments with five replicates. [Ionomycin gradient 0.1, 1 and 10 μg/ml; HT-DNA (Herring Testes DNA) gradient 0.5, 0.75 and 1 μg/ml.]
4.12 Discussion

In this chapter, we examined the role of TLR3 L412F in primary human lung fibroblasts from IPF patients in the context of: (i) TLR3-independent responses (i.e. TLR1-9), (ii) secondary-IFN responses and (iii) non-TLR-dependent responses. Previously, the Donnelly Research Group demonstrated that TLR3 L412F affected TLR3 signalling in Leu/Phe IPF lung fibroblasts which had attenuated IL-8 and RANTES protein expression following Poly(I:C)-induced TLR3 activation compared with Leu/Leu IPF fibroblasts (154). Due to the fact that the TLR superfamily members share common signalling molecules, we speculated that TLR3 L412F may affect TLR signalling through a number of different TLRs. Furthermore, we also investigated the impact of TLR3 L412F on non-TLR-dependent activation of NF-κB and IRF3 in pulmonary fibroblasts from IPF patients.

In this chapter, we have shown that TLR3 L412F affects not only TLR3 signalling but also signalling through many of the TLRs which sense bacterial PAMPs. Specifically, we saw that TLR3 L412F attenuates pro-inflammatory cytokine production through TLRs 2, 4, 5, 6 and 9 in Leu/Phe IPF fibroblasts compared with wild-type IPF patients. Each of these TLRs recognises different bacterial moieties and leads to the induction of a specific immune response against infection (99). In future studies, we would like to further explore this finding in order to determine whether this attenuated response is detrimental or beneficial during bacterial infection in IPF patients. We would also like to examine responses to LPS and live bacterial infection in our novel TLR3 L413F knock-in mouse model, which was generated using CRISPR/Cas-9 gene editing (see Chapter 6). As previously mentioned, disease pathogenesis in IPF has been associated more recently with bacterial infection (271, 288). Other researchers have demonstrated that a higher bacterial load in IPF patients at diagnosis is associated with an increased risk of mortality (288). Clinical trials for the use of the prophylactic broad spectrum antibiotic, co-trimoxazole, in combination with regular treatment in IPF are ongoing (44, 45). It could be suggested that a tailored IPF treatment including the use of antibiotics prophylactically in Leu/Phe IPF patients may be beneficial.
Given the importance of TLR3 in anti-viral immunity in the lungs in humans, here we hypothesised that TLR3 L412F may have a detrimental impact on anti-viral responses in Leu/Phe IPF fibroblasts. In support of this theory, we have already shown that Leu/Phe IPF fibroblasts have attenuated RANTES protein production following TLR3 activation. As previously described, viruses such as herpesviruses, influenza A virus, hepatitis C virus and torque tenue virus have been associated with disease progression in IPF (71, 72, 79, 82, 85) (68). Furthermore, we have found that TLR3 L412F affects the secondary interferon anti-viral response of RLRs. We have demonstrated additionally in this chapter that RANTES protein production is attenuated following TLR3- and TLR4-activation in the presence of variant TLR3 L412F in IPF fibroblasts. This is indicative of reduced IRF3 activation and type I interferon expression in Leu/Phe IPF fibroblasts. Type I interferons are crucial for anti-viral immune responses. Type I IFN production relies on a positive-feedback loop through the type I IFN receptor (120). Their expression also induces the expression of many ISGs including cytosolic intracellular receptors such as RIG-I, MDA5 and PKR (120). RLRs, RIG-I and MDA5, are vital for detection of RNA viruses intracellularly and induce the production of pro-inflammatory cytokines and type I interferons (122).

Looking beyond the TLR–dependent anti-viral pathways, here, we examined whether TLR3 L412F could affect the secondary interferon response and intracellular RLR signalling. Here, we analysed RIG-I expression in IPF fibroblasts following Poly(I:C) stimulation. As previously mentioned, viruses such as influenza A virus have been associated with IPF (85, 269). RIG-I is essential for the control of viruses such as influenza A virus by detection of the 5’-triphosphate group in their RNA structure (289). We have demonstrated in our studies that Leu/Leu IPF fibroblasts induce RIG-I expression following Poly(I:C) activation. However, Leu/Phe IPF fibroblasts are less able to upregulate RIG-I expression with some Leu/Phe IPF fibroblasts expressing high levels of RIG-I basally. This suggested dyregulation in the secondary interferon response and a potential risk of infection in Leu/Phe IPF fibroblasts. In order to confirm that the presence of the SNP dysregulates the secondary interferon response in vivo, we will perform in vivo live H1N1 infection model in our novel TLR3 L413F knock-in mice and examine RIG-I expression. In addition, in the future, we will investigate levels of RIG-I expression in
bronchoalveolar lavage (BAL) cells pellets from IPF patients in order to determine whether TLR3 L412F enhances susceptibility to viral infection.

In this chapter, we also analysed Poly(dA:dT)-induced RIG-I signalling in Leu/Leu and Leu/Phe IPF fibroblasts. Poly(dA:dT) indirectly activates the intracellular viral sensor RIG-I leading to the induction of pro-inflammatory cytokines and type I interferons (282). We found that not only was RIG-I expression affected by the TLR3 L412F SNP but Leu/Phe IPF fibroblasts also had attenuated expression of pro-inflammatory cytokines and RANTES following RIG-I activation. Taken together, these results show an inability of Leu/Phe IPF fibroblasts to mount an appropriate response to viral infection (mimicked by Poly(I:C) and Poly(dA:dT) through the RIG-I receptor.

Similarly, we found that two other anti-viral intracellular receptors, MDA5 and PKR, had reduced mRNA expression following Poly(I:C) stimulation in Leu/Phe fibroblasts compared with Leu/Leu IPF fibroblasts. At the protein level we have shown that only the Leu/Leu fibroblasts strongly upregulate MDA5 and PKR expression following TLR3 activation. Taken together, these results suggests that only wild-type IPF fibroblasts induce a robust anti-viral response following TLR3 activation and not Leu/Phe IPF fibroblasts, highlighting a potential detrimental role for viral infection in disease pathogenesis in Leu/Phe IPF patients.

In addition, in the absence of stimulation with Poly(I:C), dysregulated basal expression of intracellular receptors can be seen in Leu/Phe IPF fibroblasts. We have demonstrated that basally, RIG-I, MDA5 and PKR receptor expression is affected by the SNP. RIG-I, MDA5 and PKR basal protein levels vary between Leu/Phe IPF patient fibroblasts with a number of patients having high basal expression of receptors without prior stimulation. High basal levels of receptor expression may play a role in the inability to upregulate expression following stimulation, therefore indicating a defective anti-viral response in Leu/Phe IPF patient fibroblasts. Here we show that only Leu/Leu IPF fibroblasts induce a robust secondary interferon response following Poly(I:C)-induced TLR3 activation. This highlights a potential detrimental role for TLR3 L412F during viral infection in IPF fibroblasts. In order to further examine the effect of viral infection in Leu/Leu and
Leu/Phe IPF fibroblasts, in future studies we will infect *in vitro* IPF fibroblasts with live influenza A virus, H1N1.

We have shown that Leu/Phe IPF fibroblasts have reduced IRF3 activation and subsequently reduced RANTES and IFN-β production. Because of this, we investigated whether treatment of Leu/Phe IPF fibroblasts with recombinant IFN-β would restore the attenuated TLR responses *in vitro* and could therefore potentially be used as treatment in these IPF patients. Recombinant IFN-β treatment is currently used to treat multiple sclerosis (290). It has also been trialled in the treatment of the lung disease, acute respiratory distress syndrome (ARDS) (291). It has been shown in mice that recombinant IFN-α in combination with the TLR4 agonist LPS in the lung enhances production of pro-inflammatory cytokines and interferons compared with LPS alone (292). Similarly, IFN-β and LPS treatment has also been shown to synergize for greater production of cytokines in mice (293). In our studies, we found that treatment with recombinant IFN-β did not restore TLR1-8 responses in Leu/Phe IPF fibroblasts. Unfortunately, IL-8 and RANTES protein expression was not upregulated following TRL1-8 stimulation in combination with recombinant IFN-β. Previously, in our laboratory we have shown that TLR3-induced RANTES protein production can be restored in 412F-homozygous pulmonary sarcoidosis fibroblasts following treatment with recombinant IFN-β (168). This finding may due to the fact that pulmonary sarcoidosis is mainly seen in younger people (< 50 years) and therefore, their fibroblasts are more responsive. Furthermore, the pulmonary sarcoidosis fibroblasts used in our previous study were grown from diagnostic biopsies and not from explanted lung tissue, which we have utilised in this chapter from IPF patients. To further investigate whether recombinant IFN-β can restore RANTES expression in IPF patients, additional fibroblasts lines will be employed. Additionally, Goritzka *et al.* speculated from their results that the role of type I interferons may not be to upregulate the production of pro-inflammatory cytokines themselves but instead to amplify the production pro-inflammatory cytokines following activation of NF-κB (292). This may also apply to our results, type I interferons may not have been able to restore pro-inflammatory cytokine and type I interferon responses as activation of NF-κB and IRF3 is defective in Leu/Phe IPF fibroblasts.
In this study, we hypothesised that the inhibitory effect of TLR3 L412F on TLR3 activity could in part be due to reduced TLR3 expression in IPF fibroblasts. In our experiments, we found that TLR3 expression following Poly(I:C) treatment was greatly upregulated in Leu/Leu IPF fibroblasts while upregulation in Leu/Phe IPF fibroblasts was to a much lesser extent. We have shown that not only does TLR3 L412F affect TLR3 activity but it also affects receptor expression both at the mRNA and protein level. Western blot analysis of TLR3 expression revealed that there is dysregulated expression in Leu/Phe IPF fibroblasts, with some variants expressing very low levels of TLR3 basally and upon stimulation with Poly(I:C) while another has high basal expression. This is an important finding as it suggests one possible reason for the reduced TLR3 activity seen in Leu/Phe IPF fibroblasts. Results published by other researchers have not been consistent on this topic. Some groups have shown that the SNP does affect expression (157) while other groups have shown in different cell types that the presence of the SNP does not affect TLR3 receptor expression (176, 294). This may suggest that the effects of TLR3 L412F may be cell–type specific.

In this chapter, our work has shown for the first time that TLR3 L412F not only affects TLR3 signalling but also signalling through additional TLR family members, including TLR2, 4, 5, 6 and 9. TLRs share a number of common downstream signalling proteins and transcription factors (e.g. NF-κB and IRF3) with other non- TLR receptors, including RLRs (116). Due to this, we investigated whether TLR3 L412F was capable of impacting upon non-TLR-dependent activation of NF-κB and IRF3 in IPF fibroblasts. Here, we activated NF-κB and IRF3 in a TLR-independent manner using Poly(dA:dT), an activator of the RIG-I and cGas/STING pathway (282, 295), HT-DNA, a STING activator (284, 285), and two NF-κB activators, PMA and ionomycin, respectively. In our studies, we demonstrated that Leu/Phe IPF fibroblasts had attenuated NF-κB activation following Poly(dA:dT), PMA and HT-DNA stimulation compared with Leu/Leu wild-type IPF fibroblasts. Similarly, Leu/Phe IPF fibroblasts had attenuated IRF3 activation following Poly(dA:dT) and HT-DNA stimulation compared with Leu/Leu IPF fibroblasts. This is an important result as it demonstrates for the first times that TLR3 L412F can impact upon non-TLR-dependent signalling in pulmonary fibroblasts from IPF patients. This result provides evidence of a novel manner in
which **TLR3** L412F may hinder the ability of IPF patients to respond to non-**TLR** PAMPs during bacterial and/or viral infection. This could lead to a subsequent increase in disease progression in IPF patients, which to date has no specific treatment.

Our work has shown defective **TLR**-dependent and **TLR**-independent responses to bacterial and viral PAMPs, respectively, in Leu/Phe IPF fibroblasts. This finding could be detrimental during the immune response to infection. However, in certain bacterial and viral infections, a lack of **TLR** signalling has been shown to be protective. One example of this can be seen in protection against sepsis by a functional SNP in the Mal protein (also known as TIRAP) (296). Mal is an adaptor protein which enables signalling through **TLR**2 and 4 (104, 297). The SNP in Mal (**TIRAP** S180L) has been shown to impair **TLR**2 signalling and attenuate pro-inflammatory cytokine production. It was shown that heterozygosity for the SNP had a protective effect against malaria, bacteraemia, invasive pneumococcal disease and tuberculosis (296). Reduced cytokine production following **TLR** activation was suggested to protect patients against the excessive cytokine response seen in patients following infection. This could apply to our work in IPF fibroblasts. Here, we have shown that Leu/Phe IPF fibroblasts have attenuated upregulation of cytokine production and type I interferons following **TLR** activation. We hypothesise that this attenuated response to infection could be protective during specific infections. As previously mentioned, in order to investigate this we will virally infect our **TLR3** L413F mice with IAV H1N1 *in vivo* in order to determine whether the presence of the SNP is beneficial or pathogenic during H1N1 infection.

Further support for this hypothesis comes from Johnson *et al.* who have shown that defective **TLR** signalling is protective during *Mycobacterium leprae* (*M. leprae*) infection (298). A polymorphism in **TLR1** (**TLR1** I602S) has been shown to affect receptor trafficking and subsequent signalling. **TLR1** I602S has been associated with decreased incidence *M. leprae* infection indicating that functional **TLR1** responses may be detrimental during leprosy (298). A second group found another functional mutation in **TLR1** (**TLR1** T1805G) which also affected **TLR1** signalling and cytokine production and conferred protection against *M. leprae* infection (299). Interestingly, **TLR1** I602S has been shown to be retained at a high frequency in Caucasians almost 75% suggesting that evolutionarily it has been kept
at a high frequency as it is beneficial (298). Similarly, TLR3 L412F has been shown to be retained at high levels in the European population. Heterozygosity for this SNP has been retained 45.7% in the European population compared with 37.8% in the South Asian population and 5% in the African population (165). We hypothesize that the reason for this could be that is beneficial in the control of certain infections. Additional studies are required in order to investigate this. We currently have access to a novel TLR3 L413F knock-in mouse, which provides a suitable model in which to investigate the effects of the TLR3 SNP during bacterial or viral infection in the future.

In support of this theory, a protective role for the TLR3 L412F SNP has been shown in certain viral infections. Sironi et al. found that the presence of the SNP was protective in HIV-1 infection, with decreased incidence of the SNP associated with HIV-1 infection (176). In addition, in HSV-2 infection, the minor allele frequency of the SNP was found at higher levels in HSV-2 seronegative individuals compared with HSV-2 seronegative individuals (177).

In conclusion, we have found in this study that TLR3 L412F heterozygous IPF fibroblasts have defective responses to TLR3, 4, 5, 6, and 9-induced anti-bacterial and/or anti-viral PAMPs. Furthermore, TLR3 L412F heterozygous IPF fibroblasts have defective secondary interferon response following TLR3 activation compared with wild-type IPF fibroblasts. We have also demonstrated that non-TLR pathways, which activate NF-κB and IRF3, are also affected in TLR3 L412F heterozygous IPF fibroblasts. These results provide evidence for novel mechanisms by which the TLR3 L412F SNP may have an impact on viral and/or bacterial infection, disease progression and increased risk of mortality in IPF patients.
Figure 4.18. **TLR3 signalling pathway in Leu/Leu IPF patients.** TLR3 is activated by dsRNA and activates a downstream signalling cascade through the adaptor protein TRIF. TRIF associates with TRAF6 and RIP-1 to initiate the downstream signalling cascade resulting in NF-κB activation and pro-inflammatory cytokine expression. TRIF also interacts with TRAF3 to activate the transcription factor IRF3 and type I interferon expression. IFN-β acts back on the cell through the IFNAR1 to induce expression of many ISGs including cytosolic receptors; RIG-I, MDA5 and PKR. In Leu/Leu IPF fibroblasts, there is low basal expression of RIG-I, MDA5 and PKR which is upregulated following Poly(I:C)-induced TLR3 activation. Adapted from (99, 151).
Figure 4.19. TLR3 signalling pathway in Leu/Phe IPF patients. TLR3 activation by dsRNA is affected by the presence of TLR3 L412F. TLR3 L412F is thought to affect ligand binding due to the bulky nature of the phenylalanine substitution. This results in defective signal transduction and upregulation production of pro-inflammatory cytokines and type I interferons in Leu/Phe IPF fibroblasts following TLR3 activation. Reduced expression of type I interferons results in attenuation of the secondary interferon response and reduced expression of RIG-I, MDA5 and PKR following TLR3 activation. In Leu/Phe IPF fibroblasts, there is high basal expression of RIG-I, MDA5 and PKR which can not be upregulated following Poly(I:C)-induced TLR3 activation. Adapted from (99, 151).
Chapter 5

Characterisation of the effect of TLR3 L412F on autophagy responses in wild-type and variant primary human lung fibroblasts
Chapter 5 Characterisation of the effect of TLR3 L412F on autophagy responses in wild-type and variant primary human lung fibroblasts

5.1 Introduction

TLR3 is important in immune responses to infection and acts by recognising double-stranded RNA from viruses, bacteria, helminths and mRNA released from necrotic cells (142-145). It induces the production of pro-inflammatory cytokines and type I interferons in response to infection. Activation of TLR3 also upregulates expression of secondary viral sensors such as RIG-I, MDA5 and PKR (257). Previously, our group has shown that TLR3 L412F affects TLR responses in Leu/Phe IPF primary lung fibroblasts. We demonstrated that TLR3 signalling was attenuated in Leu/Phe IPF fibroblasts compared with wild-type Leu/Leu fibroblasts as there was reduced expression of pro-inflammatory cytokines and type I interferons following TLR3 activation (154). In addition to this, in Chapter 4 we have shown that Leu/Phe IPF fibroblasts have attenuated anti-viral responses due reduced expression of intra-cellular anti-viral receptors, RIG-I, MDA5 and PKR.

In addition to the role of TLR3 L412F in IPF, our research group has also previously established a role for this TLR3 SNP in another related interstitial lung disease (ILD), pulmonary sarcoidosis. Pulmonary sarcoidosis is a multisystem, chronic granulomatous disease of unknown cause (169). It is an inflammatory disease which is predominantly dominant within the lung but can present in many other organs. Previously, we demonstrated that 412F-homozygous, Phe/Phe primary human lung fibroblasts from sarcoidosis patients showed reduced NF-κB and IRF3 activation following Poly(I:C) stimulation compared with wild-type patients (168). In summary, we have demonstrated that TLR3 L412F attenuates TLR3-induced pro-inflammatory and type I interferon responses in primary lung fibroblasts from two interstitial lung diseases, namely IPF and pulmonary diseases. An interesting and outstanding question addresses how TLR3 L412F impacts upon TLR3-responses in pulmonary fibroblasts during live viral infection in ILD patients.

In this study, the aim was to investigate the role of the TLR3 L412F during live viral infection in both pulmonary sarcoidosis and IPF primary lung fibroblasts. Following on from the work done using the TLR3 synthetic agonist, Poly(I:C) (168),
our group examined the effect of live influenza A virus (IAV) [Strain A/Texas/36/1991(H1N1)] infection on pro-inflammatory cytokine and type I interferon expression in vitro (Unpublished, Armstrong et al.). They showed that homozygous Phe/Phe primary human lung fibroblasts from pulmonary sarcoidosis patients had attenuated expression of IL-8, IFN-β and RANTES mRNA expression following live viral infection with IAV H1N1 compared with wild-type lung fibroblasts from pulmonary sarcoidosis patients. Interestingly, even though there was defective signalling through TLR3 following live H1N1 infection, primary human lung fibroblasts from homozygote Phe/Phe pulmonary sarcoidosis patients had lower levels of H1N1 nucleoprotein compared with wild-type sarcoidosis patients. The lower levels of H1N1 nucleoprotein in Phe/Phe fibroblasts, compared with wild-type Leu/Leu fibroblasts, could not be explained. We hypothesized that the cellular mechanism, autophagy, could play a role in the increased viral clearance.

Autophagy is a complex, multi-stage cellular mechanism which plays a role in a number of cellular functions including the recycling of misfolded proteins and damaged organelles, antigen presentation, control of inflammation and the clearance of bacteria and viruses (199). Autophagy is essential in times of cellular stress to promote survival. Autophagy can be induced by many different stimuli, including those also activated during viral infection. TLR activation, type I interferon expression and nutrient deprivation have all been shown to induce autophagy in cells (190, 191, 203, 210). Other researchers have previously demonstrated that there are decreased levels of autophagy in IPF lungs, which may be a factor in the promotion of pro-fibrotic responses seen in IPF (27, 215, 216). Defective autophagy seen in IPF patients has been attributed to a number of factors including aging and mTOR activation (27, 215).

In this study, we have examined the induction of autophagy in primary human lung fibroblasts from pulmonary sarcoidosis patients and IPF patients using a number of standard stimuli. Specifically, we analysed autophagy induction following: (i) live IAV H1N1 infection of pulmonary sarcoidosis fibroblasts, (ii) under starvation conditions in pulmonary IPF fibroblasts and (iii) through activation of TLRs in pulmonary IPF fibroblasts, respectively. By qPCR we have analysed the expression of a range of early to late stage autophagy genes, Beclin 1, Atg5 and
LC3B. We further analysed autophagy in the cell by measuring LC3-II levels by western blot in IPF lung fibroblasts with and without stimulation. LC3-II protein expression is indicative of increased autophagy in the cell and the ratio of LC3-I: LC3-II protein expression, in the presence of degradative inhibitors such as bafilomycin A1, is routinely used in experiments to analyse autophagic flux (300, 301). Furthermore, we examined the role of the mTOR protein in TLR3 signalling and autophagy in IPF fibroblasts by western blot analysis and downstream cytokine production.

In this chapter, we provide evidence of increased levels of LC3-II in Leu/Phe IPF fibroblasts compared with Leu/Leu fibroblasts following bafilomycin A1 treatment. We have also shown that IPF fibroblasts do not induce autophagy following treatment with a range of stimuli including Poly(I:C), LPS and rapamycin, highlighting a potential defect in the autophagy pathway. Finally, we found that mTOR activation is important for TLR3-induced cytokine production in IPF fibroblasts. In addition we have found that mTOR is constitutively activated in IPF fibroblasts and may play a role in the defective autophagy signalling.
5.2 Primary human lung fibroblasts from Phe/Phe pulmonary sarcoidosis patients exhibit reduced NF-κB and IRF3 activation and lower viral H1N1 nucleoprotein following infection

Wild-type (Leu/Leu) and homozygous (Phe/Phe) pulmonary sarcoidosis lung fibroblasts were previously infected with influenza A virus (Strain A/Texas/36/1991(H1N1)) (Armstrong et al, unpublished). We have shown that homozygote Phe/Phe sarcoidosis fibroblasts have reduced RANTES (Fig. 5.1C) and IFN-β (Fig. 5.1B) mRNA expression following infection for 24 h compared with Leu/Leu sarcoidosis fibroblasts. Similarly, IL-8 mRNA expression is attenuated in Phe/Phe sarcoid fibroblasts compared with wild-type (Fig. 5.1A). Attenuated expression of RANTES, IFN-β and IL-8 mRNA expression is indicative of a blunted response through transcription factors IRF3 and NF-κB respectively. Interestingly, it was found that Phe/Phe sarcoid fibroblasts had lower H1N1 viral load following 24hr infection (Fig. 5.1D).

Here, using these samples, we went on to investigate H1N1 viral load in a separate independent experiment. Again we found that Phe/Phe sarcoid fibroblasts had lower H1N1 viral load following 24hr infection. This second independent experiment confirmed that Phe/Phe fibroblasts from pulmonary sarcoidosis patients had a lower viral load than wild-type fibroblasts following 24hr infection (Fig. 5.2).

5.3 Primary human lung fibroblasts from Phe/Phe pulmonary sarcoidosis patients exhibit increased levels of autophagy markers following H1N1 infection

Leu/Leu and Phe/Phe pulmonary sarcoidosis lung fibroblasts were previously infected with H1N1 (Armstrong et al, unpublished). In our work, we went on to investigate the mRNA expression of autophagy genes in these cells by qPCR. As previously mentioned, autophagy is a complex pathway with many different stages. We analysed mRNA expression of three genes; Beclin-1 (an early stage autophagy protein), Atg5 (a middle stage protein), and LC3B (a late stage autophagy protein). At 24 h post H1N1 infection, there was increased mRNA expression of the early-, middle- and late stage-autophagy genes; Beclin 1, Atg5 and LC3B, respectively, in
Phe/Phe lung fibroblasts from sarcoidosis patients (Fig. 5.3B, D, F) compared with medium only. In Leu/Leu cells there was a modest induction in Beclin 1 and LC3B expression (Fig. 5.3A, E) compared with medium only. Atg5 levels were unchanged (Fig 5.3C). Taken together these results suggest that there was greater induction of autophagy gene expression in Phe/Phe fibroblasts from sarcoidosis patients following H1N1 infection which may indicate increased autophagy levels in Phe/Phe fibroblasts.
Figure 5.1. Effect of TLR3 L412F on transcription factors NF-κB and IRF3 and H1N1 viral nucleoprotein levels in Leu/Leu and Phe/Phe primary human lung fibroblasts from pulmonary sarcoidosis patients following 24 h live viral infection with MOI-2 Influenza A virus [Strain A/Texas/36/1991(H1N1)]. Primary human lung fibroblasts from Leu/Leu pulmonary sarcoidosis induce expression of IL-8 (A), IFN-β (B) and RANTES (C) at 24 h post-infection with MOI-2 H1N1. H1N1 nucleoprotein levels are higher in Leu/Leu patients compared with Phe/Phe sarcoidosis patients (D) at 24 h post infection with H1N1, as quantitated by qPCR analysis. Results shown are the mean +/- S.E.M of three replicate samples from one experiment. (Unpublished data, generated by the Donnelly research group previously).
Figure 5.2. H1N1 viral nucleoprotein levels in Leu/Leu and Phe/Phe primary human lung fibroblasts from pulmonary sarcoidosis patients following 24 h live viral infection with MOI-2 Influenza A virus [Strain A/Texas/36/1991(H1N1)]. H1N1 nucleoprotein levels are higher in Leu/Leu wild-type patients (A) compared with Phe/Phe sarcoidosis patients (B) at 24 h post infection with H1N1, as quantitated by qPCR analysis. Results shown are the mean +/- S.E.M of three replicate samples from one experiment.
Figure 5.3. Effect of TLR3 L412F on autophagy marker-mRNA expression in Leu/Leu and Phe/Phe primary human lung fibroblasts from pulmonary sarcoidosis patients following 24 h live viral infection with MOI-2 Influenza A virus [Strain A/Texas/36/1991(H1N1)]. H1N1 upregulates Beclin 1, ATG5 and LC3B mRNA expression in primary human lung fibroblasts from Leu/Leu sarcoidosis patients (A, C, E) and Phe/Phe patients (B, D, F) at 24 h post infection, as quantitated by qPCR analysis. Results shown are the mean +/- S.E.M of four replicate samples from one experiment.
5.4 Primary lung fibroblasts from Leu/Phe IPF patients upregulate mRNA expression of autophagy markers in response to nutrient deprivation following HBSS-treatment

In order to examine the level of autophagy in TLR3 L412F IPF fibroblasts, cells were treated with a potent autophagy inducer, Hank’s Balanced Salt Solution (HBSS). HBSS induces nutrient starvation which in turn promotes autophagy (302). When nutrients are plentiful in a cell, activated mammalian target of rapamycin (mTOR) prevents the induction of autophagy by inhibiting the ULK1 protein complex with Beclin-1 (196, 197). In times of nutrient stress and starvation activated AMPK inhibits mTOR to allow for the induction of autophagy. In Leu/Leu IPF fibroblasts there was a significant induction of Beclin 1 mRNA (*p<0.05, Fig. 5.4A) and Atg5 mRNA (*p<0.05, Fig. 5.4C) expression following 100% HBSS-treatment for 24 h compared with medium only. Similarly, Beclin 1 mRNA (Fig. 5.4B) and Atg5 (Fig. 5.4D) mRNA levels increased in Leu/Phe IPF fibroblasts following 100% HBSS-treatment for 24 h compared with medium only, however this was not significant. The late stage gene, LC3B expression was significantly induced in the Leu/Leu IPF fibroblasts following 100% HBSS-treatment for 24 h compared with medium only (5.2 fold, **p<0.01, Fig. 5.4E). However, in 100% HBSS-treated Leu/Phe IPF fibroblasts there was a greater upregulation of LC3-II expression compared with medium only. Leu/Phe IPF fibroblasts significantly upregulated (84.2 fold, ***p<0.001, Fig. 5.4F) LC3B gene expression after 24 h compared with medium only. These findings indicate potentially higher levels of autophagy in IPF Leu/Phe IPF patients than Leu/Leu IPF patients following nutrient deprivation.
Figure 5.4. Effect of TLR3 L412F on autophagy marker-mRNA expression in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients following 24 h treatment with HBSS. HBSS induces Beclin 1, ATG5 and LC3B mRNA expression in primary human lung fibroblasts from Leu/Leu (A, C, E) and Leu/Phe IPF patients (B, D, F) at 24 h post-treatment, as quantitated by qPCR analysis. Higher levels of LC3B mRNA expression are seen in Leu/Phe IPF fibroblasts (F) compared with Leu/Leu IPF fibroblasts (E). A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: HBSS treatment compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from three cell lines per genotype.
5.5 Leu/Phe IPF fibroblasts express higher basal levels of LC3-II protein expression than wild-type fibroblasts

One of the standard methods for examining autophagy is by analysing LC3-II protein expression by western blot (300). LC3B is a late phase autophagy protein which cycles between two forms; LC3-I and LC3-II. LC3-I is converted to LC3-II through lipiddation during autophagy when it is conjugated to the autophagosome. The conversion of LC3-I to LC3-II is a recognized hallmark of autophagy induction. Due to the cyclical nature of the autophagy pathway, autophagy inhibitors (such as bafilomycin A1) are used to promote build-up of LC3B proteins to analyse autophagy flux. Autophagic flux can be measured using an autophagy inhibitor such as bafilomycin A1 to determine the LC3—I—II turnover rate in unstimulated cells and following treatment. Bafilomycin A1 acts by inhibiting vacuolar H+ ATPase (V-ATPase) which neutralises the lysosomal pH. Bafilomycin A1 treatment in IPF fibroblasts prevents LC3-I and LC3-II degradation by blocking lysosomal fusion with autophagosomes. If autophagy is occurring in the cell, there will be difference in the amount of LC3-II in the presence and absence of inhibitors. If autophagy flux is occurring, the amount of LC3-II will be higher in the presence of the inhibitor (in our case, bafilomycin A1) (301). Induction of autophagy by a treatment can also be assessed by the use of bafilomycin A1. If levels of LC3-II are higher with treatment in combination with bafilomycin A1 compared with treatment alone as well as bafilomycin A1 alone, the additive effect in LC3-II levels may suggest that the treatment enhances autophagic flux (301).

In this study, we examined the levels of LC3-II protein expression, as an indicator of autophagy activity, in Leu/Leu and Leu/Phe IPF fibroblasts by Western blot. All densitometry analysis was performed using Image J and the data is relative to WT medium + DMSO.

LC3-II expression +/- bafilomycin A1 treatment

In Fig. 5.5 we demonstrate that Leu/Phe IPF fibroblasts have similar basal levels of LC3-II expression compared with Leu/Leu IPF fibroblasts after 12 h unstimulated. No consistent difference in LC3-II expression was found between the genotypes in unstimulated IPF fibroblasts. In the presence of bafilomycin A1, LC3-II
expression is increased in Leu/Leu IPF fibroblasts compared with unstimulated Leu/Leu IPF fibroblasts. Similarly LC3-II expression is increased in bafilomycin A1-treated Leu/Phe IPF fibroblasts compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts. Higher LC3-II expression is seen following bafilomycin A1 treatment in Leu/Phe IPF fibroblasts compared with bafilomycin A1-treated Leu/Leu IPF fibroblasts. Higher LC3-II accumulation with bafilomycin treatment is indicative of higher levels of autophagy, therefore this result suggests that Leu/Phe IPF fibroblasts may have higher levels of autophagy than Leu/Leu fibroblasts.
Figure 5.5: Basal expression of LC3-II protein in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. LC3-II protein expression was assessed by Western blot in the presence and absence of the autophagy inhibitor, bafilomycin. Higher levels of LC3-II protein expression are seen in primary human lung fibroblasts from Leu/Phe IPF patients compared with Leu/Leu IPF patients at 12 h in the presence of bafilomycin. Western blot image is representative of 3 independent experiments with similar results. Densitometry analysis was performed using Image J software. Results shown are the mean +/- S.E.M of three cell lines per genotype. [Bafilomycin- 50 nM]
5.6 Effect of TLR3 and TLR4 activation on LC3-II production in Leu/Leu and Leu/Phe IPF fibroblasts

Following the observation that Leu/Phe IPF fibroblasts have higher levels of LC3-II accumulation following bafilomycin A1 treatment alone, the level of LC3-II expression following stimulation was assessed by western blot. As previously mentioned, TLR3 is activated by the synthetic double-stranded RNA, Poly(I:C), and TLR4 is activated by LPS. Other researchers have previously shown that many TLRs including TLR3, 4, and 7 induce autophagy in macrophages (198). Similarly, TLR3 and TLR4 activation has been shown to induce autophagy in lung cancer cells (203). Zhan et al. found that TLR3 and 4 activation, by their synthetic ligands; Poly(I:C) and LPS respectively, induced autophagy via the downstream signalling protein TRAF6. TLR3 signals through the downstream adaptor protein TRIF while TLR4 can signal through either TRIF or MyD88 (96). LPS-induced TLR4 has been shown to induce autophagy via a TRIF dependent, MyD88 independent pathway (203, 303). Additionally, Gao et al. demonstrated in cultured rat myocardiocytes that TLR3 activation induces autophagy and increases protein levels of LC3-II (304).

Poly(I:C): Basal LC3-II expression (Medium + DMSO)

Leu/Leu and Leu/Phe IPF fibroblasts were stimulated with Poly(I:C) (50μg/ml) in the presence or absence of bafilomycin A1 for 12 h. All densitometry analysis was performed using Image J and the data is relative to WT medium + DMSO. We found in Fig. 5.6 that Leu/Phe IPF fibroblasts had similar levels of LC3-II expression compared with Leu/Leu IPF fibroblasts, unstimulated at 12 h.

Poly(I:C): LC3-II expression following bafilomycin A1 treatment

Following 12 h bafilomycin A1 treatment, there was an increase in LC3-II protein accumulation in Leu/Leu IPF fibroblasts compared with unstimulated Leu/Leu IPF fibroblasts. Similarly, there was an increase in LC3-II protein accumulation in Leu/Phe IPF fibroblasts treated with bafilomycin A1 compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts. As previously seen in Fig. 5.5, there were higher levels of LC3-II protein accumulation Leu/Phe IPF fibroblasts compared L412F-wild-type following 12 h bafilomycin A1 treatment.
**Poly(I:C): LC3-II expression following Poly(I:C) treatment**

Poly(I:C) treatment for 12 h did not induce LC3-II expression in Leu/Leu IPF fibroblasts compared with unstimulated Leu/Leu IPF fibroblasts. LC3-II expression levels remained similar in Leu/Phe IPF fibroblasts compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts following Poly(I:C) stimulation for 12 h.

**Poly(I:C): LC3-II expression following Poly(I:C) and bafilomycin A1 treatment**

Poly(I:C) treatment in combination with bafilomycin A1 treatment induced greater levels LC3-II protein accumulation in Leu/Leu IPF fibroblasts compared with unstimulated Leu/Leu IPF fibroblasts. There was a modest increase in LC3-II protein accumulation in Poly(I:C) and bafilomycin A1 treated Leu/Leu IPF fibroblasts compared with bafilomycin A1-alone treated Leu/Leu IPF fibroblasts.

Poly(I:C) treatment in combination with bafilomycin A1 treatment induced LC3-II protein accumulation in Leu/Phe IPF fibroblasts compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts. However, there was no increase in LC3-II protein accumulation in Poly(I:C) and bafilomycin A1 treated Leu/Phe IPF fibroblasts compared with bafilomycin A1-alone treated Leu/Phe fibroblasts.

This result suggests that Poly(I:C) did not induce autophagy in these IPF fibroblasts as LC3-II protein levels were similar following bafilomycin treatment alone and with Poly(I:C). If Poly(I:C) drove autophagy, we would have expected higher levels of LC3-II protein accumulation in Poly(I:C) and bafilomycin A1 treated IPF fibroblasts compared with bafilomycin A1 treatment alone.

**LPS: Basal LC3-II expression (Medium + DMSO)**

TLR4-induced autophagy was also assessed in IPF fibroblasts. Leu/Leu and Leu/Phe IPF fibroblasts were stimulated with LPS (1 μg/ml) in the presence or absence of bafilomycin A1 for 12 h. All densitometry analysis was performed using Image J and the data is relative to WT medium + DMSO. We found in Fig. 5.7 that basally, Leu/Phe IPF fibroblasts had similar levels of LC3-II expression compared with Leu/Leu IPF fibroblasts, unstimulated at 12 h.
**LPS: LC3-II expression following bafilomycin A1 treatment**

LC3-II protein accumulation was increased following 12 h bafilomycin A1 treatment alone in Leu/Leu IPF fibroblasts compared with unstimulated Leu/Leu IPF fibroblasts. Similarly, there was an increase in LC3-II expression in Leu/Phe IPF fibroblasts compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts. As previously seen in Fig. 5.5 and 5.6, there were higher levels of LC3-II protein accumulation in Leu/Phe IPF fibroblasts compared with Leu/Leu following 12 h bafilomycin A1 treatment.

**LPS: LC3-II expression following LPS treatment**

LPS stimulation for 12 h did not induce LC3-II expression in Leu/Leu IPF fibroblasts compared with unstimulated Leu/Leu IPF fibroblasts. LC3-II expression levels showed a modest increase in Leu/Phe IPF fibroblasts compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts following LPS treatment for 12 h.

**LPS: LC3-II expression following LPS and bafilomycin A1 treatment**

LPS treatment in combination with bafilomycin A1 increased LC3-II protein accumulation in Leu/Leu IPF fibroblasts compared with unstimulated Leu/Leu IPF fibroblasts. There was no increase in LC3-II protein accumulation in LPS and bafilomycin A1 treated Leu/Leu IPF fibroblasts compared with bafilomycin A1-alone treated Leu/Leu IPF fibroblasts. Similarly, LPS treatment in combination with bafilomycin A1 treatment increased LC3-II protein accumulation in Leu/Phe IPF fibroblasts compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts. There was no increase in LC3-II protein accumulation in LPS and bafilomycin A1 treated Leu/Phe IPF fibroblasts compared with bafilomycin A1-alone treated Leu/Phe IPF fibroblasts.

This result suggests that LPS did not induce autophagy in these IPF fibroblasts as LC3-II protein levels were similar following bafilomycin treatment alone and with LPS.
Figure 5.6. **Effect of TLR3 L412F on LC3-II protein production in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients following Poly(I:C) stimulation.** LC3-II protein expression following Poly(I:C) stimulation for 12 h was assessed by Western blot in the presence and absence of the autophagy inhibitor, bafilomycin. In both genotypes, bafilomycin treatment induced a build-up of LC3-II basally. Higher levels of LC3-II protein expression are seen in primary human lung fibroblasts from Leu/Phe IPF patients compared with Leu/Leu IPF patients at 12 h post-treatment in the presence of bafilomycin. Poly(I:C) stimulation did not induce LC3-II expression. Western blot image is representative of 3 independent experiments with similar results. Densitometry analysis was performed using Image J software. Results shown are the mean +/- S.E.M of three cell lines per genotype. [Poly(I:C)- 50 μg/ml and Bafilomycin- 50 nM]
Figure 5.7. Effect of TLR3 L412F on LC3-II protein production in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients following LPS stimulation. LC3-II protein expression following LPS stimulation for 12 h was assessed by Western blot in the presence and absence of the autophagy inhibitor, bafilomycin. In both genotypes, bafilomycin treatment induced a build-up of LC3-II basally. Higher levels of LC3-II protein expression are seen in primary human lung fibroblasts from Leu/Phe IPF patients compared with Leu/Leu IPF patients at 12 h post-treatment in the presence of bafilomycin. Western blot image is representative of 3 independent experiments with similar results. Densitometry analysis was performed using Image J software. Results shown are the mean +/- S.E.M of three cell lines per genotype. [LPS-1 μg/ml and Bafilomycin- 50 nM]
5.7 Effect of rapamycin treatment on LC3-II production Leu/Leu and Leu/Phe IPF fibroblasts

Rapamycin: Basal LC3-II expression (Medium + DMSO)

Rapamycin is a standard method for inducing autophagy in cells. As previously described, rapamycin targets and inhibits the signalling protein mTOR which leads to the induction of autophagy through activation of the downstream protein ULK-1 (196). In order to examine autophagy induction independent of the TLR pathway, we stimulated IPF fibroblasts with rapamycin with or without bafilomycin A1 for 12 h (Fig. 5.8). Here, we show that under basal conditions there a modest increase LC3-II expression in Leu/Phe IPF fibroblasts compared with Leu/Leu IPF fibroblasts after 12 h. In our results we have found no consistent differences between unstimulated TLR3 genotypes in IPF lung fibroblasts. This may be due to the cyclical nature of the autophagy pathway. Further time point studies will need to be completed to examine LC3-II protein expression in unstimulated IPF fibroblasts.

Rapamycin: LC3-II expression following bafilomycin A1 treatment

Bafilomycin A1 treatment increased LC3-II protein accumulation in wild-type Leu/Leu cells compared with unstimulated Leu/Leu IPF fibroblasts. Similarly, there was an increase in LC3-II protein accumulation in Leu/Phe IPF fibroblasts compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts.

Rapamycin: LC3-II expression following rapamycin treatment

A modest increase in LC3-II protein expression was seen following rapamycin stimulation for 12 h in Leu/Leu IPF fibroblasts compared with unstimulated Leu/Leu IPF fibroblasts. LC3-II expression levels did not increase in Leu/Phe IPF fibroblasts compared with unstimulated Leu/Phe IPF fibroblasts following rapamycin treatment alone for 12 h.

Rapamycin: LC3-II expression following rapamycin and bafilomycin A1 treatment

Rapamycin treatment in combination with bafilomycin A1 treatment induced a modest increase in LC3-II protein accumulation in Leu/Leu IPF fibroblasts
compared with unstimulated IPF fibroblasts and rapamycin-alone treated Leu/Phe IPF fibroblasts. However, there was no difference in LC3-II protein accumulation in rapamycin and bafilomycin A1-treated Leu/Leu IPF fibroblasts compared with bafilomycin A1-alone treated Leu/Leu IPF fibroblasts.

Rapamycin treatment in combination with bafilomycin A1 induced increased LC3-II protein accumulation in Leu/Phe IPF fibroblasts compared with unstimulated Leu/Leu and Leu/Phe IPF fibroblasts. However, there was no increase in LC3-II protein accumulation in rapamycin and bafilomycin A1 treated Leu/Phe IPF fibroblasts compared with bafilomycin A1-alone treated Leu/Phe IPF fibroblasts. Therefore rapamycin, like Poly(I:C) and LPS, did not induce autophagy in these IPF fibroblasts as LC3-II protein levels were similar following bafilomycin treatment alone and with rapamycin.

Taken together these results have shown that Poly(I:C), LPS and rapamycin do not induce LC3-II expression, and therefore autophagy, in IPF fibroblasts of either genotype. However, treatment with bafilomycin A1 revealed that unstimulated Leu/Phe IPF fibroblasts have higher expression of LC3-II protein levels compared with Leu/Leu which may suggest increased dysregulation of the autophagy pathway.
**Figure 5.8:** Effect of *TLR3* L412F on LC3-II protein production in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients following rapamycin stimulation. LC3-II protein expression following rapamycin stimulation for 12 h was assessed by Western blot in the presence and absence of the autophagy inhibitor, Bafilomycin. Leu/Leu IPF fibroblasts treated with bafilomycin had a modest build-up of LC3-II basally which did not increase following rapamycin stimulation. Similarly, Leu/Phe IPF fibroblasts treated with bafilomycin had a greater build-up of LC3-II basally than Leu/Leu IPF fibroblasts and following rapamycin stimulation LC3-II production did not increase. Western blot image is representative of 3 independent experiments with similar results. Densitometry analysis was performed using Image J software. Results shown are the mean +/- S.E.M of three cell lines per genotype. [Rapamycin- 500 nM and Bafilomycin- 50 nM].
5.8 Effect of TLR3 L412F on Poly(I:C)-induced IL-8, IL-6 and RANTES protein production in the presence of bafilomycin A1 in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients

Our studies and others have shown that the autophagy inhibitor, bafilomycin A1, inhibits autophagy through blocking lysosomal degradation of LC3-II causing an intra-cellular build-up of the protein. Bafilomycin A1 has been shown previously to inhibit TLR3 signalling and subsequent cytokine production following Poly(I:C) treatment by blocking lysosomal maturation (305). During this study, we examined cytokine production following bafilomycin A1 treatment. Following treatment of Leu/Leu and Leu/Phe IPF fibroblasts with Poly(I:C) with or without bafilomycin A1 for 12 h, supernatants were harvested for ELISA. Leu/Leu IPF fibroblasts stimulated with Poly(I:C) (10 and 50 μg/ml) significantly upregulated production of IL-8 (**p<0.01, Fig. 5.9A), IL-6 (**p<0.001, Fig. 5.9C) and RANTES (*p<0.05, **p<0.01 Fig. 5.9E) compared with medium only. Leu/Phe IPF fibroblasts had attenuated production of IL-8 (Fig. 5.9B), IL-6 (Fig. 5.9D) and RANTES (Fig. 5.9F) following Poly(I:C) (10 and 50 μg/ml) stimulation compared with medium only.

In Leu/Leu IPF fibroblasts bafilomycin A1 treatment in combination with Poly(I:C) attenuated IL-8 (Fig. 5.9A), IL-6 (Fig. 5.9C) and RANTES (Fig. 5.9E) cytokine production in Leu/Leu IPF fibroblasts compared with Poly(I:C) treatment alone in Leu/Leu IPF fibroblasts. In Leu/Phe IPF fibroblasts, the modest induction of IL-8, IL-6 and RANTES seen following Poly(I:C) stimulation was even further reduced following combined treatment of bafilomycin A1 and Poly(I:C) (Fig. 5.9 B, D, F).
Figure 5.9. Effect of TLR3 L412F on IL-8, IL-6 and RANTES protein production in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. Higher levels of IL-8, IL-6 and RANTES protein production following Poly(I:C) stimulation are seen in primary human lung fibroblasts from Leu/Leu IPF (A, C, E) patients compared with Leu/Phe IPF patients (B, D, F) at 12 h post-treatment, as quantitated by ELISA. In both genotypes, bafilomycin treatment attenuated IL-8, IL-6 and RANTES protein production following Poly(I:C) stimulation. Results shown are the mean +/- S.E.M of three cell lines per genotype with five to six replicates (A, C, E). Results shown are the mean +/- S.E.M of five cell lines per genotype with five to six replicates. (B, D, F). A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: Poly(I:C)/Bafilomycin treatment compared with medium-only treatment. [Poly(I:C)- 10 and 50 μg/ml, Bafilomycin- 50 nM].
5.9 Effect of TLR3 L412F on LPS and rapamycin-induced IL-8, IL-6 and RANTES protein production in the presence of bafilomycin A1 in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients

In order to determine whether bafilomycin A1 also affected signalling through TLR4. Cytokine production was assessed in IPF fibroblasts stimulated with LPS (1 μg/ml) in combination with bafilomycin A1 for 12 h. Leu/Leu IPF fibroblasts stimulated with LPS significantly upregulated production of IL-8 (**p<0.001, Fig. 5.10A) and IL-6 (**p<0.001, Fig. 5.10C) combined to medium only. RANTES was not produced in response to LPS stimulation after 12hrs (Fig. 5.10E). In contrast to the results seen in Fig. 5.9, bafilomycin A1 treatment did not affect the levels of LPS-induced IL-8 and IL-6 production in Leu/Leu fibroblasts (Fig. 5.10A, C). In Leu/Phe IPF fibroblasts a modest increased in IL-8 protein production was seen after 12 h. IL-6 was significantly upregulated (**p<0.001, Fig. 5.10D) compared with medium only in Leu/Phe IPF fibroblasts and RANTES was not upregulated following LPS stimulation (Fig. 5.10F). Similar to the results seen in Leu/Leu IPF fibroblasts, combined treatment of LPS and bafilomycin A1 did not attenuate IL8 (Fig. 5.10B) or IL6 (Fig. 5.10D) protein production in Leu/Phe IPF fibroblasts. These results prove that bafilomycin A1 treatment only affected TLR3 signalling in IPF fibroblasts and not TLR4.

Cytokine expression was also examined following 12 h rapamycin treatment in IPF fibroblasts in the presence of bafilomycin A1. In Leu/Leu IPF fibroblasts rapamycin induced modest but significant levels of IL-8 (*p<0.05, Fig. 5.10A) and IL-6 (*p<0.05, Fig. 5.10C) compared with medium only. Similarly, in Leu/Phe IPF fibroblasts rapamycin induced modest but significant levels of IL-6 (*p<0.05, Fig. 5.10D) but not IL-8 (Fig. 5.10B). Rapamycin did not induce significant levels of RANTES expression in either genotype of IPF fibroblasts (Fig. 5.10E, F).
**Figure 5.10.** Effect of TLR3 L412F on IL-8, IL-6 and RANTES protein production in Leu/Leu and Leu/Phe primary human lung fibroblasts from IPF patients. Higher levels of IL-8 and IL-6 protein production following LPS stimulation are seen in primary human lung fibroblasts from Leu/Leu IPF (A, C) patients compared with Leu/Phe IPF patients (B, D) at 12 h post-treatment, as quantitated by ELISA. LPS and rapamycin did not induce RANTES protein production at 12 h post treatment in either genotype (E, F). Rapamycin induced minimal levels of IL-8 and IL-6 protein expression in both genotypes. Bafilomycin treatment did not attenuate IL-8 or IL-6 protein production following LPS or rapamycin stimulation. Results shown are the mean +/- S.E.M of three cell lines per genotype with five to six replicates (A, C, E). Results shown are the mean +/- S.E.M of five cell lines per genotype with five to six replicates. (B, D, F). A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.001: Treatment compared with medium-only treatment. [LPS- 1 μg/ml, Rapamycin- 500nM, Bafilomycin- 50 nM].
5.10 Effect of TLR3 L412F on mTOR activity and autophagy induction in IPF lung fibroblasts.

In the final part of this study we evaluated the role of mTOR in the induction of autophagy in IPF fibroblasts. Signalling through the mTOR plays a role in a number of different important cellular process such as cell proliferation, autophagy and cellular homeostasis. As previously stated, rapamycin targets and inhibits the signalling protein mTOR which leads to the induction of autophagy through activation of the downstream protein ULK-1 (196). mTOR also affects TLR3 signalling. Zhao et al. found that mTOR signalling regulates TLR3-induced cytokine production in keratinocytes (306). It was shown that rapamycin treatment in combination with Poly(I:C)-induced activation of TLR3 attenuated production of pro-inflammatory cytokines (via NF-κB) and IFN-β (via IRF3) (306). Here, we examined total mTOR protein and mTOR activity in order to determine the effect of mTOR on autophagy induction and TLR3-induced cytokine production in Leu/Leu and Leu/Phe IPF fibroblasts.

We examined the total amount of mTOR protein expressed in Leu/Leu and Leu/Phe IPF fibroblasts by western blot. IPF fibroblasts were stimulated with rapamycin (500nM) and/or Poly(I:C) (50μg/ml) for 12hrs to induce autophagy. mTOR activation leads to the phosphorylation of many sites on the protein. We analysed the phosphorylation of mTOR at site Ser2448 as a measure of activity of the mTOR protein. Leu/Leu and Leu/Phe IPF fibroblasts were analysed on separate blots. All densitometry analysis was performed using Image J and the data is relative to WT medium only.

Phospho-mTOR and total mTOR: Leu/Leu IPF fibroblasts

In Fig. 5.11 it can be seen that phospho-mTOR levels are high basally in Leu/Leu IPF fibroblasts. Phospho-mTOR expression levels reduced following rapamycin treatment for 12 h compared with medium only. Following Poly(I:C) treatment, Leu/Leu IPF fibroblasts had similar levels of phospho-mTOR protein compared with medium only. A combination of rapamycin treatment and Poly(I:C) treatment for 12 h also reduced expression of phospho-mTOR compared with medium only. Total mTOR was highly expressed in Leu/Leu IPF fibroblasts and
these levels remained the same following rapamycin, Poly(I:C) or Poly(I:C) and rapamycin treatment. Densitometry analysis allowed us to measure mTOR activity in Leu/Leu IPF fibroblasts. A ratio of phospho-mTOR : total mTOR protein was used to determine mTOR activity, the higher the ratio, the more mTOR activity. It was found that following rapamycin treatment mTOR activity was reduced compared with medium only. Poly(I:C) treatment did not change mTOR activity compared with medium only but a combined treatment of rapamycin and Poly(I:C) reduced mTOR activity.

**Phospho-mTOR and total mTOR: TLR3 L412F heterozygote IPF fibroblasts**

In Leu/Phe IPF fibroblasts, similar results are observed. It can be seen that phospho-mTOR levels are high basally in Leu/Phe IPF fibroblasts. Phospho-mTOR levels reduced following rapamycin treatment for 12 h compared with medium only. Following Poly(I:C) treatment, Leu/Phe IPF fibroblasts had a modest increase phospho-mTOR expression compared with medium only. A combination of rapamycin treatment and Poly(I:C) treatment for 12 h also reduced expression of phospho-mTOR compared with medium only.

Total mTOR was highly expressed in Leu/Phe IPF fibroblasts and these levels remained the same following rapamycin, Poly(I:C) or Poly(I:C) and rapamycin treatment. mTOR activity was also assessed in Leu/Phe IPF fibroblasts. A ratio of phosphor-mTOR : total mTOR protein was used to determine mTOR activity. It was found that mTOR activity no differences were seen following rapamycin, Poly(I:C) or a combination of rapamycin and Poly(I:C) treatment compared with medium only.

Taken together these results show that in Leu/Leu and Leu/Phe IPF fibroblasts, mTOR is constitutively active basally as there are high levels of phospho-mTOR in unstimulated Leu/Leu and Leu/Phe IPF fibroblasts. We have also shown that expression of total mTOR protein is unaffected by treatment with Poly(I:C) and rapamycin. In order to further elucidate the effect of the TLR3 L412F SNP on mTOR activity, downstream signalling proteins of the mTOR pathway will be examined in future studies.
Fig 5.11. Phospho-mTOR and Total mTOR protein levels are similar in TLR3 L412F wild-type and heterozygote cell lines following treatment with rapamycin and/or Poly(I:C). Primary IPF human lung fibroblasts from TLR3 L412F wild-type and heterozygote express similar levels of Phospho-mTOR and Total mTOR protein levels following 12 h stimulation with rapamycin in the presence or absence of Poly(I:C). Western blot image is representative of 3 independent experiments with similar results. Densitometry analysis was performed using Image J software. Results shown are the mean +/- S.E.M of three cell lines per genotype. [Poly(I:C)] - 50 μg/ml and Rapamycin - 500 nM.
5.11 Effect of rapamycin treatment on TLR- induced cytokine production in Leu/Leu and Leu/Phe IPF lung fibroblasts.

Rapamycin targets mTOR to inhibit mTOR activation and downstream signalling. mTOR activation is important for signalling through TLR3 and induction of interferons stimulated genes (ISGs) (243). During this study, we examined TLR-induced cytokine production following rapamycin treatment in IPF primary human lung fibroblasts. Following treatment of Leu/Leu and Leu/Phe IPF fibroblasts with Poly(I:C) with or without rapamycin for 12 h, supernatants were harvested for ELISA. Leu/Leu IPF fibroblasts stimulated with Poly(I:C) (50 μg/ml) upregulated production of IL-6 (Fig. 5.12A), IL-8 (Fig. 5.12C) and RANTES (*p<0.05, Fig. 5.12E) compared with medium only. Leu/Phe IPF fibroblasts also upregulated production of IL-6 (**p<0.01, Fig. 5.12B), IL-8 (***p<0.001, Fig. 5.12D) and RANTES (Fig. 5.12F) following Poly(I:C) (50 μg/ml) stimulation compared with medium only.

In Leu/Leu IPF fibroblasts, rapamycin treatment in combination with Poly(I:C) attenuated IL-8 (Fig. 5.12C) and RANTES (Fig. 5.12E) but not IL-6 (Fig. 5.12A) cytokine production in Leu/Leu IPF fibroblasts compared with Poly(I:C) treatment alone in wild-type IPF fibroblasts. In Leu/Phe IPF fibroblasts, rapamycin treatment did not affect induction of IL-6 (Fig. 5.12B), IL-8 (Fig. 5.12D) protein production but a modest decreases was seen in RANTES expression reduced following combined treatment of rapamycin and Poly(I:C) (Fig. 5.12F). These results show that mTOR activity is important in TLR3 signalling in IPF fibroblasts, inhibition of this pathway results in attenuated production of pro-inflammatory cytokines and type I interferons following TLR3 activation.

We also examined the effect of the inhibition of mTOR on TLR4 signalling. Leu/Leu and Leu/Phe IPF fibroblasts were treated with LPS in the presence or absence of rapamycin for 12 h, supernatants were harvested for ELISA. Leu/Leu IPF fibroblasts stimulated with LPS (1 μg/ml) upregulated production of IL-6 (Fig. 5.12A), IL-8 (Fig. 5.12C) but minimal levels of RANTES (Fig. 5.12E) compared with medium only. Leu/Phe IPF fibroblasts induce a modest increase in production
of IL-6 (Fig. 5.12B), IL-8 (Fig. 5.12D) and RANTES (Fig. 5.12F) following LPS (1 μg/ml) stimulation compared with medium only.

In Leu/Leu fibroblasts rapamycin treatment in combination with LPS did not affect IL-6 (Fig. 5.12A), IL-8 (Fig. 5.12C) and RANTES (Fig. 5.12E) cytokine production in Leu/Leu IPF fibroblasts compared with LPS treatment alone. Similarly, in Leu/Phe IPF fibroblasts, rapamycin treatment did not affect induction of IL-6 (Fig. 5.12B), IL-8 (Fig. 5.12D) or RANTES expression (Fig. 5.12F) following combined treatment of rapamycin and LPS compared with LPS alone.

These results show that mTOR activity is important in TLR3 signalling but not TLR4 signalling in IPF fibroblasts, inhibition of this pathway results in attenuated production of pro-inflammatory cytokines and type I interferons following TLR3 activation only.
**Figure 5.12.** Effect of rapamycin on IL-6, IL-8 and RANTES protein production in Leu/Leu and Leu/Phe primary human lung fibroblasts following TLR3 and TLR4 activation. IL-6, IL-8 and RANTES protein production is induced following Poly(I:C) and LPS stimulation in primary human lung fibroblasts from Leu/Leu IPF patients (A, C, E) compared with medium only. TLR3 L412F attenuates IL-6, IL-8 and RANTES in Leu/Phe IPF patients (B, D, F) at 12 h post-treatment, as quantitated by ELISA. Rapamycin treatment attenuated IL-6, IL-8 and RANTES protein production following Poly(I:C) stimulation in primary human lung fibroblasts from Leu/Leu IPF patients but not LPS stimulation (A, C, E). Rapamycin treatment did not attenuate IL-6, IL-8 and RANTES protein production following Poly(I:C) and LPS stimulation in primary human lung fibroblasts from Leu/Phe IPF patients (B, D, F). A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences.*p<0.05, **p<0.01, ***p<0.001: Poly(I:C)/LPS/Rapamycin treatment compared with medium-only treatment. Results shown are the mean +/- S.E.M of three to six replicates from a representative of two separate experiments [Poly(I:C) - 50 μg/ml, LPS - 1 μg/ml and Rapamycin - 500 nM].
5.12 Proposed mechanism of action in Leu/Leu and Leu/Phe IPF primary human lung fibroblasts.

In Fig. 5.13 and 5.14 we have illustrated our hypothesis for Leu/Leu and Leu/Phe IPF primary human lung fibroblasts. As previously shown in Chapters 3 and 4, Leu/Leu IPF fibroblasts can upregulate expression of pro-inflammatory cytokines and RANTES following activation of TLR3 by Poly(I:C). We have found that mTOR activity is important in the production of pro-inflammatory cytokines and RANTES following TLR3 activation and inhibition of this pathway attenuates this. Additionally, Leu/Leu fibroblasts exhibit persistent mTOR activation which has been shown to be detrimental in models of fibrosis. Leu/Leu IPF fibroblasts express lower levels of LC3-II protein compared with Leu/Phe IPF fibroblasts but also show an inability to upregulate autophagy following stimulation with Poly(I:C), LPS and rapamycin, suggesting dysregulation of the autophagy process potentially due to persistent mTOR activation in these cells.

We have demonstrated that Leu/Phe IPF fibroblasts have attenuated upregulation of pro-inflammatory cytokine and RANTES expression following activation of TLR3 by Poly(I:C) but have high basal cytokines and RANTES production. Similar to Leu/Leu fibroblasts, Leu/Phe IPF fibroblasts exhibit persistent mTOR activation and are less responsive to inhibition of mTOR with rapamycin treatment. Leu/Phe IPF fibroblasts express higher levels of LC3-II protein compared with Leu/Leu IPF fibroblasts, indicative of higher autophagy in these cells. However, they also show an inability to upregulate autophagy following stimulation with Poly(I:C), LPS and rapamycin, suggesting defective autophagy in both Leu/Leu and Leu/Phe IPF fibroblasts.
In Leu/Leu IPF primary human lung fibroblasts

Viral dsRNA
mRNA from necrotic cells
Human viral dsRNA
Bacterial dsRNA

TLR3

PIPS → PIPS
PI3K
Akt
TSC1 TSC2
Rhdb ← Rhdb
Rapamycin
mTORC1
Persistant Activation

ULK-1 complex

Initiation
Cytosolic cargo
Phago-Atg12 complex
LC3-II
Fusion with Lysosome
Degradation and Nutrient Recycling

PAS
Autophagosome

Dysregulated Autophagy - High LC3-II accumulation following benflumetol treatment but no induction of autophagy

Nucleus

ISRE
↑ IFN-α

↑ IL-8

↑ IL-6
Figure 5.13. **Autophagy in Leu/Leu IPF patients.** TLR3 is activated by dsRNA and activates a downstream signalling cascade resulting in pro-inflammatory cytokine and type I interferon expression. TLR3 can induce autophagy through TRAF6 ubiquitination of autophagy proteins Beclin-1 and ULK1. TRIF can also directly interact with Beclin-1 to induce autophagy. We have demonstrated that bafilomycin treatment induces the accumulation of the late phase autophagy protein, LC3-II, in Leu/Leu IPF fibroblasts. However, in Leu/Leu IPF fibroblasts, Poly(I:C)-activation of TLR3 did not induce autophagy. We have found that mTOR exhibits persistent activation in Leu/Leu IPF fibroblasts. Persistent activation of mTOR may play a role in defective autophagy responses seen in Leu/Leu IPF fibroblasts. TLR3 induces mTOR activity though the PI3K. PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) to act as a second messenger that induces the phosphorylation of Akt on Thr308. Akt phosphorylates and inhibits the heterodimer tuberous sclerosis complex 1 (TSC1)/TSC2. This in turn activates Rheb and in turn mTORC1 activation occurs. Adapted from (99, 190, 191).
In Leu/Pha IPF primary human lung fibroblasts

Viral dsRNA

TLR3-4 RIG-I may affect lisfand binding and signal transduction

Cytoplasm

PIPK2 ↔ PIP3

PASK

Akt

TRIF

TRAP3

TRAF6

DIP-1

IKKγ/1IKKα

TAK1

TA82

TA83

p65

p50

p65

p50

p65

p50

NF-κB

Phosphore

Autophagosome

Dysregulated autophagy - High LC3-II accumulation following bafilomycin treatment but no induction of autophagy

PAS

Lysosome

Degradation and nutrient Recycling

Cytoplasmic cargo Envelopment

Atg5-Atg12 complex

LC3-II

Fusion with lysosome

Inhibition

P62

Phosphore

Autophagosome

Nucleus

DIP3

p65

p50

IFN-β

IFN-β

IL-6

IL-6

ISRE

↓ ISRE
**Figure 5.14. Autophagy in Leu/Phe IPF patients.** TLR3 activation by dsRNA is affected by the presence of TLR3 L412F. This results in defective signal transduction and upregulation production of pro-inflammatory cytokines and type I interferons in Leu/Phe IPF fibroblasts following TLR3 activation. TLR3 can induce autophagy through TRAF6 ubiquitination of autophagy proteins Beclin-1 and ULK1. TRIF can also directly interact with Beclin-1 to induce autophagy. We have demonstrated that bafilomycin treatment induces greater accumulation of the late phase autophagy protein, LC3-II, in Leu/Phe IPF fibroblasts compared with Leu/Leu IPF fibroblasts. However, in Leu/Phe IPF fibroblasts, Poly(I:C)-activation of TLR3 did not induce autophagy. We have found in Leu/Phe IPF fibroblasts that mTOR exhibits persistent activation. Persistent activation of mTOR may play a role in defective autophagy responses seen in Leu/Leu IPF fibroblasts. TLR3 induces mTOR activity though the PI3K. PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) to act as a second messenger that induces the phosphorylation of Akt on Thr308. Akt phosphorylates and inhibits the heterodimer tuberous sclerosis complex 1 (TSC1)/TSC2. This in turn activates Rheb and in turn mTORC1 activation occurs. Adapted from (99, 190, 191).
5.13 Discussion

In this chapter, we investigated the effects of TLR3 L412F on the induction of autophagy and regulation of its associated proteins in pulmonary fibroblasts following: (i) live IAV H1N1 infection of pulmonary sarcoidosis fibroblasts, (ii) under starvation conditions in pulmonary IPF fibroblasts and (iii) through activation of TLRs in pulmonary IPF fibroblasts, respectively. In our initial experiments in this chapter, we utilised primary human lung fibroblasts from pulmonary sarcoidosis patients (a related ILD) which had been infected previously by us with live IAV H1N1 for 24 h. Complementary-DNA (cDNA) from these cells was available for use in this chapter. We previously set-up a collaboration to look at live viral infection of IPF lung fibroblasts with live IAV H1N1 infection. However, these experiments will now be performed at a later stage due to logistical reasons. In the context of the autophagy experiments performed in this chapter using IPF lung fibroblasts, induction of autophagy was investigated HBSS-induced nutrient deprivation, Poly(I:C) and LPS treatments, and rapamycin treatment. In this chapter we have demonstrated that live H1N1 infection attenuates IL-8, IFN-β and RANTES mRNA expression in Phe/Phe sarcoidosis fibroblasts compared with wild-type cells (Unpublished, Armstrong et al). Interestingly, these effects were accompanied by a reduction in H1N1 viral load in Phe/Phe fibroblasts. In this chapter, we established that there was an increase in transcription of the autophagy proteins Beclin 1, ATG5 and LC3B in Phe/Phe sarcoidosis fibroblasts compared with their wild-type counterparts. In this chapter, we also demonstrated in IPF Leu/Phe fibroblasts that there was an increase in transcription of several autophagy proteins compared with wild-type IPF fibroblasts. However, here we established that autophagy responses were defective overall in wild-type and Leu/Phe primary lung fibroblasts from IPF patients. We have also shown that there are high levels of LC3-II expression following bafilomycin A1 treatment coupled with an inability to upregulate LC3-II expression following stimulation with Poly(I:C), LPS and rapamycin. In addition, we have shown that mTOR activation is essential for TLR3 signalling but not TLR4 signalling in IPF fibroblasts. Furthermore, we have demonstrated that Leu/Leu and Leu/Phe IPF fibroblasts have persistent mTOR activation which could play a role in the defective autophagy responses.
Our initial experiments in this chapter demonstrated that IAV H1N1-infected Phe/Phe lung fibroblasts from sarcoidosis patients had reduced IL-8, RANTES, IFN-β mRNA expression, as well as reduced viral load compared with wild-type sarcoidosis cells. We initially hypothesised that this decrease in viral load may be result of the effect of the TLR3 SNP on autophagy in Phe/Phe cells. Previously, the TLR3 L412F SNP has been shown to be protective during certain viral infections and as the frequency of the SNP has been retained at a high level of heterozygosity in the European population (45.7%) (165). Here, we suggest that the SNP is beneficial in the control of certain viral infections. It has been shown in HIV patients that those who carried the Leu/Phe allele had lower viral nucleoprotein levels following infection than wild-type patients (176). Autophagy is an important anti-viral mechanism in cells, because of this many viruses including influenza A virus (IAV) have been shown to manipulate and target the autophagy pathway for their survival. IAV can induce autophagy and it has been shown to be beneficial for the virus as it leads to greater viral replication. Inhibition of autophagy reduces viral protein load in the cell (92). IAV has previously been found to disrupt the autophagy pathway by preventing autophagosome fusion with the lysosome (236). This pro-survival IAV mechanism also enhances the viral protein load in the cell (307). Taking this information into account, in this study we wanted to analyse whether increased levels of autophagy or defective autophagy caused the reduced viral nucleoprotein levels seen in Phe/Phe pulmonary sarcoidosis fibroblasts. Our initial results in IAV H1N1 infection, demonstrated that there was an increase in transcription of the autophagy proteins Beclin 1, ATG5 and LC3B in Phe/Phe sarcoidosis fibroblasts compared with their wild-type counterparts. However, as we were unable to carry out additional live IAV H1N1 infection studies in either sarcoidosis or IPF lung fibroblasts, in all future experiments additional stimuli were used to induce autophagy, these included: nutrient deprivation, rapamycin treatment, and using Poly(I:C) and LPS treatments.

In order to investigate the effects of TLR3 L412F on autophagy in IPF primary lung fibroblasts, we initially examined autophagy induction in Leu/Leu and Leu/Phe IPF fibroblasts following nutrient deprivation. Nutrient deprivation-induced autophagy by HBSS in IPF fibroblasts was assessed by qPCR. Interestingly, LC3B mRNA expression was significantly induced in Leu/Phe IPF fibroblasts, however, it
was not as strongly induced in Leu/Leu fibroblasts. This was a significant finding as, for a second time, we demonstrated differences in mRNA expression of autophagy markers between genotypes and therefore, potential differences in autophagy responses between Leu/Leu and Leu/Phe IPF patients. Analysis of LC3B mRNA expression by qPCR indicated that autophagy responses in Leu/Leu and Leu/Phe patients may be different and so, to continue this work and add to this finding we examined LC3B protein expression by western blot, a classical method of analysing autophagy in the cell (300).

Along with our colleagues (27, 215, 216, 308), in this chapter we provide evidence to suggest that the autophagy pathway is dysregulated in IPF primary human lung fibroblasts which may contribute to IPF pathogenesis but may increase protection in IAV H1N1 infection. By western blot we found no consistent difference in LC3-II protein expression between unstimulated Leu/Leu and Leu/Phe IPF fibroblasts. Following bafilomycin treatment, we have shown greater accumulation of LC3-II protein in Leu/Phe IPF lung fibroblasts compared with Leu/Leu fibroblasts by western blot. This indicated greater levels of autophagy flux in Leu/Phe IPF fibroblasts compared with Leu/Leu IPF fibroblasts. High levels of LC3-II expression in cells can be caused by induction of autophagy or a dysregulation of the autophagy pathway to prevent LC3-II degradation (301).

Throughout this chapter we have examined whether the high LC3-II expression levels, seen in IPF lung fibroblasts with bafilomycin treatment, are due to high levels of autophagy or dysregulation of the process.

Our work has shown that IPF fibroblasts did not induce greater levels of autophagy following TLR3 or TLR4 activation. However, TLR activation has been shown to drive autophagy in a number of different cell types (198, 203). One group found that in macrophages, TLRs3, 4 and 7 significantly induced autophagy compared with unstimulated macrophages (198). In our work we have shown that LC3-II protein expression was not induced following treatment with TLR agonists, Poly(I:C) and LPS, and rapamycin. Following Poly(I:C), LPS and rapamycin treatment in the presence of bafilomycin treatment, we also did not see greater accumulation of LC3-II protein in comparison to bafilomycin treatment alone in either genotype indicating that Poly(I:C), LPS and rapamycin treatment had no effect on the induction LC3-II expression in IPF fibroblasts. Taken together these results
indicated that autophagy could not be upregulated in Leu/Leu or Leu/Phe IPF fibroblasts. Failure to induce autophagy in our IPF primary human fibroblasts may have been due to the diseased nature of the cells or due to the existing high basal levels of autophagy. Further experiments would be needed to confirm whether explant IPF primary human lung fibroblasts are unresponsive to autophagy upregulation. These results provide evidence to suggest that the autophagy pathway is defective in IPF primary human lung fibroblasts as there are high levels of basal LC3-II expression with bafilomycin treatment coupled with an inability to upregulate LC3-II expression following stimulation with Poly(I:C), LPS and rapamycin.

We have shown that there is a defective autophagy response upon stimulation with known autophagy inducers; Poly(I:C), LPS and rapamycin. Here, we have demonstrated using an autophagy inhibitor, bafilomycin, that LC3-II expression is increased basally in Leu/Phe IPF fibroblasts compared with Leu/Leu IPF fibroblasts. The reason for this is unclear, however, a potential reason for this could be the high basal levels of cytokines in Leu/Phe IPF fibroblasts. We have previously shown that Leu/Phe IPF primary human lung fibroblasts have significantly higher basal levels of pro-inflammatory cytokines such as IL-6 and IL-8 as well as RANTES. Many pro-inflammatory cytokines such as IL-6 have the ability to induce autophagy (309). It could be postulated that the high basal levels of cytokines such as IL-6 are contributing to the higher LC3-II expression in Leu/Phe IPF fibroblasts compared with Leu/Leu fibroblasts. In support of this, we have already shown in Chapter 4, that high RANTES basal levels in Leu/Phe IPF fibroblasts is associated with high basal levels of interferon-induced receptors such as RIG-I and MDA5. Similar to LC3-II, these receptors are kept at high levels basally in Leu/Phe IPF fibroblasts and do not upregulate expression following stimulation. It could be suggested that the high basal levels of pro-inflammatory cytokines in the Leu/Phe IPF fibroblasts result in high LC3-II expression, however, upon stimulation there is a dysregulation in the autophagy process preventing further upregulation of LC3-II expression.

We believe that while there are high levels of LC3-II basally in the IPF lung fibroblasts, the inability to upregulate LC3-II expression in either genotype upon stimulation with known autophagy inducers proves that autophagy is insufficient. This is supported by other researchers who have previously found that there is insufficient autophagy in the IPF lung and in particular lung fibroblasts (27, 216). As
previously mentioned, unfortunately, in the time frame of this PhD we were not able to source normal healthy lung fibroblasts to run concurrently with the \textit{TLR3} \textit{L412F} wild-type and heterozygous primary human lung fibroblasts. In future studies, we will examine autophagy induction in primary human lung fibroblasts using the cell line, CCD19-Lu. It has been shown previously by Romero \textit{et al.} that autophagy can be induced in healthy lung fibroblasts from both young and old donors \cite{27}. It would be important to analyse the effect of the SNP on the autophagy response in both healthy and IPF human lung fibroblasts.

This is a significant finding in the pathogenesis of IPF in Leu/Phe IPF patients. In IPF fibroblasts, defective autophagy in response to TLR3 and 4 stimulation or infection may decrease susceptibility to viral infection in Leu/Phe IPF patients as viral replication may be impaired. In our live viral infection studies, one limiting factor was access to live virally infected fibroblasts. Previously, our lab infected Leu/Leu and Phe/Phe primary lung fibroblasts from sarcoidosis patients with IAV H1N1, however in the timeframe of this PhD we were unable to repeat this experiment in IPF primary human lung fibroblasts. Because of this, we were able to do a limited number of experiments in sarcoidosis fibroblasts and the rest in IPF fibroblasts. The Donnelly group has previously shown that primary lung fibroblasts from sarcoidosis patients and IPF patients respond similarly following Poly(I:C) stimulation in the presence of the SNP \cite{154, 168}. In the presence of the SNP, lung fibroblasts from IPF patients and sarcoidosis patients have reduced NF-\textit{kB} and IRF3 activation following Poly(I:C) stimulation \cite{154, 168}. Here, we have shown that primary sarcoidosis fibroblasts have a trend towards increased autophagy markers by qPCR analysis following live viral infection with IAV H1N1. However, increased RNA expression could be caused by either increased autophagic flux or a defect in the autophagy pathway resulting in a build-up of autophagy proteins. To date there is limited information available on the role of autophagy in sarcoidosis. In order to determine whether autophagy is also defective in primary sarcoidosis fibroblasts, we will measure autophagic flux by performing Western blot analysis of LC3-II in the presence and absence of the autophagy inhibitor, bafilomycin.

Previous studies have shown that the influenza A virus requires a competent autophagy pathway to replicate successfully \cite{92, 236}. Here, in our studies, we have mimicked infection using TLR3 and 4 synthetic agonists; Poly(I:C) and LPS, and
rapamycin in Leu/Leu and Leu/Phe IPF primary human lung fibroblasts and we have shown that the autophagy pathway is not inducible. In addition, we have also shown that Leu/Phe IPF fibroblasts have consistently higher basal levels of LC3-II protein expression, suggesting increased dysregulation in the Leu/Phe IPF fibroblasts compared with Leu/Leu IPF lung fibroblasts. This could suggest that IAV H1N1 would not replicate successfully in Leu/Leu and Leu/Phe primary IPF fibroblasts and this could in part account for the lower viral titres seen in the sarcoidosis fibroblasts.

In order to confirm this, we will live-virally infect IPF primary human lung fibroblasts with IAV H1N1 in vitro. Using real-time PCR and Western blot, we hope to analyse the effect of the SNP on viral titres and IAV-induced autophagy in primary IPF fibroblasts. We have already seen in vitro that IAV H1N1 viral load is lower in Phe/Phe fibroblasts from pulmonary sarcoidosis patients compared with wild-type sarcoidosis fibroblasts. In further support of this theory, two independent studies have demonstrated that inhibition of the autophagy pathway by several methods led to reduced IAV viral protein load compared with autophagy-competent cells, proving that autophagy is crucial for viral replication (92, 236). Unfortunately in the timeframe of this PhD we were unable to examine the effect of TLR3 L412F following live H1N1 infection. Future work will be undertaken to characterise the role of TLR3 L412F during live viral infection with H1N1 in IPF primary human lung fibroblasts in vitro and in vivo in our novel TLR3 L413F knock-in mouse.

While defective autophagy may be beneficial in controlling certain viral infections, in contrast to this, it may be detrimental to fibrosis. We have demonstrated that following LPS stimulation, Leu/Phe IPF fibroblasts have attenuated production of pro-inflammatory cytokines and type I interferons as well as an inability to upregulate autophagy. It is worth noting that another research group has shown TLR4-induced autophagy to be protective in the murine bleomycin model of fibrosis (310).

Others researchers have demonstrated a role for autophagy in TLR3 and TLR4-induced cytokine and type I interferon production (167, 203). However, in our study we provide evidence that endosomal acidification but not autophagy is important for TLR3 signalling in IPF fibroblasts. We have found that the use of the autophagy inhibitor bafilomycin caused attenuated pro-inflammatory cytokine production (IL-8 and IL-6) following Poly(I:C)-induced TLR3 activation.
Bafilomycin treatment blocks endosomal acidification, an essential process for TLR3 signalling. We have found that inhibition of endosomal acidification in Leu/Leu and Leu/Phe IPF fibroblasts caused a reduction in pro-inflammatory cytokine production following Poly(I:C) and bafilomycin treatment. We did not, however, see a decrease in pro-inflammatory cytokine production following Poly(I:C) treatment alone in Leu/Leu and Leu/Phe IPF fibroblasts. This result is important as it demonstrates that endosomal acidification is vital for TLR3 signalling, however autophagy is not as induction of autophagy by high concentrations of Poly(I:C) did not have an impact on TLR3 signalling and downstream pro-inflammatory cytokine production in Leu/Leu IPF fibroblasts.

In contrast to Zhan et al. (203), we did not find a decrease in pro-inflammatory cytokine expression following combined bafilomycin and LPS-induced TLR4 activation in IPF lung fibroblasts suggesting that TLR4 activation is not affected by inhibition of endosomal acidification or autophagy.

Similarly, we have shown that mTOR also plays an important role in cytokine production. mTOR regulates TLR3-induced cytokine production in keratinocytes (306). Rapamycin treatment in combination with Poly(I:C)-induced activation of TLR3 attenuated production of pro-inflammatory cytokines (via NF-kB) and IFN-β (via IRF3) (306). In our work we have shown that mTOR activation is necessary for optimal TLR3 signalling in IPF primary lung fibroblasts but not TLR4 signalling. It has been found that the PI3K/Akt/mTOR pathway is important in the expression of IFN-stimulated gene (ISG) expression (243, 244). We have shown that mTOR inhibition by rapamycin treatment coupled with Poly(I:C)-induced TLR3 activation attenuates IL-8 and RANTES protein expression in Leu/Leu IPF primary human lung fibroblasts. This result demonstrated that inhibition of mTOR affected both the NF-κB and IRF3 arms of the TLR3 signalling pathway in Leu/Leu IPF fibroblasts. Previously, using bafilomycin treatment, we have shown that endosomal acidification was crucial for TLR3-induced cytokine production. Additionally, we have demonstrated autophagy to be defective in both Leu/Leu and Leu/Phe IPF fibroblasts. It is likely that inhibitory effects of rapamycin on TLR3-induced IL-8 and RANTES protein production are due to the effect of rapamycin on the downstream signalling pathway of TLR3 and not on its ability to induce autophagy.
This was not seen in LPS-induced TLR4 activation and rapamycin treatment indicating that mTOR was only important for TLR3 signalling in IPF cells. We have shown that inhibition of mTOR (by rapamycin) and inhibition of autophagy (by bafilomycin and rapamycin) does not affect TLR4 signalling and downstream cytokine production in IPF primary human lung fibroblasts.

In our studies, we have shown that mTOR is constitutively activated in IPF fibroblasts under basal conditions in both Leu/Leu and Leu/Phe genotypes. As previously mentioned mTOR is a potent driver of autophagy. Previously, Romero et al. found that IPF fibroblasts exhibited persistent mTOR activation and decreased levels of mTOR inhibition following starvation compared with normal healthy fibroblasts which correlated with reduced induction of autophagy and increased apoptosis resistance in IPF fibroblasts (27). Here, we have cultured primary lung fibroblasts from IPF lung tissue following lung transplantation. The average of age of diagnosis in IPF patients is 60 years (5) and as our samples have been obtained from patients at the end-stage of their disease, it is likely that the aging process has also had an impact on the persistent mTOR activity we have found in this study.

We suggest that persistent basal mTOR activation may be detrimental in Leu/Leu and Leu/Phe IPF fibroblasts. It has been found that sustained activation of mTOR plays a detrimental role in the murine models of kidney fibrosis (250, 251). Furthermore, inhibition of the mTOR pathway has also been shown to be beneficial in two lung fibrosis murine models (215, 247). Taken together, these results suggest that persistent mTOR activation could play a role in the development of fibrosis in these patients. Inhibitors of the PI3K/mTOR pathway have become potential anti-fibrotic therapies for use in the treatment of IPF. A drug developed by GlaxoSmithKline (GSK2126458), has become the first PI3K/mTOR inhibitor to complete a Phase 1 clinical trial in IPF (222). A good example of why this may be beneficial can be seen in Lymphangioleiomyomatosis (LAM). Hyperactivation of mTOR and low induction of autophagy has been shown to be detrimental in (LAM), a progressive cystic lung disease seen in females (248). Rapalogues (derivatives of rapamycin) targeting mTOR activity have been trialled for the treatment of LAM (249) and are now given as part of LAM treatment to patients. However, we have found that Leu/Leu IPF fibroblasts may be more responsive to inhibition of mTOR by rapamycin than Leu/Phe IPF fibroblasts. We have shown that following
rapamycin treatment, Leu/Leu fibroblasts has a modest reduction in mTOR activity compared with unstimulated Leu/Leu IPF fibroblasts. Rapamycin did not have as noticeable a difference in the inhibition of mTOR activity in the Leu/Phe IPF fibroblasts in comparison to unstimulated Leu/Phe IPF fibroblasts. In addition to this, we found in Leu/Phe IPF fibroblasts that there is attenuated Poly(I:C)-induced IL8 and RANTES protein production. Rapamycin and Poly(I:C) treatment in these cells did not further reduce IL-8 and RANTES expression, suggesting that Leu/Phe IPF fibroblasts may not respond as well to mTOR inhibition as Leu/Leu IPF fibroblasts. It could be argued that use of mTOR inhibitors in the treatment of IPF may be more beneficial to wild-type IPF patients than Leu/Phe IPF patients. In future studies, analysis of downstream signalling protein expression must be done in order to further elucidate the impact of mTOR signalling on Leu/Leu and Leu/Phe IPF patients.

One limitation of this study has been the ability to compare IPF fibroblast responses to normal healthy control fibroblasts. This has been due to limited access to healthy control fibroblasts. Human lung fibroblasts are obtained during a procedure known as a VATS biopsy during a bronchialveolar lavage. Due to the invasive nature of this procedure, healthy people will not undergo this procedure therefore limiting access to normal healthy control fibroblasts. In other studies, some groups have used fibroblasts cultured from lung cancer patients. These, however, can also not be considered “healthy” as they are cultured from fibroblasts taken from around the tumour region, indicating that they will also have phenotypic changes compared with healthy lung fibroblasts. In future studies we would like to use a primary transformed lung fibroblast cell line (CCD-19Lu) as a comparison for our IPF cell lines. We have previously genotyped this cell line and found they are wild-type. This, however, is only one cell line. It is important that we source other primary healthy lung fibroblast cell lines to use in our studies. In this study we have shown that IPF primary human lung fibroblasts from Leu/Leu and Leu/Phe patients do not induce autophagy following stimulation with high concentrations of Poly(I:C), LPS and rapamycin. Under normal healthy conditions, all three stimuli should induce autophagy and in future studies we hope to show this response using healthy control fibroblasts. Similarly, we and others have shown that IPF fibroblasts exhibit persistent mTOR activation basally and following rapamycin treatment. In
future studies, it would be interesting to determine the responsiveness of healthy lung fibroblasts to mTOR inhibition by rapamycin and compare levels mTOR activation basally and stimulation in IPF fibroblasts compared with healthy controls. In assessing the role of mTOR in IPF, it is important that cancer associated “healthy” lung fibroblasts are not used as a control as dysregulation of the mTOR signalling pathway is strongly associated with many human cancers. Primary cell lines such as CCD-19Lus would possibly be a more suitable comparison for assessing mTOR activity.

In conclusion we have provided evidence to suggest that IPF lung fibroblasts have defective autophagy in Leu/Leu and Leu/Phe IPF patients. We have demonstrated through the use of the autophagy inhibitor bafilomycin that Leu/Phe IPF patients have higher levels of LC3-II expression compared with Leu/Leu fibroblasts. In addition, we have shown that mTOR plays a vital role in TLR3 signalling in IPF fibroblasts. Inhibition of mTOR attenuates TLR3-induced pro-inflammatory cytokine and type I interferon responses. Finally, we have shown that Leu/Leu and Leu/Phe IPF fibroblasts have persistent mTOR activation which could play a role in the defective autophagy responses and the development of fibrosis.
Chapter 6

Characterisation of the functionality of the *TLR3* L413F knock-in mouse
Chapter 6 Characterisation of the functionality of the $TLR3$ L413F knock-in mouse

6.1 Introduction

In chapters 4 and 5, we have reported a number of novel ways in which the $TLR3$ L412F SNP manifests its effects in primary human lung fibroblasts from IPF patients. Namely, we have shown for the first time that $TLR3$ L412F also reduces the ability of these fibroblasts to respond to a number of anti-bacterial and anti-viral PAMPs for TLR2, 4, 5, 6 and 9. Subsequently, $TLR3$ L412F variant IPF fibroblasts also had a reduced ability to generate a secondary type I interferon response compared with wild-type fibroblasts, as demonstrated by the dysregulation observed in RIG-I, PKR and MDA-5 expression and reduced RANTES production. $TLR3$ L412F variant IPF fibroblasts also had a reduced ability to respond to the non-TLR agonists Poly(dA:dT), PMA and HT-DNA. Finally, although we observed a reduced ability of $TLR3$ L412F wild-type and variant IPF fibroblasts to undergo autophagy, high levels of the late-phase autophagy protein, LC3-II, accumulation were observed following treatment of Leu/Phe IPF fibroblasts with bafilomycin compared with wild-type cells. A question remains as to what effects these novel observations have in the 412F-variant IPF patient?

In order to model the effect of the human $TLR3$ L412F SNP in vivo, we commissioned the generation of a $TLR3$ L413F knock-in mouse (Taconic Biosciences Inc., Köln, Germany), using CRISPR-Cas-9 gene editing. CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated 9) is a technique developed to target and manipulate specific gene elements by editing the gene sequence (311). Using this technique, gene sequences can be precisely and efficiently altered by deletion, insertion or substitution at targeted gene sites (311). CRISPR-Cas9 has revolutionised gene editing. It has been used in both prokaryotic and eukaryotic organisms, with the hope of being used in the future for treatment of diseases such as cancer (312). Other authors have shown that there is a high degree of homology between human and murine species in the context of the $TLR3$ L412F polymorphism (C1234T) and the corresponding amino acid substitution, Leu412Phe (157). $TLR3$ L412F has been shown by Ranjith-Kumar et al. to be conserved
throughout many difference species including mammals and fish (157). The human Leu412Phe amino acid substitution corresponds to Leu413Phe in mice (TLR3 L413F knock-in mouse).

In this chapter, we describe the characterisation of the functionality of this mouse using *in vitro* analysis of TLR3-activation in a number of primary cell types including: murine lung fibroblasts, splenocytes, intraperitoneal (i.p.) macrophages and bone marrow derived macrophages (BMDMs). Our future aim is to examine the effect of the TLR3 SNP in fibrosis *in vivo* using the TLR3 L413F knock-in mouse. Bleomycin treatment is the gold standard method for inducing fibrosis in the lungs in murine models. Previously our lab showed that TLR3 knockout (TLR3−/−) mice develop increased levels of pulmonary fibrosis *in vivo* following bleomycin challenge compared with wild-type mice (154).

While a lot of research has been conducted into defective signalling and infection in TLR3 knockout mice, to the best of our knowledge there are no other TLR3 knock-in mouse models published. Evidence from other studies suggests that TLR3 L412F has a dual role in viral infection. It has been shown to be protective some viral infections while pathogenic in others. TLR3 L412F has been associated with protection against HIV-1 and HSV-2 infection (176, 177). However, it has been associated with increased risk of other viral infections such as chronic HBV, TBE and human CMV (179, 181, 183, 185). We are specifically interested in examining the relationship between TLR3 L412F and IAV H1N1. Our data has demonstrated that Leu/Phe IPF fibroblasts do not mount an appropriate response to Poly(I:C) or Poly(dA:dT), the synthetic agonists for TLR3 and RIG-I, respectively. TLR3 and RIG-I are important viral sensors for induction of an anti-viral response against IAV H1N1 (289, 313). In future studies, we will examine the role of the TLR3 SNP *in vivo* during live IAV H1N1 infection *in vivo* using our novel TLR3 L413F mouse. In the first published study using TLR3 knockout mice in 2001, these mice were shown to have reduced responses to Poly(I:C) and reduced production of pro-inflammatory cytokines such as IL-6 (142). More recently, other researchers have demonstrated that TLR3−/− TLR7−/− double-knockout mice infected with IAV H1N1 had significantly reduced survival compared with wild-type mice (314).
While the TLR pathway is largely conserved in mice and humans, some distinct differences have been identified. Previously, it was found that TLR3 expression differed in mice and humans (315, 316). Other researchers previously identified that there were differences within the promoter regions, and associated response elements, of mouse and human TLR3 genes. This may explain, in part, the differences in TLR3 expression patterns seen in humans and mice (315). With regards to signalling differences, Sun et al. performed a large screen of many TLR signalling pathway proteins in humans and mice (317). They found that murine and human TLRs relied on different TLR signalling molecules, IL-1 Receptor-Associated Kinases (IRAKs), for MyD88 signal transduction. They suggested that in murine macrophages IRAKs 2 and 4 were required more than IRAK1 in the formation of Myddosome complex and subsequent signal transduction while in human macrophages IRAK1 was the crucial molecule in Myddosome signalling (317).

Therefore, as a result of the reported differences in human and murine TLR3 expression and signalling, in this chapter we characterised the effect of the TLR3 SNP in primary cells derived from TLR3 L413F knock-in mice. Specifically, murine lung fibroblasts, splenocytes, i.p. macrophages and bone marrow derived macrophages (BMDMs), were isolated, cultured and analysed. Characterisation studies performed in murine cells were comparable to those carried out previously by us in Chapters 3, 4 and 5. Specifically, KC [a murine homolog of IL-8; (318)] and RANTES were used as readouts for NF-κB and IRF3 activation, respectively. RANTES and KC were chosen as readouts for the IRF3 and NF-κB pathways. In addition, analysis of MIP2 mRNA expression [another murine homolog of IL-8; (319, 320)] was also performed.
6.2 Primary lung fibroblasts from Leu/Phe and Phe/Phe mice show reduced RANTES and KC protein production compared with Leu/Leu mice following Poly(I:C) treatment

In this study, the main cell type of interest was the pulmonary fibroblast. Here, we isolated and cultured primary murine lung fibroblasts from TLR3 L413F Leu/Leu, heterozygote Leu/Phe and homozygote Phe/Phe mice in vitro. Cells were subsequently treated with increasing doses of Poly(I:C) for 24 h (0.1, 1, 10 μg/ml). Poly(I:C) (1 and 10 μg/ml) significantly upregulated RANTES protein production in the Leu/Leu mice (**p<0.001, Fig. 6.1A), this upregulation is not seen in the Leu/Phe or Phe/Phe mice (Fig 6.1B-C). Following Poly(I:C) treatment, reduced RANTES production was observed in Leu/Phe (Fig. 6.1B) and Phe/Phe (Fig. 6.1C) lung fibroblasts compared with Leu/Leu fibroblasts (Fig. 6.1 A).

Similarly, KC protein expression was induced in Leu/Leu murine lung fibroblasts (Fig. 6.1D). KC protein production was attenuated in Leu/Phe (Fig. 6.1E) and Phe/Phe (Fig. 6.1F) lung fibroblasts compared with Leu/Leu murine lung fibroblasts (Fig. 6.1 D) following Poly(I:C) stimulation for 24 h.

This result was important as for the first time, we proved that the TLR3 L412F SNP had an effect in both human and murine lung fibroblasts.
Figure 6.1. Effect of TLR3 L413F on TLR3-induced RANTES and KC protein production in Leu/Leu, Leu/Phe and Phe/Phe primary murine lung fibroblasts. TLR3 L413F Leu/Leu murine lung fibroblasts induce both RANTES (A) and KC (D) protein expression following 24 h treatment with Poly(I:C). TLR3 L413F attenuates Poly(I:C)-induced RANTES and KC production in primary murine lung fibroblasts from Leu/Phe fibroblasts (B, E) and Phe/Phe fibroblasts (C, F) compared with Leu/Leu fibroblasts (A, D) as quantitated by ELISA. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. ***p<0.001: Poly(I:C) treatments compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments.
6.3 Primary lung fibroblasts from Leu/Phe and Phe/Phe mice show reduced RANTES and MIP2 mRNA expression compared with Leu/Leu mice following Poly(I:C) treatment

A similar trend is seen in RANTES mRNA expression (Fig. 6.2). RANTES mRNA expression is significantly induced in Leu/Leu murine pulmonary fibroblasts following Poly(I:C) stimulation (1 and 10 μg/ml) (**p<0.01, ***p<0.001, Fig. 6.2A). TLR3 L412F attenuates RANTES mRNA expression in Leu/Phe and Phe/Phe murine lung fibroblasts, however, it is still significantly induced in Leu/Phe (***p<0.001, Fig. 6.2B) and Phe/Phe (***p<0.001, Fig. 6.2C) murine fibroblasts following Poly(I:C) stimulation.

However, a different expression pattern is seen in MIP2 mRNA expression. MIP2, a murine homolog of IL-8, mRNA expression is significantly upregulated in Leu/Leu mice following Poly(I:C) stimulation (1 and 10 μg/ml) (***p<0.001, Fig. 6.3A). TLR3 L413F reduces MIP2 mRNA expression in the Leu/Phe (Fig 6.3B) mice but not in Phe/Phe murine lung fibroblasts which express similar levels of MIP2 to Leu/Leu mice following Poly(I:C) stimulation (Fig 6.3C).

6.4 Effect of TLR3 L413F on TLR3 and TLR4 mRNA expression in Leu/Leu, Leu/Phe and Phe/Phe mice following Poly(I:C) treatment

TLR3 mRNA expression is significantly upregulated in Leu/Leu mice following Poly(I:C) stimulation (10 μg/ml) (***p<0.001, Fig. 6.4A). Poly(I:C) significantly induces TLR3 mRNA expression in Leu/Phe murine lung fibroblasts (**p<0.01, Fig 6.4B). However, a modest reduction is observed in Leu/Phe mice compared with Leu/Leu mice (Fig 6.4A). Phe/Phe murine lung fibroblasts significantly upregulate TLR3 expression upon Poly(I:C) (10μg/ml) stimulation and express similar levels of TLR3 (***p<0.001, Fig 6.4 C) when compared with Leu/Leu mice (Fig 6.4A).

Leu/Leu and Leu/Phe murine lung fibroblasts express comparable levels of TLR4 which are not upregulated following 24 h stimulation with Poly(I:C) (Fig 6.4D, E). Phe/Phe murine lung fibroblasts significantly upregulate TLR4 expression
(*p<0.05, Fig. 6.4F). Higher levels of TLR4 expression following Poly(I:C) stimulation are seen in Phe/Phe murine lung fibroblasts compared with both Leu/Leu and Leu/Phe fibroblasts.
Figure 6.2. Effect of TLR3 L413F on Poly(I:C)-induced RANTES mRNA expression in Leu/Leu, Leu/Phe and Phe/Phe primary murine lung fibroblasts. Poly(I:C)-induced RANTES mRNA expression is upregulated in Leu/Leu primary murine lung fibroblasts (A). Leu/Phe (B) and Phe/Phe (C) fibroblasts express lower levels of RANTES mRNA compared with (A) Leu/Leu fibroblasts at 24 h post-treatment, as quantitated by qPCR analysis. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: Poly(I:C) treatment compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments.
Figure 6.3. Effect of TLR3 L413F on TLR3-induced MIP-2 mRNA expression in Leu/Leu, Leu/Phe and Phe/Phe primary murine lung fibroblasts. MIP2 mRNA expression is induced in Leu/Leu (A) murine lung fibroblasts following treatment with Poly(I:C). TLR3 L413F attenuates Poly(I:C)-induced MIP-2 mRNA expression in primary murine lung fibroblasts from (B) Leu/Phe fibroblasts compared with (A) Leu/Leu fibroblasts at 24 h post-treatment, as quantitated by qPCR analysis. The effect of TLR3 L413F is not seen in (C) Phe/Phe fibroblasts compared with (A) Leu/Leu fibroblasts. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: Poly(I:C) treatments compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments.
Figure 6.4. Effect of TLR3 L413F on Poly(I:C)-induced TLR3 and TLR4 mRNA expression in Leu/Leu, Leu/Phe and Phe/Phe primary murine lung fibroblasts. Similar levels of TLR3 and TLR4 mRNA expression are found in primary murine lung fibroblasts from Leu/Leu fibroblasts (A, D) and Leu/Phe fibroblasts (B, E) at 24 h post-treatment, as quantitated by qPCR analysis. Highest levels of TLR3 and TLR4 are seen in Phe/Phe fibroblasts (C, F) at a concentration of 10 μg/ml Poly(I:C). A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, **p<0.01, ***p<0.001: Poly(I:C) treatments compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments.
6.5 Primary lung fibroblasts from Leu/Phe mice but not Phe/Phe mice show reduced RANTES and KC protein expression compared with Leu/Leu mice following LPS treatment

In order to elucidate the effect of LPS treatment on TLR3 L413F mice, we stimulated primary murine lung fibroblasts for 24 h with an LPS gradient (1, 10 and 100 ng/ml) \textit{in vitro}. RANTES protein production is significantly induced in Leu/Leu mice following LPS stimulation (10 and 100 ng/ml) (**p<0.001, Fig. 6.5A). TLR3 L413F attenuates RANTES protein expression in Leu/Phe fibroblasts (Fig. 6.5B) compared with Leu/Leu fibroblasts. However, RANTES protein expression was seen to be significantly upregulated in Leu/Phe and Phe/Phe mice following 24 h stimulation with the highest concentration of LPS (100 ng/ml) (**p<0.001, Fig 6.5B, C). Comparable levels of RANTES protein expression were observed in Phe/Phe mice (Fig 6.5C) compared with Leu/Leu mice (Fig 6.5A).

A similar trend is seen in KC protein expression. KC protein expression is significantly upregulated in Leu/Leu mice following LPS stimulation (10 and 100 ng/ml) (**p<0.001, Fig. 6.5D). TLR3 L413F reduces KC protein production in Leu/Phe mice (Fig 6.5E). An increase in KC protein was observed in Phe/Phe mice (Fig 6.5F) following 24 h stimulation with 100 ng/ml LPS compared with Leu/Leu mice (**p<0.001, Fig 6.5D).
Figure 6.5. Effect of TLR3 L413F on LPS-induced RANTES and KC production in Leu/Leu, Leu/Phe and Phe/Phe primary murine lung fibroblasts. LPS-induced RANTES and KC protein production is seen in Leu/Leu primary murine lung fibroblasts (A, D). TLR3 L413F attenuates RANTES and KC protein expression in Leu/Phe (B, E) fibroblasts compared with Leu/Leu fibroblasts (A, D) at 24 h post-treatment, as quantitated by ELISA. No difference in expression levels of either RANTES or KC is seen in Phe/Phe (C, F) fibroblasts and compared with Leu/Leu (A, D) fibroblasts at 24 h post-treatment. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. **p<0.01, ***p<0.001: LPS treatments compared with medium-only treatment. Results shown are the mean +/- S.E.M of six replicates from a representative of two separate experiments.
6.6 The effect of TLR3 L413F is cell-type specific

To test investigate the functionality of TLR3 L413F further and to ensure that the effect of the SNP was not cell-specific, we isolated primary murine splenocytes and intra-peritoneal (i.p.) macrophages from L413F mice and set-up respective in vitro cultures, which were subsequently treated with Poly(I:C) and LPS. Previously in section 6.2 we have found that primary lung fibroblasts from Leu/Phe and Phe/Phe mice have reduced RANTES protein expression compared with Leu/Leu mice following 24 h Poly(I:C) stimulation (10 μg/ml) (Fig. 6.1). Here, we observed that Leu/Leu murine splenocytes significantly upregulate RANTES protein expression (1 and 10 μg/ml, ***p<0.001, Fig. 6.6A) following 24 h stimulation with Poly(I:C). Primary murine splenocytes from Leu/Phe and Phe/Phe mice also significantly upregulated RANTES expression (1 and 10 μg/ml, ***p<0.001, Fig. 6.6B and C). However, TLR3 L413F attenuated RANTES protein expression in vitro in both Leu/Phe and Phe/Phe murine splenocytes compared with Leu/Leu splenocytes (Fig. 6.6A). This is an important result as we have shown in a second cell type that Poly(I:C)-induced RANTES expression was attenuated in the presence of the SNP.

KC protein expression was not significantly induced in any genotype following Poly(I:C) stimulation in murine splenocytes (Fig. 6.6D-F).

Following LPS stimulation (100 ng/ml) for 24 h, Leu/Leu murine splenocytes induced RANTES expression (Fig. 6.7A). A marginal reduction RANTES protein expression was observed in Phe/Phe splenocytes (Fig. 6.7 C) compared with Leu/Leu splenocytes (Fig. 6.7A). Interestingly, Leu/Phe splenocytes have increased RANTES protein production (Fig. 6.7B) compared with both wild-type and homozygote splenocytes. KC protein expression was significantly induced in Leu/Leu murine splenocytes following LPS stimulation (***p<0.001, Fig. 6.7D). Leu/Phe murine splenocytes expressed comparable levels of KC to Leu/Leu splenocytes (Fig. 6.7E) and Phe/Phe splenocytes had marginally lower levels of KC protein expression (Fig. 6.7F).

Intraperitoneal (I.P.) macrophages were also isolated and cultured in vitro and stimulated with Poly(I:C) and LPS. Leu/Leu i.p. macrophages significantly induced RANTES protein expression following Poly(I:C) treatment (***p<0.001, Fig. 6.8A). Phe/Phe murine i.p. macrophages (Fig. 6.8C) had a modest reduction in
RANTES protein expression following Poly(I:C) treatment compared with wild-type mice (Fig. 6.8A). Interestingly here, we observed increased RANTES protein expression in Leu/Phe i.p. macrophages (Fig. 6.8B) compared with both Leu/Leu and Phe/Phe i.p. macrophages. KC protein levels were comparable in all genotypes following Poly(I:C) stimulation (Fig 6.8D-F).

Following LPS stimulation, Leu/Leu i.p. macrophages upregulated RANTES (**p<0.001, Fig. 6.8A) and KC (*p<0.05, Fig. 6.8D) protein expression compared with media only. We observed higher levels of RANTES and KC protein following LPS stimulation in i.p. macrophages isolated from Leu/Phe (Fig. 6.8B, ***p<0.001 6.8E) and Phe/Phe (**p<0.001, Fig. 6.8C, *p<0.05, 6.8F) mice compared with wild-type mice (Fig. 6.8A, 6.8C). This result indicates an important difference between the human and murine studies.
Figure 6.6.  Effect of TLR3 L413F on Poly(I:C)-induced RANTES and KC production in Leu/Leu, Leu/Phe and Phe/Phe primary murine splenocytes. Poly(I:C) treatment induces RANTES production in Leu/Leu splenocytes (A). TLR3 L413F attenuates Poly(I:C)-induced RANTES production in primary murine Leu/Phe splenocytes (B) and Phe/Phe splenocytes (C) compared with Leu/Leu splenocytes (A) at 24 h post-treatment, as quantitated by ELISA. KC production is not induced in Leu/Leu, Leu/Phe and Phe/Phe splenocytes following 24 h treatment with Poly(I:C) (D-F). A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. ***p<0.001: Poly(I:C) treatments compared with medium-only treatment. Results shown are the mean +/- S.E.M of four to six separate experiments with six replicates.
Figure 6.7. Effect of TLR3 L413F on LPS-induced RANTES and KC protein expression in Leu/Leu, Leu/Phe and Phe/Phe primary murine splenocytes. TLR3 L413F attenuates LPS-induced RANTES production in primary murine Phe/Phe splenocytes (C) compared with Leu/Leu splenocytes (A). Conversely, TLR3 L413F augments RANTES production in Leu/Phe splenocytes (B) compared with Leu/Leu splenocytes (A) at 24 h post-treatment, as quantitated by ELISA. LPS stimulation significantly induces KC protein expression in Leu/Leu splenocytes (D). Leu/Phe (E) fibroblasts express similar levels of KC to Leu/Leu. TLR3 L413F attenuates KC expression in Phe/Phe fibroblasts (F). A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. ***p<0.001: LPS treatments compared with medium-only treatment. Results shown are the mean +/- S.E.M of four separate experiments with six replicates [LPS (TLR4) 100ng/ml].
**Figure 6.8.** Effect of TLR3 L413F on Poly(I:C) and LPS-induced RANTES and KC production in Leu/Leu, Leu/Phe and Phe/Phe primary intra-peritoneal (I.P.) macrophages. RANTES protein expression was induced by both Poly(I:C) and LPS at 24 h post-treatment in Leu/Leu I.P. macrophages (A). Higher levels of RANTES protein are induced by Poly(I:C) in Leu/Phe (B) but not Phe/Phe (C) I.P. macrophages. Conversely, following LPS treatment RANTES protein expression is higher in both Leu/Phe (B) and Phe/Phe (C) I.P. macrophages compared with Leu/Leu (A). KC protein expression is not induced by Poly(I:C) in any genotype (D-F). LPS treatment induces RANTES protein expression in Leu/Leu (D) I.P. macrophages but higher levels of LPS are seen in both Leu/Phe (E) and Phe/Phe (F) I.P. macrophages. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. \(*p<0.05, \,**p<0.01, \,***p<0.001\): Poly(I:C) or LPS treatment compared with medium-only treatment. Results shown for TLR3 L413F wild-type and heterozygote mice are the mean +/- S.E.M of four separate experiments with n=2 replicates per experiment. Result shown for TLR3 L413F homozygote mice is the mean +/- S.E.M of three separate experiments with two replicates per experiment. [Poly(I:C) (TLR3) 10 μg/ml, LPS (TLR4) 100 ng/ml]
6.7 RANTES protein expression from primary bone marrow derived macrophages (BMDM) from Leu/Leu, Leu/Phe and Phe/Phe mice following Poly(I:C) and LPS treatment

Further *in vitro* work was undertaken using bone marrow derived macrophages (BMDMs). BMDMs were cultured *in vitro* and stimulated with Poly(I:C) (0.1, 1 and 10 μg/ml) and LPS (1, 10 and 100 ng/ml) for 24 h. RANTES protein expression was analysed by ELISA. It was observed that RANTES protein expression was significantly upregulated in Leu/Leu mice following Poly(I:C) stimulation (**p<0.001, 1 and 10 μg/ml, Fig. 6.9A). Similarly, Leu/Phe and Phe/Phe cells significantly induced RANTES protein expression (**p<0.001, 1 and 10 μg/ml, Fig. 6.9B, C). RANTES protein expression was similar in all three genotypes following LPS stimulation (Fig. 6.9A-C).

Following LPS stimulation for 24 h, Leu/Leu BMDMs significantly upregulated RANTES protein production (**p<0.001, 10 and 100 ng/ml) (Fig. 6.10A). We found that Phe/Phe BMDMs (Fig. 6.10B) have attenuated RANTES responses to LPS (100 ng/ml) when compared with Leu/Leu BMDMs (Fig. 6.10A). This was in contrast to RANTES expression observed in lung fibroblasts and i.p. macrophages but similar to RANTES expression seen in the splenocytes. Similar levels of RANTES protein were observed in Leu/Phe BMDMs (Fig 6.10B) and wild-type BMDMs.
Figure 6.9. Effect of TLR3 L413F on Poly(I:C)-induced RANTES production in Leu/Leu, Leu/Phe and Phe/Phe primary bone marrow-derived macrophages (BMDMs). Poly(I:C)-induced RANTES production was similar in all three genotypes of primary murine BMDMs (A-C) at 24 h post-treatment, as quantitated by ELISA. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. *p<0.05, ***p<0.001: Poly(I:C) treatment compared with medium-only treatment. Results shown are the mean +/- S.E.M of three to six replicates from four separate experiments.
Figure 6.10. Effect of TLR3 L413F on LPS-induced RANTES production in Leu/Leu, Leu/Phe and Phe/Phe primary bone marrow-derived macrophages (BMDMs). TLR3 L413F attenuates LPS-induced RANTES production in primary murine BMDMs from Phe/Phe BMDMs (C) compared with Leu/Leu BMDMs (A) but not Leu/Phe BMDMs (B) at 24 h post-treatment, as quantitated by ELISA. A Kruskal-Wallis Test with a Dunn’s multiple comparison post-hoc test was used to test for statistical differences. **p<0.01, ***p<0.001: LPS treatment compared with medium-only treatment. Results shown are the mean +/- S.E.M of three to six replicates from four separate experiments.
6.8 Discussion

In this chapter, we characterised the functionality of our novel CRISPR/Cas-9 TLR3 L413F knock-in mice. In our work to date, we have examined TLR3-induced NF-κB- and IRF3-activation, respectively, in TLR3 L412F wild-type and heterozygote IPF primary human lung fibroblasts. We have demonstrated that TLR3-induced activation of NF-κB and IRF3 was reduced in Leu/Phe IPF lung fibroblasts compared with Leu/Leu IPF fibroblasts. In this chapter, we examine the effects of the TLR3 SNP on TLR3- and TLR4-activation in a number of primary cell types from TLR3 L413F knock-in mice. As pulmonary fibroblasts play a major role in the pathogenesis of IPF, our work in both humans and mice has focused primarily on primary lung fibroblasts.

Characterisation experiments in mice revealed that TLR3 L413F attenuates RANTES and KC protein production (a murine IL-8 homologue) following Poly(I:C) treatment in both Leu/Phe and Phe/Phe lung fibroblasts. This result is in keeping with the effects of the TLR3 SNP which we observed in primary human lung fibroblasts. Furthermore, we determined that the TLR3 SNP attenuated TLR3-induced RANTES protein production from splenocytes from Leu/Phe and Phe/Phe mice, which is comparable to the results seen in both murine and human lung fibroblasts. However, the effects of the TLR3 SNP on LPS-treated murine lung fibroblasts were not comparable to the effects of the SNP in Poly(I:C)-treated human lung fibroblasts. Furthermore, the effects of the TLR3 SNP in other murine cells following either Poly(I:C) or LPS treatment, respectively, was different in murine i.p. macrophages and BMDMs compared with the effects of the SNP in murine lung fibroblasts. Specifically, in i.p. macrophages, TLR3 L413F attenuated RANTES protein production in Phe/Phe mice but surprisingly it was higher in Leu/Phe mice compared with wild-type mice. In addition, in BMDMs the presence of TLR3 L413F had no effect on RANTES production following Poly(I:C) treatment in either Leu/Phe or Phe/Phe cells.

Therefore, in this chapter, we obtained results which suggest that TLR3 L412F appears to act in a cell-specific and/or species-dependent manner in humans and mice. Interestingly, the effects of the SNP on TLR3-activation are comparable in murine and human lung fibroblasts, which are our specific cells of interest in this
study. The results in this chapter which describe the divergent effects of the TLR3 SNP on specific cell types, underline the need to examine the activity and function of the SNP in an *in vivo* setting. In this way, we will obtain a definitive idea of how *TLR3* L413F functions at an organismal level. This work is the primary focus of our future studies. We are currently breeding *TLR3* L413F mice in order to investigate the effect of the SNP in mice in response to a systemic administration of Poly(I:C) and LPS, respectively. We will also shortly investigate the effect of the SNP in *TLR3* L413F knock-in mice following live infection with IAV H1N1.

We have shown that there is reduced NF-κB and IRF3 activation following Poly(I:C) stimulation in murine lung fibroblasts and splenocytes from Leu/Phe and Phe/Phe mice compared with Leu/Leu. There are potentially a number of reasons why this is the case. In regards to activation of the receptor, as previously mentioned, *TLR3* L412F is a leucine (Leu) to phenylalanine (Phe) substitution in the extracellular domain of the TLR3 monomer. Previously, it was suggested that as phenylalanine is a large amino acid with a benzene ring, this substitution may affect ligand binding and glycosylation of nearby residues (157). Recently, there is more evidence to suggest that the mutation has similar ligand binding ability to wild-type TLR3 (321), thus defective responses through TLR3 may be through a different mechanism. This suggests that Leu/Leu, Leu/Phe and Phe/Phe mice would have similar ability to bind dsRNA. The presence of the SNP could also affect protein expression through interference of correct protein folding and exportation from the Golgi. While a number of groups have analysed wild-type and variant TLR3 expression in human cell lines (157), there is no information available as to whether the TLR3 protein undergoes correct protein folding and transport to the cell membrane and endosomal membrane to date. Incorrect folding or reduced exportation of the TLR3 receptor to the cell and endosomal membranes could also lead to the defective TLR3 responses seen in primary murine lung fibroblasts and splenocytes seen in Leu/Phe and Phe/Phe mice.

We have shown many cell-type specific differences in our murine characterisation following Poly(I:C) stimulation. In our studies we have shown that murine lung fibroblasts and splenocytes respond similarly following Poly(I:C) treatment in the presence of the SNP, however, two populations of macrophages had different responses following Poly(I:C) stimulation (interperitoneal and bone marrow
derived macrophages). This may in part due to the expression of TLR3 in different cell types. TLR3 is highly expressed in the brain and lungs (142). However, in other cells such as macrophages, expression may be less compared with lung fibroblasts resulting in different responses following Poly(I:C)-induced activation of TLR3. In order to investigate this further, we will examine the expression of TLR3 in each by Western blot.

Secondly, cell-type specific differences may be due to reliance on and activation of other intracellular receptors such as MDA5. MDA5 is activated by intracellular Poly(I:C). In cells such as I.P. macrophages and BMDMs we do not see differences in RANTES protein production following Poly(I:C) treatment. As these cells are endocytic cells it may be due to additional activation of MDA5 as well as TLR3 therefore giving different responses to those seen in human lung fibroblasts and splenocytes. In order to further elucidate differences in TLR3 activation in the presence and absence of the SNP in each cell type, it would be beneficial to activate the TLR3 receptor in a more TLR3-specific manner. As Poly(I:C) activates TLR3 and MDA5 it would be important to use, for example, TLR3 specific activating antibody to further confirm the role of the SNP in TLR3 activation in each cell type.

Additionally, cell-type specific differences may be due to differences in TLR signalling pathways in mice and humans. Previously, it was shown that the murine and human TLR3 genes contain different response elements within their promotor regions, which would lead to differential expression of TLR3 in different cell-types and species (315). It has also been shown that TLR3 signalling relies on different IRAKs in mice and humans (317). Finally, it may also be due to the fact that our work to date has focused on human cells in a diseased state (IPF), whereas in the mouse they are normal healthy lung fibroblasts.

To date, there is limited information on the role of TLR3 L412F in immune cells such as macrophages or monocytes. Research groups have used immortalised cell lines, HEK293s and Cos 7 cells, for in vitro analysis (157, 167). In this thesis, we have focused on the role of TLR3 L412F in fibrosis and we have predominantly focused on primary human lung fibroblasts from IPF patients and TLR3 L413F knock-in mice. We have focused on the fibroblast response to elucidate the protective role of TLR3 and its induction of type I IFNs in IPF. It would be interesting in future studies to further examine the role of inflammation in IPF by
examining primary human alveolar macrophage responses to TLR stimulation such as Poly(I:C) and LPS. A significant limitation to using alveolar macrophages is obtaining primary alveolar macrophages from IPF patients as the number of new IPF patients per month is low. Approximately one new IPF patient per month will undergo a BAL procedure. If the patient is exhibiting signs of an acute exacerbation a BAL procedure will not be performed. IPF patients will on average only undergo a BAL at diagnosis and not thereafter. Secondly, alveolar macrophages need to be collected from IPF BAL fluids, used immediately and are more difficult to grow in vitro. For this reason, the TLR3 L413F knock-in mouse may be useful in elucidating the role of alveolar macrophages in the chronic inflammatory response seen during fibrosis.

In our human fibroblast studies, we found that the TLR3 L412F reduced the responses of primary lung fibroblasts to LPS. Furthermore, lung fibroblasts from Leu/Phe IPF patients had also defective responses to TLR2, 5, 6 and 9-agonists (see Chapter 4). In our murine studies, we have found that responses to LPS-treatment in TLR3 L413F lung fibroblasts differed in specific cell types. In murine lung fibroblasts, LPS- induced KC and RANTES protein production was attenuated in the presence of the SNP in Leu/Phe mice, but not in Phe/Phe fibroblasts, compared with wild-type fibroblasts. In contrast, results following Poly(I:C) treatment in the presence of the TLR3 SNP was comparable in human and murine fibroblasts. In murine splenocytes, LPS-induced KC and RANTES production was significantly less in Phe/Phe cells compared with wild-type cells. Surprisingly, the highest level of LPS-induced RANTES production was observed in Leu/Phe splenocytes, with KC levels being comparable to those from wild-type fibroblasts. Experiments using i.p. macrophages showed that levels of LPS-induced KC and RANTES production were unexpectedly increased in Leu/Phe and Phe/Phe cells compared with those from wild-type mice. In BMDMs, levels of LPS-induced KC and RANTES production was less in Phe/Phe cells but not Leu/Phe cells. These results demonstrate the differences in effects of TLR3 L412F in two separate primary macrophage cell lines.

In chapter 4, we examined the effect of the TLR3 L412F on TLR3 mRNA and protein expression in primary human lung fibroblasts. We found that Leu/Phe fibroblasts from IPF patients had a decreased ability to upregulate TLR3 mRNA and protein expression following Poly(I:C), LPS or CpG treatment compared with wild-
type IPF patients. In this chapter, we investigated the effect of the TLR3 SNP on TLR3 mRNA expression in murine lung fibroblasts from the TLR3 L413F knock-in mice following Poly(I:C) treatment. Previously, it has been demonstrated that there is a close relationship between TLR3 and TLR4 in mice and that these receptors can act synergistically in anti-viral and anti-bacterial immunity. It has been demonstrated that TLR4-MyD88 signalling in murine alveolar macrophages can induce TLR3 expression (322). Similarly, i.p. administration of LPS has been shown to upregulate TLR3 expression in the murine lungs (142). In this chapter we demonstrated that Poly(I:C) induced TLR3-transcription was reduced in Leu/Phe murine fibroblasts compared with cells from wild-type mouse. This result is comparable to that seen in our human lung fibroblasts. However, levels of TLR3-transcription observed in Phe/Phe murine fibroblasts were comparable to those seen in wild-type cells. Previously, we demonstrated that Poly(I:C)-induced TLR3 mRNA expression was markedly reduced in lung fibroblasts from Phe/Phe patients with pulmonary sarcoidosis (168). Here, we hypothesise that the differences in regulation of TLR3 transcription in murine versus human lung fibroblasts may be due to differences in TLR expression in mice and humans. In addition, this difference could also be due to the fact that our murine cells were harvested from healthy animals. In contrast, our human cells were harvested from diseased patients.

In order to confirm the functionality of the TLR3 SNP definitively in vivo, we will use our novel TLR3 L413F knock-in mouse in a number of animal experiments. Firstly, we will investigate the effects of the SNP: (1) during systemic administration of Poly(I:C) and LPS, respectively, (2) using the bleomycin model of lung fibrosis and (3) using live viral infection with IAV H1N1. Mice are currently being bred for these experiments which will commence shortly. Furthermore, in order to characterise fully the effects of TLR3 L413F in vivo, we will also include TLR3 knockout mice in these experiments.

Previously, we used TLR3 knockout mice (TLR3\(^{-/-}\) mice) in the bleomycin model of lung fibrosis (154). We demonstrated that TLR3\(^{-/-}\) mice experienced increased pulmonary fibrosis and reduced survival compared with TLR3 wild-type mice. This effect was accompanied by increased levels of IL-4, IL-13, TGF-\(\beta\) and hydroxyproline in the lungs after bleomycin administration compared with wild-type mice. In future work, using the bleomycin model, we hypothesise that we may
observe a similar detrimental effect in TLR3 L413F knock-in mice due to the fact that human and murine lung fibroblasts which are heterozygous for the TLR3 SNP exhibit a similar phenotype to TLR3 knockout cells.

Previously, our collaborators also examined the role of TLR3 production in a Schistosoma mansoni egg-induced pulmonary granuloma model (155). In this study, these authors showed that TLR3 was protective, as lungs from TLR3+/− mice had larger granulomas and greater collagen deposition compared with wild-type mice. This study highlighted a role for TLR3 in protection against Th2-driven granulomas and pro-fibrotic Th2 responses (155). Similarly, other authors have shown that in skin and lung fibroblasts that Poly(I:C)-induced TLR3 activation and subsequent type I interferon expression causes a downregulation of pro-fibrotic genes, including α-smooth muscle actin and collagen (323). In addition, this group also demonstrated a role for RIG-I and MDA5-induced type I IFN expression in protection against fibrosis (323).

In this chapter, we demonstrated in vitro that murine lung fibroblasts from Leu/Phe and Phe/Phe mice have attenuated Poly(I:C)-induced NF-κB and IRF3 responses compared with wild-type mice. This finding is also comparable to results observed by us in human lung fibroblasts from IPF and pulmonary sarcoidosis patients. In the context of patients’ responses to viral infection, we hypothesise that the presence of the TLR3 SNP may be beneficial during IAV H1N1 infection. In support of this theory, Le Goffic et al. previously demonstrated that TLR3−/− mice have an advantage during influenza A viral infection (IAV) compared with wild-type mice. They demonstrated that the reduced cytokine and RANTES expression, observed in TLR3−/− mice, correlated with reduced T cell infiltration and longer survival in TLR3−/− mice (324). In addition, other researchers have shown that TLR3−/− mice have reduced sickness behaviour following IAV challenge compared with wild-type mice (325).

In conclusion, in this chapter we have obtained results which suggest that TLR3 L412F in general appears to act in a cell-specific and/or species-dependent manner in humans and mice. Interestingly, the effects of the SNP on TLR3-activation are comparable in murine and human lung fibroblasts, which is important given their role in pulmonary fibrosis. In addition, these are our specific cells of interest in this study. The results in this chapter also underline the need to examine
the activity and function of the SNP in an in vivo setting. The availability of our novel TLR3 L413F knock-in mice provides a useful model in which to do this.
Chapter 7

Discussion and Future Work
In this Ph.D. thesis, we describe novel mechanisms of action of the TLR3 L412F SNP in driving disease progression in IPF patients. Previously, the Donnelly research group demonstrated that TLR3 L412F increased mortality risk and accelerated the decline in lung function (FVC) in IPF patients (154). These clinical effects were associated with an inability on 412F-heterozygote (Leu/Phe) and – homozygote (Phe/Phe) primary lung fibroblasts from IPF patients to mount appropriate pro-inflammatory and anti-viral responses compared with wild-type patients (Leu/Leu) following treatment with the synthetic TLR3-agonist, Poly(I:C). In this thesis, we demonstrated for the first time that the TLR3 L412F SNP has additional effects on TLR2, 4, 5, 6 and 9 responses of lung fibroblasts from Leu/Phe IPF patients. In addition, we have also shown that TLR3 L412F can also impact upon non-TLR responses (Poly(dA:dT), HT-DNA and PMA) in lung fibroblasts from Leu/Phe IPF patients. We have also determined that both wild-type and Leu/Phe lung fibroblasts from IPF patients have defective autophagy responses. Furthermore, the levels of dysregulation in autophagy would appear to be greater in Leu/Phe compared with wild-type lung fibroblasts from IPF patients. In future studies, we will investigate the impact of these TLR3 L412F-mediated effects in vivo using our novel TLR3 L413F knock-in mice. In this thesis, we have confirmed that primary murine lung fibroblasts from these knock-in mice have comparable responses to 412F-heterozygote primary lung fibroblasts from IPF patients.

In this thesis, we analysed the responses in IPF primary lung fibroblasts from IPF patients to a panel of TLR agonists (TLR1-9) in the presence of the TLR3 L412F SNP. TLR3 is activated by dsRNA which initiates a downstream signalling cascade via the TRIF adaptor molecule, activating transcription factors NF-κB and IRF3 and leading to the production of pro-inflammatory cytokines and type I interferon, IFN-β (142). In this thesis, we demonstrated in Leu/Phe IPF fibroblasts that there is attenuated TLR3 activation and subsequently blunted expression of pro-inflammatory cytokines, IL-8 and IL-6, and type I interferons. This also correlated with reduced TLR3 mRNA expression and dysregulated TLR3 protein expression in Leu/Phe IPF fibroblasts. In our work, we have found that the SNP affects not only TLR3 signalling but signalling through TLRs 2, 4, 5, 6 and 9. This was a surprising
result. The TLR3 L412F SNP is a single base pair (C>T) change which leads to an amino acid change, leucine to phenylalanine (157) and is located in the extracellular domain of the TLR3 receptor.

Unexpectedly, in this thesis we found that TLR3 L412F affected not only TLR3 signalling but also caused dysregulation of a number of other signalling pathways, both TLR-dependent and –independent. This resulted in reduced NF-κB- and IRF3-activation in Leu/Phe IPF patients irrespective of whether fibroblasts were stimulated with TLR-dependent or –independent agonists. Previously, it had been shown in immortalised cell lines that TLR3 L412F reduced NF-κB and IRF3 activation in luciferase assays (157, 167). We have shown in primary fibroblasts from a related ILD, pulmonary sarcoidosis, that TLR3 L412F results in attenuated NF-κB and IRF3 activation and blunted pro-inflammatory cytokine and type I interferon expression (168). Here, we have shown that the dysregulation seen in Leu/Phe IPF fibroblasts is at the TLR level and downstream in the secondary interferon response. Additionally, using non-TLR agonists, including Poly(dA:dT), HT-DNA and PMA, we have demonstrated that NF-κB and IRF3 activation is affected independently of TLR activation in Leu/Phe IPF fibroblasts. Non-TLR receptors such as RIG-I and STING initiate the same downstream signalling cascades as TLRs to activate NF-κB and IRF3, we found that these pathways were affected in the presence of the SNP upon stimulation with their respective ligands, Poly(dA:dT) and HT-DNA. Interestingly, we also used PMA for this study, an NF-κB activator that signals through a distinct signalling pathway to TLRs. PMA activates PKC which subsequently leads to the activation of transcription factor NF-κB (326, 327). Following PMA stimulation of Leu/Phe IPF fibroblasts we also found dysregulation of NF-κB activation. This indicates that dysregulation of NF-κB occurs independently of the TLR and RLR signalling pathway in Leu/Phe IPF fibroblasts. If NF-κB mRNA and protein expression are dysregulated in Leu/Phe IPF fibroblast, this may result in blunted NF-κB activation by all pathways irrespective of the TLRs.

In order to identify where the TLR3 signalling pathway becomes dysregulated, we would like to examine this pathway in Leu/Leu and Leu/Phe IPF fibroblasts using Western blot and RNA sequencing (RNA-Seq). We have shown that there is dysregulation of TLR3 expression in the presence of the SNP. In
Leu/Phe IPF fibroblast we have shown that there is reduced expression of the TLR3 receptor basally and following stimulation with Poly(I:C). However, to date, it is not known whether other proteins involved in the TLR3 pathway are affected by the presence of the SNP. Recently, it has been discovered that ligand binding is not majorly affected in the presence of the SNP (321). Because of this, it is important to examine the downstream signalling proteins. TLRs and RLRs share many downstream signalling proteins resulting in the activation of NF-κB and IRF3 and subsequent upregulation in expression of proteins associated with the TLR pathway (115). We will determine whether basally protein expression of NF-κB and IRF3 differs between Leu/Leu and Leu/Phe IPF fibroblasts and following TLR3 activation. We would also like to examine upstream proteins such as the TLR3 adaptor protein, TRIF, TRAF6 and the IKK complex basally and following TLR3 activation in order to determine whether protein expression and upregulation is affected in the presence of the SNP.

In addition to this, we will examine the TLR3 pathway at the RNA level by RNA-seq. While RNA-Seq is expensive, this would be highly beneficial as it facilitates the examination of many different genes in the TLR signalling pathway simultaneously. Using RNA-Seq we would be able to compare gene expression profiles in IPF fibroblasts in the presence and absence of TLR3 L412F both basally (untreated cells) and following TLR3 activation.

Furthermore, we have found differences between Leu/Leu and Leu/Phe IPF fibroblasts basally. We have shown that Leu/Phe IPF fibroblasts express high levels of pro-inflammatory cytokines and RANTES basally, resulting in attenuated upregulation following TLR activation. A contributing factor to the high IL-6, IL-8 and RANTES basal expression seen may be the aging process. High basal levels of pro-inflammatory cytokines such as IL-6 and TNF-α have been associated with chronic inflammation and dysregulation of the immune response seen in aged individuals (328, 329). In these studies we have analysed basal cytokine expression levels in IPF primary human lung fibroblasts from explanted lung tissue. IPF patients are on average more than 60 years old at diagnosis (9). Therefore the high basal cytokine levels of seen in these patients may be due to aging as well as disease progression. It is worth noting, that it is the Leu/Phe IPF patients who display significantly higher levels of IL-8, IL-6 and RANTES compared with Leu/Leu wild-
type patients. This suggests that the presence of the SNP in the Leu/Phe fibroblasts may be associated with an accelerated aging phenotype compared with Leu/Leu IPF patients. In support of this, the Donnelly lab have previously shown that IPF patients who were Leu/Phe and Phe/Phe were 5 times more likely to die at 12 and 24 months post-diagnosis compared with Leu/Leu wild-type patients which was further associated with faster lung decline (154). This accelerated disease progression in combination with the aging process may result in the higher cytokine/chemokine expression seen in Leu/Phe IPF patients.

The presence of the SNP has also been shown in other diseases to be associated with higher basal levels of pro-inflammatory cytokines. The Donnelly lab has recently shown that Leu/Phe cystic fibrosis patients have higher basal IL-6, IL-8 and IL-1β levels in serum compared with wild-type patients (unpublished data). Similarly, Sironi et al. have shown higher IL-6 levels in HIV-infected patients in the presence of the SNP compared with wild-type patients (176). This suggests that dysregulation of the TLR signalling pathway caused by the presence of the SNP is also a contributing factor to high basal cytokine levels.

In addition, we have found dysregulation in the secondary interferon response in Leu/Phe IPF fibroblasts at both the mRNA and protein level. We propose that high level basal levels of RANTES, indicative of high basal type I IFN expression, causes dysregulation of the secondary interferon response. High basal type I IFN expression induces dysregulation of the secondary interferon response resulting in high basal expression of secondary intracellular sensors, we have found dysregulated basal expression of IFN-induced intracellular secondary sensors, RIG-I, MDA5 and PKR, in Leu/Phe IPF patients. In a number of patients RIG-I, MDA5 and PKR levels are higher basally in Leu/Phe IPF fibroblasts compared with Leu/Leu IPF fibroblasts. However, they are unable to upregulate their expression upon TLR activation. This highlights a potential detrimental role for TLR3 L412F during viral infection in Leu/Phe IPF fibroblasts.

Lack of competent TLR signalling in IPF fibroblasts led us to question whether Leu/Phe IPF patients may be more susceptible to certain viral infections while possibly being protected against others, such as IAV H1N1. Viral infection has been strongly associated with IPF pathogenesis. In murine models of fibrosis it has
been shown that viruses such as gammaherpes viruses can exacerbate fibrosis (78). Viruses have been proposed by some researchers as a “second-hit” in the development of fibrosis due to persistent antigenic stimulation leading to further disease progression (68, 69). Here, we have shown that in the presence of the SNP many viral sensors including TLR3, TLR9, RIG-I, MDA5, PKR and STING are affected in Leu/Phe IPF fibroblasts. We have shown that there is reduced NF-κB and IRF3 activation and subsequent pro-inflammatory cytokine and type I interferon expression using synthetic agonists for TLR3, RIG-I and STING in Leu/Phe IPF fibroblasts demonstrating a blunted anti-viral response. Interestingly, we have also shown that basal expression of TLR3, RIG-I, MDA5 and PKR is dysregulated in Leu/Phe IPF fibroblasts with many Leu/Phe patients unable to upregulate their expression following Poly(I:C) stimulation. Taken together all of these results suggest a blunted TLR3-induced anti-viral response in Leu/Phe IPF fibroblasts.

However, we have found in a related-ILD, pulmonary sarcoidosis, patient cohort that Phe/Phe fibroblasts had lower viral load when infected in vitro with IAV H1N1 compared with Leu/Leu patients. This result was coupled with attenuated pro-inflammatory cytokine production and type I interferons in the presence of the SNP compared with wild-type patients, similar to what we have seen in IPF fibroblasts. This led us to question which cellular mechanism was involved in protecting Phe/Phe patients from a higher H1N1 viral load. We hypothesized that autophagy may play a role in this as autophagy is an important cellular mechanism for the clearance of intracellular viruses and bacteria (199). In our work, we have shown that the autophagy pathway is dysregulated in IPF fibroblasts. Basally, we have shown that Leu/Phe fibroblasts have high levels of LC3-II expression when bafilomycin treatment is used to accumulate the protein. This could indicate higher levels of autophagy, however, following Poly(I:C) activation, we found we could not induce greater levels of autophagy in IPF fibroblasts suggesting dysregulation of the autophagy process. This may in part be due to persistent mTOR activation we have found in IPF lung fibroblasts. In support of this, persistent mTOR activation has been shown to be associated with lower levels of autophagy and fibrosis in IPF fibroblasts (27).

Importantly, this dysregulation may be beneficial in the control of certain viruses including IAV H1N1. IAV H1N1 can manipulate the autophagy pathway for
replication and survival. IAV H1N1 can induce autophagy through inhibition of the Akt/mTOR pathway (235). IAV induces the formation of autophagosomes and it has been shown that the M2 protein can block autophagosome fusion with the lysosome to prevent degradation (236). Defective autophagy has been shown to result in an inability of IAV H1N1 to replicate, leading to a reduction in viral load in the host cell (92, 236). Unfortunately, in the time frame of this PhD, we were unable to live virally infect IPF fibroblasts in vitro with IAV H1N1. We are currently organising a collaboration to perform this experiment in the coming months. Following infection of these cells, we will determine whether dysregulation of the autophagy pathway in IPF fibroblasts is protective in H1N1 infection by examining viral protein load by qPCR and autophagy protein expression by western blot. Furthermore, we have set up a collaboration with Prof. Xiao Su in the Institut Pasteur of Shanghai to virally infect TLR3 L413F knock-in mice with IAV H1N1 in order to further determine whether the presence of the SNP is beneficial in the control of IAV H1N1 in mice.

We have shown that anti-viral TLR and non-TLR responses to synthetic ligands are attenuated in Leu/Phe IPF patient. As viruses have been strongly associated with the development of fibrosis, in future studies we would like to analyse IPF patients ex vivo for viral signatures in the presence and absence of the SNP. Many different methods have been used previously to detect viral infection in IPF patients using serum and lung tissue samples. These include assessing levels of virus-specific antibodies by ELISA and western blot in serum, measuring viral DNA titres and viral antigen levels in lung tissue or serum samples by real-time PCR and visualising the localisation of viruses in lung tissue by immunohistochemistry (73, 81, 82).

In our work we have found that Leu/Phe IPF fibroblasts are defective in both anti-bacterial and anti-viral responses to infection through the TLR receptors. Control of bacterial infection in the cell relies upon signalling through TLRs 1, 2, 4, 5, 6 and 9 (99). Different TLRs are used to elicit responses to Gram-negative and Gram-positive bacterial infection. Gram-positive bacteria are recognised by TLR2 which dimerises with either TLR1 or 6. Gram-negative bacteria are recognised by TLR4 (275). TLR5 recognises bacterial flagellin (276). We have shown using synthetic agonists in vitro that bacterial TLRs 2, 4, 5 and 6 have defective signalling following stimulation in Leu/Phe IPF fibroblasts. In future studies, we would like to
further examine the role of the SNP in vitro in IPF fibroblasts during live Gram-negative bacterial infection, using *Haemophilus influenzae* (*H. influenzae*) and during live Gram-positive infection, using *Streptococcus pneumoniae* (*S. pneumoniae*). The role of bacterial infection in IPF is widely being studied now. Previously, it was thought that viral infection played a role in disease progression in IPF and to a lesser extent bacterial infection. Work undertaken by the Maher lab in Imperial College London has shed light on the role of bacterial infection in IPF (288). They have shown that IPF patients carry a higher bacterial load than both healthy individuals and patients suffering from COPD. High bacterial load was correlated with increased risk of mortality in IPF patients. They have also provided evidence to suggest that the lung microbiome is colonised with distinct population of bacteria compared with healthy individuals (288). It is important to us to expand on this finding in the context of the TLR3 L412F SNP. It is our hope that in future we will be able to use BAL fluids from Leu/Leu, Leu/Phe and Phe/Phe IPF patients to analyse the lung microbiome for bacterial and viral signatures. From our work to date in IPF fibroblasts, it could be argued that TLR3 L412F could be beneficial in stratifying IPF patients into those with consistent higher bacterial loads or distinct populations of bacteria in their lung microbiome. For example, *H. influenzae* sp. has been shown to be present at significantly higher levels in the IPF lung compared with healthy lungs and TLR4 responses have been proven to be important in the clearance of this bacteria (288, 330). In our studies in IPF lung fibroblasts, we have shown a consistent defective TLR4 response in Leu/Phe patients to LPS. It could be suggested that TLR3 L412F heterozygote IPF patients could be more susceptible to infections like *H. influenzae* sp. than wild-type IPF patients. In order to determine whether the presence of the SNP impacts on responses to live *H. influenzae* sp. infection, in future studies, we would like to examine bacterial responses in vitro in IPF lung fibroblasts and in our TLR3 L413F knock-in mice.

In future studies, we would like to examine the role of the SNP during live infection of both Gram-negative and Gram-positive bacteria in vitro in IPF primary human fibroblasts and in vivo using our TLR3 L413F knock-in mouse. As previously mentioned, we would like to analyse responses to the Gram-negative bacteria, *H. influenzae*. In regards to Gram-positive bacteria, we would like to examine *S. pneumoniae*. *S. pneumoniae* sp. have been associated with disease progression in
IPF. Molyneaux and colleagues have showed that *S. pneumoniae* is one of the major bacterial species present in the lungs of IPF patients and found it was significantly associated with poorer outcomes (88, 89, 288). TLRs 2, 4 and 9 are crucial for defence against pneumococcal infection (331-333). Here, we have shown attenuated activation of TLRs 2, 4 and 9 in Leu/Phe IPF fibroblasts, suggesting that these patients may be more susceptible to gram-positive bacteria such as *S. pneumoniae*. In order to confirm this, we would like to examine responses to *S. pneumoniae, in vitro* in IPF lung fibroblasts and in our *TLR3* L413F knock-in mice.

In addition, there is increasing evidence of bacterial infection and changes in the respiratory microbiome during acute exacerbations in IPF (AE-IPF) (91). It would be interesting to genotype and analyse bacterial burden in IPF patients both during the normal course of IPF and during an AE-IPF in order to elucidate whether the presence of *TLR3* L412F influences bacterial burden or species present. One limitation to this experiment is access to BAL fluids of IPF patients during AE-IPF. While routinely BALs are performed on IPF patients at diagnosis. The procedure is invasive and may have the potential to further exacerbate patient’s condition. Because of this patients won’t be sent for BALs during an AE-IPF and therefore we may only be able to analyse patients by less invasive methods such as sputum analysis or nasoopharangeal swabs.

Defective TLR signalling may be beneficial in certain bacterial infections. Heterozygosity for the *TLR3* L412F SNP has been kept at high genotype frequency globally, and in particular in the European population (45.7%) (165). We suggest that the SNP has been highly conserved within the European population as it is beneficial during certain infections. In a number of infections, excessive inflammation following TLR activation has been shown to be detrimental. Defects in TLR signalling have been shown to be beneficial in reducing the inflammation induced by infection. For example, defective TLR1 signalling has been shown to be protective in leprosy. A SNP in TLR1 has been shown to attenuate TLR1 signalling. In addition, a SNP in the TLR2 and 4 adaptor protein, Mal, has been shown to be protective during infections such as malaria, TB and sepsis (296). TIRAP S180L blunts TLR 2 and 4-induced NF-κB activation and pro-inflammatory cytokine expression (296). Due to the excessive inflammatory response seen following TLR activation in these infections, defective TLR2 and 4 signalling was thought to be
protective in dampening the excessive inflammatory response. We have shown that Leu/Phe IPF fibroblasts have blunted pro-inflammatory cytokine production following activation of TLRs 2, 3, 4, 5, 6 and 9. Therefore it is possible that the presence of the TLR3 L412F may confer a protective benefit during certain bacterial infections and sepsis. Because of this, it is important that we examine both Gram-negative and Gram-positive bacterial infections in IPF fibroblasts in vitro, in BAL fluids from IPF patients and in vivo in TLR3 L413F mice.

It has not previously been shown whether defective TLR3 signalling could be protective during certain viral infections, such as influenza A virus. Secondary bacterial infections following IAV infection are common worldwide and greatly enhance patient’s risk of mortality. Shahanigan et al. have demonstrated that induction of type I IFNs following viral infection leaves the host more susceptible to secondary bacterial infection with Streptococcus pneumoniae (334). They performed in an in vivo model of infection in which mice were infected with influenza followed by S. pneumoniae or S. pneumoniae alone. It was found that viral infection-induced type I IFN expression inhibited the expression of essential chemokines such as MIP-2 during bacterial infection leading to worsened outcome for the doubly infected mice (334). This demonstrated that the immune response to the viral infection hindered the ability of the host to clear the subsequent bacterial infection resulting in a greater mortality. It could be suggested that in Leu/Phe and Phe/Phe IPF patients, defective TLR3/IRF3 activation and reduced type I interferon production following IAV infection may be protective during secondary bacterial infections in IPF patients. While the immune response to viral infection in these patients may be reduced, it could result in enhanced bacterial clearance due to the absence of type I IFNs. In order to investigate this further, the TLR3 L413F mouse could be employed to investigate the effects of influenza A viral infection and subsequent S. pneumoniae infection in vivo.

Furthermore, we have shown that the autophagy response is defective in both Leu/Leu and Leu/Phe IPF lung fibroblasts. We have found that Leu/Phe IPF fibroblasts in particular have greater accumulation of LC3-II protein than Leu/Leu IPF fibroblasts, indicative of greater levels of dysregulation. So far we have examined the role of autophagy in IPF fibroblasts in the context of TLR3 and TLR4 agonists, and viral infection. However, autophagy is responsible not only for the
clearance of intracellular viruses but also bacteria (335). In our work in IPF fibroblasts, we have shown that autophagy is not induced by a number of different stimuli including LPS. As previously mentioned, it has been shown that IPF patients have reduced autophagy in the lung (215, 216). It is possible that lower levels of autophagy could contribute to higher bacterial loads seen in IPF patients. In future work, we would like to examine autophagy protein expression in vitro following live Gram-negative, such as *H. influenzae*, and Gram-positive bacterial infection, such as *S. pneumoniae*, in IPF fibroblasts. Following on from this, we would examine in vivo in our TLR3 L413F knock-in mouse autophagy induction following Gram-negative and Gram-positive bacterial infection using whole lung homogenates. The EME-TIPAC clinical trial is currently ongoing for the use of the prophylactic broad spectrum anti-biotic, co-trimoxazole, in combination with regular treatment, in IPF (44, 45). We believe here we have added to the body of evidence to suggest that a tailored IPF treatment including the use of anti-biotics prophylactically may be beneficial in IPF patients especially in Leu/Phe IPF patients.

Following on from the lung microbiome, it would be interesting to examine the gut microbiome in the context of the TLR3 L412F SNP to examine the bacterial populations. In order to further elucidate the effect of TLR3 L412F systemically in IPF patients, it would be interesting to examine whether the SNP also affected the gut microbiome in IPF patients. It has previously been shown that IPF patients have distinct bacterial populations in the lung microbiome compared with healthy controls and this can change further during AE-IPF (89, 91, 288). The gut microbiome consists of many different commensal bacterial species and it has been shown that factors such as smoking, aging and use of anti-biotics can alter the different species present (336). As the average age of diagnosis in IPF patients is above 60 years and most patients have a history of smoking (5), it could be suggested that gut microbiome may also be altered in these patients. It has been suggested that there is a relationship between the gut microflora and bacterial clearance in the lungs. Others have found that the presence of gut bacterial populations enhanced ability of the lungs to clear *E. coli pneumonia*. Depletion of gut bacteria by anti-biotic treatment led to blunted defence and increased bacterial counts in the lungs in mice (337, 338). Interestingly, the effects of commensal bacterial depletion were reversed with LPS treatment, suggesting a role for TLR4 in this process (337, 338). In future studies, it
would be interesting to examine the gut microbiome in IPF patients to determine whether there are differences in the presence and absence of the SNP and whether this impacts the lung microbiome in IPF patients. Faecal DNA samples are routinely used to examine the gut microbiome, unlike obtaining lung BALs and surgical biopsies of lung fibroblasts, it would be much easier in this study to obtain samples for analysis.

Throughout this study, we have mainly focused on the effect of TLR3 L412F on IPF primary human lung fibroblasts cultured from explanted lung tissue following lung transplantation. One limitation of this study was access to IPF lung samples and normal healthy control fibroblasts. Lung fibroblast samples are obtained by VATS biopsy, a surgical procedure, which has been associated recently with a risk to exacerbations of IPF. Because of this, VATS biopsies, previously used in the diagnosis of IPF are not performed in newly diagnosed IPF patients. Therefore, our access to samples we believe is limited to people who have reached the end-stage of their disease and lived long enough to receive a lung transplant, i.e. more mild-moderate patients than severe patients who have not survived long enough to receive a transplant. In support of this, our lab have previously shown that at 12 and 24 months Leu/Phe and Phe/Phe IPF patients are five times more likely to die than Leu/Leu IPF patients. Furthermore, in a total of 14 explant lung fibroblast lines we have cultured, no Phe/Phe IPF patients were found. This has meant for our study that we have predominantly examined mild-moderate IPF patients (Leu/Leu and Leu/Phe) and not the most severe. In order to overcome this issue and expand on our studies, we would like to in the future isolate PBMCs and serum from whole blood from IPF patients and cells collected from IPF BALs. It is important to elucidate whether the effects of TLR3 L412F are seen only in the lung or whether the SNP manifests itself systemically. In both of these cell types we would examine TLR responses in vitro and in the serum examine basal levels of pro-inflammatory cytokines, such as IL-8 and IL-6, and RANTES in the periphery to gain insight into whether TLR3 L412F has a role systemically as well as in lung fibroblasts. If TLR3 L412F was shown to have an effect in the periphery as well as in lung fibroblasts, this could be beneficial clinically in treatment of IPF patients as they could use PBMC and serum responses to determine disease progression. In regards to access to control normal healthy fibroblasts, we have shown that use of an immortalised cell
line such as CCD-191lu is not beneficial for comparison, furthermore it is only one normal cell line. It is important to obtain access to more normal cell lines in future studies. Other publications have used “normal” lung fibroblasts taken from around tumour biopsies from lung cancer patients. Again these are not an optimal control fibroblast as they are located in the tumour microenvironment and therefore may have phenotypic changes as well.

In order to further examine the role of TLR3 L412F in fibrosis and infection, we have commissioned the generation of the TLR3 L413F knock in mouse using CRISPR/Cas9 gene editing. One of the primary aims of this thesis was to characterise the mouse for future use in fibrosis and live infection models in vivo. Here, we have found that the effects of TLR3 L412F to be cell-specific. In murine lung fibroblasts and splenocytes in vitro we have found that TLR3 L413F attenuated Poly(I:C)-induced pro-inflammatory cytokine, KC, and RANTES expression in Leu/Phe and Phe/Phe murine lung fibroblasts. This indicated that similar to human IPF Leu/Phe lung fibroblasts, murine Leu/Phe and Phe/Phe lung fibroblasts have defective signalling through TLR3 and attenuated KC and RANTES responses. However, in other cell types and following LPS stimulation we have seen that Leu/Phe and Phe/Phe mice respond differently to IPF human lung fibroblasts. This may be due to species differences between mice and humans.

Unfortunately, in the time frame of this PhD, we were unable to perform in vivo characterisation of the TLR3 L413F mice. In the coming weeks we will be completing this experiment to examine determine whether TLR3 L413F affects Poly(I:C) and LPS-induced responses systemically in the mice. Following on from this, we will run the bleomycin-induced fibrosis model in the TLR3 L413F mice. We have previously found using the bleomycin-induced fibrosis model in TLR3+/− mice that absence of TLR3 signalling results in increased fibrosis in the lungs (154). Similarly, in Schistosoma mansoni egg-induced pulmonary granuloma model, researchers established that TLR3+/− mice had greater collagen deposition than wild-type mice, demonstrating a protective role for TLR3 in Th2-driven pro-fibrotic responses (155). Taking into account that TLR3+/− mice have been shown to have worsened fibrosis in bleomycin and Schistosoma mansoni models of fibrosis, we hypothesize that TLR3 L412F may play a detrimental role in fibrosis and similar results may be seen in TLR3 L413F mice. In support of this, we have now shown in
*vitro* in TLR3 L413F knock in mice that there is defective TLR3 signalling and attenuated KC and RANTES protein expression in Leu/Phe and Phe/Phe murine lung fibroblasts.

In conclusion, there is an unmet clinical need for novel diagnostic and prognostic biomarkers for use in the treatment of IPF. Considerable work has been undertaken to develop novel biomarkers. The most well documented candidate diagnostic biomarker to date is the MUC5B gene (46). Currently, there is no method clinically to stratify IPF patients based on their disease progression. Stratification of IPF patients into slow and rapid progressors will allow physicians to tailor treatment plans for each individual patient and fast track rapid progressors for lung transplantation. In this study, we believe, we have added evidence to support the use of TLR3 L412F as a candidate prognostic biomarker. Our research group has previously shown that Leu/Phe and Phe/Phe IPF patients are 5 times more likely to die at 12 and 24 months than wild-type IPF patients. This correlates with a significant decline in lung function (FVC) in Leu/Phe and Phe/Phe patients (154). In this study, we have also demonstrated additionally that the presence of the SNP results in defective cellular responses to TLR2, 3, 4, 5, 6 and 9 PAMPs. Furthermore, Leu/Phe IPF patients also appear to have defective cellular responses to the non-TLR agonists such as Poly(dA:dT), PMA, and HT-DNA. In this thesis, we have also characterised the effects of the TLR3 L412F SNP in autophagy responses in IPF patients and provided evidence to suggest that the autophagy pathway is defective in IPF primary human lung fibroblasts. These finding suggests that the mechanism in which TLR3 L412F accelerates mortality and decline in lung function in IPF patients involves mechanisms which are both TLR3-dependent and – independent. These findings warrant further investigation in IPF patients.
Chapter 8 Bibliography
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